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PREDICTION OF PRE-ECLAMPSIA

A THESIS SUBMITTED FOR CONSIDERATION FOR THE DEGREE OF DOCTOR IN MEDICINE TO THE UNIVERSITY OF DUBLIN, TRINITY COLLEGE

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CONTENTS

DECLARATION **5**

ACKNOWLEDGEMENTS **6**

PUBLICATIONS **7**

SUMMARY **9**

1. PREDICTION OF PRE-ECLAMPSIA : AN INTRODUCTION **10**

1.1 INTRODUCTION 10

1.2 OBJECTIVES 18

1.3 REFERENCES 19

2. MATERNAL – FETAL CELL TRAFFICKING AND PRE-ECLAMPSIA **22**

2.1 FETAL CELLS IN THE MATERNAL CIRCULATION 22

2.2 MATERNAL FETAL CELL TRAFFICKING AND COMPLICATIONS OF PREGNANCY 23

2.3 FETAL CELLS IN MATERNAL PLASMA 24

2.4 CLEARANCE OF FETAL CELLS 29

2.5 MATERNAL FETAL CELL TRAFFICKING AND PRE-ECLAMPSIA 31

2.6 MATERNAL FETAL CELL TRAFFICKING PRIOR TO THE ONSET OF PRE-ECLAMPSIA 34

2.7 MATERNAL FETAL CELL TRAFFICKING OF THE RH "D" GENE 37

2.8 REFERENCES 41

3. METHODOLOGY - PART I **45**

3.1 SRY SOLUTION PHASE PCR 45

3.2 CLONING 47

3.3 TAQMAN PCR 52

3.4 SRY TAQMAN PCR 58

3.5 EXPERIMENT : OPTIMIZATION OF SRY ASSAY 63

3.6 RHESUS " D" TAQMAN PCR 66

3.7 REFERENCES 69

4. INCREASED FETAL DNA IN THE MATERNAL CIRCULATION IN EARLY PREGNANCY IS ASSOCIATED WITH AN INCREASED RISK OF PRE-ECLAMPSIA **70**

4.1 INTRODUCTION 70

4.2 METHODS 72

4.3 RESULTS 74

4.4 DISCUSSION 79

4.5 REFERENCES 83

5. INCREASED MATERNAL FETAL CELL TRAFFICKING AND RH "D" GENE IMMUNIZATION IN PRE-ECLAMPSIA **86**

5.1 INTRODUCTION 86

5.2 METHODS 88

5.3 RESULTS 90

5.4 DISCUSSION 93

5.5 REFERENCES 96

6. FETAL CELLS IN MATERNAL PLASMA 98

6.1 INTRODUCTION 98

6.2 METHODS 100

6.3 RESULTS 102

6.4 DISCUSSION 104

6.5 EXPERIMENT 1: TO COMPARE THE YIELD OF DNA EXTRACTED FROM WHOLE BLOOD VERSUS PLASMA USING DIFFERENT VOLUMES 106

6.6 EXPERIMENT 2: TO DETECT PCR INHIBITION IN DNA EXTRACTED FROM PLASMA VERSUS DNA EXTRACTED FROM WHOLE BLOOD 108

6.7 EXPERIMENT 2A: TAQMAN PCR FOR MEASLES VIRUS F GENE IN DNA EXTRACTED FROM WHOLE BLOOD VS PLASMA SPIKED WITH MEASLES VIRUS PLASMID. 109

6.8 EXPERIMENT 2B: TAQMAN PCR FOR MEASLES VIRUS F GENE IN DNA EXTRACTED FROM WHOLE BLOOD VS PLASMA SPIKED WITH MEASLES VIRUS PLASMID PRIOR TO DNA EXTRACTION. 111

6.9 REFERENCES 114

7. HOMOCYSTEINE : A RISK FACTOR FOR PRE-ECLAMPSIA 115

7.1 STRUCTURE AND PROPERTIES OF HOMOCYSTEINE 115

7.2 EVIDENCE FOR A PATHOGENIC ASSOCIATION BETWEEN HOMOCYSTEINE AND VASCULAR DISEASE 120

7.3 HOMOCYSTEINE LEVELS IN PREGNANCY 122

7.4 HOMOCYSTEINE AND PRE-ECLAMPSIA 123

7.5 PRE-ECLAMPSIA AND MTHFR POLYMORPHISM 125

7.6 REFERENCES 130

8. METHODOLOGY - PART II 134

8.1 SAMPLE COLLECTION 134

8.2 MATERIALS 135

8.3 THEORY OF OPERATION OF HOMOCYSTEINE ASSAY 137

8.4 MEASUREMENT OF PLASMA HOMOCYSTEINE 139

8.5 PLASMA FOLATE MICROBIOLOGICAL ASSAYS 140

8.6 VITAMIN B12 ASSAY 141

8.7 GENOTYPE ANALYSIS OF THE MTHFR C677T VARIANT 143

8.8 REFERENCES 147

9. ELEVATED PLASMA HOMOCYSTEINE IN EARLY PREGNANCY: A RISK FACTOR FOR THE DEVELOPMENT OF PRE-ECLAMPSIA 148

9.1 INTRODUCTION 148

9.2 SEVERE PRE-ECLAMPSIA 150

9.3 NON-SEVERE PRE-ECLAMPSIA 161

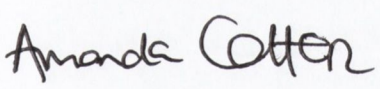
9.4 REFERENCES 170

DECLARATION

I, Amanda Cotter, declare that the work in this thesis is entirely my own.

Approval was obtained from the investigational review board of the Coombe Women's Hospital, Dublin.

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Amanda Cotter

December 2002

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PUBLICATIONS

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PAPERS

Cotter AM, Molloy AM, Scott JM, Daly SF. Elevated plasma homocysteine in early pregnancy: a risk factor for the development of severe pre-eclampsia. *American Journal of Obstetrics and Gynecology* 2001: 185(4); 781-5.

Cotter AM, Molloy AM, Scott JM, Daly SF. Elevated plasma homocysteine in early pregnancy: a risk factor for the development of severe pre-eclampsia. (Letter reply) *American Journal of Obstetrics and Gynecology* 2002: 186(5); 1108.

ABSTRACTS

Cotter AM, Molloy AM, Scott JM, Daly SF. Elevated plasma homocysteine in early pregnancy: a risk factor for the development of severe pre-eclampsia. *American Journal of Obstetrics and Gynecology* 2001: 184(1); S11.

Cotter AM, Molloy AM, Scott JM, Daly SF. Elevated plasma homocysteine in early pregnancy: a risk factor for the development of mild-moderate pre-eclampsia. *American Journal of Obstetrics and Gynecology* 2002 : 185(6); S191.

Cotter AM, Martin CM, O'Leary JJ, Daly SF. Increased maternal-fetal cell trafficking in early pregnancy is associated with an increased risk of pre-eclampsia. *American Journal of Obstetrics and Gynecology* 2002 : 185(6); S192.

Cotter AM, Martin CM, O'Leary JJ, Daly SF. Increased maternal-fetal cell trafficking and Rh "D" gene immunization in pre-eclampsia. *American Journal of Obstetrics and Gynecology* 2002 : 185(6); S191.

Cotter AM, Martin CM, O'Leary JJ, Daly SF. Increased maternal-fetal cell trafficking in early pregnancy is associated with an increased risk of pre-eclampsia. *Hypertension in Pregnancy* 2002 : P86.

Cotter AM, Martin CM, O'Leary JJ, Daly SF. Increased maternal-fetal cell trafficking and Rh "D" gene immunization in pre-eclampsia. *Hypertension in Pregnancy* 2002 : P86A.

SUMMARY

Pre-eclampsia occurs in 2-10% of pregnancies and is a leading cause of maternal and perinatal morbidity and mortality. The serious shortcoming associated with management is that the underlying aetiology is unknown. To date little research has focussed on the investigation of women in early pregnancy who subsequently develop pre-eclampsia but rather on women with the disease. To investigate aetiology and susceptibility to pre-eclampsia, this thesis focusses on two key objectives:

1. To determine if in early pregnancy there is increased fetal DNA in the maternal circulation in asymptomatic women who will subsequently develop pre-eclampsia and to correlate the quantity of fetal DNA with severity of disease.
2. To investigate if plasma homocysteine levels are elevated in early pregnancy in those women who develop pre-eclampsia.

A bank of stored blood samples obtained in early pregnancy was used. Cases were asymptomatic women who subsequently developed pre-eclampsia. Controls were women who remained normotensive and without proteinuria throughout pregnancy.

This thesis demonstrates that women who develop both severe and non-severe pre-eclampsia have higher plasma homocysteine levels in early pregnancy when compared to women who remain normotensive throughout. Also increased fetal DNA can be detected in early pregnancy in the maternal circulation of women who subsequently develop pre-eclampsia and is associated with an 8 fold increased risk of developing pre-eclampsia which appears to be a graded response.

CHAPTER 1.

PREDICTION OF PRE-ECLAMPSIA : AN INTRODUCTION

1.1 INTRODUCTION

Although pregnancy and childbirth have never been as safe for the mother and her child, pre-eclampsia still occurs in 2–10% of pregnancies and is a leading cause of maternal and perinatal morbidity and mortality. The number of maternal deaths from hypertension in pregnancy has fallen steadily over the past few decades (CEMD 1999) as have the complication rates (Leitch et al 1997). Despite some therapeutic progress, pre-eclampsia is still one of the leading causes of maternal and fetal mortality especially in the developing world (Duley 1992).

Pre-eclampsia which usually occurs late in the second trimester or more frequently in the third trimester is characterised by the occurrence of pregnancy induced hypertension, proteinuria and oedema after twenty weeks gestation in a woman with no prior incidence of these sequelae. A serious shortcoming is the fact there is no reliable test to identify those women at risk of developing the disorder early enough in their pregnancies to permit preventative treatment. Current treatment is thus restricted to symptomatic management with drugs such as anti-hypertensives, benzodiazepines or magnesium sulphate following full onset of the disease. Rapid delivery of the fetus often is the only way to resolve the disorder frequently resulting in preterm delivery, of a compromised growth restricted fetus leading to expensive prolonged treatment in the Neonatal Intensive Care Unit.

The underlying aetiology of pre-eclampsia remains unclear, thus effective primary prevention is not possible at this stage. Research in the past decade has identified some major risk factors for pre-eclampsia and manipulation of these factors might result in a decrease in its frequency. Prevention of pre-eclampsia would mean a big step forward in prenatal care.

The term prevention can have three different meanings: primary, secondary or tertiary. Primary prevention means avoiding occurrence of a disease. Secondary prevention in the context of pre-eclampsia means interrupting the pathophysiological process before development of the clinically recognisable disease. Tertiary prevention implies prevention of complications caused by the disease process and is thus synonymous with treatment (Dekker & Sibai 2001).

The optimal way to manage a disease is to prevent its occurrence, and this can only be achieved if the underlying aetiology is understood and if it is feasible to avoid or manipulate those causes. The presence of a placenta is both necessary and sufficient to cause the disorder. Pre-eclampsia can occur with a hydatidiform mole so the presence of fetus is not required. Pre-eclampsia may develop in an abdominal pregnancy so a uterus is not necessary. Delivery is central to management thereby removing the causative organ, the placenta.

Shallow endovascular cytotrophoblastic invasion in the spiral arteries, an exaggerated inflammatory response and inappropriate endothelial cell activation are key features in the pathogenesis of pre-eclampsia. However the mechanisms behind these features are unknown. Although endothelial cell dysfunction appears to be the final common pathway in the pathogenesis of pre-eclampsia, the aetiology is still unknown. Genetic factors are involved,

however it is unlikely that there is one major gene but several genetic factors that are associated with maternal susceptibility (Dekker & Sibai 1998).

Secondary prevention of a disease is only possible if three requirements are met: knowledge of pathophysiological mechanisms, availability of methods for early detection and means of intervention and correction of the pathophysiological changes. Many tests have been proposed to predict later development of the disease. Measurement of blood pressure or second trimester mean arterial pressure are not useful for early diagnosis of pre-eclampsia (Sibai 1998). Uric acid levels can be used as an indicator of disease severity in established pre-eclampsia but have a low sensitivity (Dekker & Sibai 1991). Poor sensitivity and specificity and the fact that proteinuria is a late feature of pre-eclampsia make routine use of dipsticks in a normotensive low risk population as ineffective at pre-eclampsia prediction (Halligan et al 1999) as measuring weight gain (Dawes & Grudzinskas 1991).

To date the results of preventative strategies have been disappointing. In the early 1990s, Aspirin was thought to be the wonder drug for secondary prevention of pre-eclampsia but the results of a large trial showed that if there is an indication for Aspirin use, it is in the patient at very high risk of developing severe early onset disease (CLASP 1994). A second study conducted in the Caribbean (Rotchell et al 1998) recruited relatively low risk women while another conducted in the USA (Caritis et al 1998) recruited high risk women. The Barbados trial, BLASP, used a 75g slow release aspirin while the others used standard 60g aspirin. Overall aspirin was associated with a 10% reduction in the incidence of pre-eclampsia. CLASP showed a trend towards a greater reduction in pre-eclampsia the more preterm the delivery. This resulted in a belief that

starting aspirin at 12 to 20 weeks might have benefits for the small but important sub-group of women at high risk of severe early onset disease. However this has not been confirmed. The results of trials using calcium supplements (Levine et al 1997) and fish oils (Olsen et al 2000) were similarily disappointing. Without doubt, intensive antenatal care is the mainstay of tertiary prevention and the aim of treatment is the prevention of complications.

The prevention of pre-eclampsia would have a significant impact on maternal and perinatal outcome worldwide. While the signs and symptoms of pre-eclampsia become apparent as late as the third trimester, the underlying cause of the pathophysiological mechanisms appears to occur much earlier in pregnancy possibly between 8 and 18 weeks gestation. It therefore seems logical to search for earlier indicators of this disorder and a multitude of tests have been proposed as a means of predicting the later development of the disease.

Accurate early detection of pre-eclampsia has remained elusive because a single model has been unable to predict the occurrence of a mixture of disorders (Dekker & Sibai 1991). Reviewing the extensive literature on this subject reveals some major problems. The terminology used to define or to diagnose pre-eclampsia is inconsistent. It is also a significant problem that there are no internationally accepted criteria to judge the usefulness of a predictive test. The ideal predictive test should be simple and easy to perform early in pregnancy. A test should be reproducible and non-invasive with both a high sensitivity and a high positive predictive value. However to date there is no test that fulfills these criteria. The actual value of an individual test should be evaluated in the context of the clinical or research setting in which that particular test is used. Detailed longitudinal studies may carry a significant burden for study subjects and are possible

only in small groups of high-risk patients selected by a test with a high predictive value. In such a setting it is of less importance if the particular test is invasive, time consuming or has only a moderate sensitivity. On the other hand for a test to identify a large high risk group suitable for studying the effects of therapeutic intervention, the test needs to be inexpensive, simple to perform as well as non-invasive and to express a high sensitivity. In these circumstances an average predictive value is acceptable. More than 100 clinical, biophysical and biochemical test have been recommended for predicting the development of pre-eclampsia. The findings of these numerous studies have been inconsistent and contradictory owing to the heterogeneity of the populations studied and variations in the definitions used for hypertensive disorders during pregnancy and in the methods used to express results.

It has been proposed that the placental component of pre-eclampsia is mediated by reduced placental perfusion which in turn may be due to abnormal placentation with failure of the trophoblast to induce the physiologic dilatation and remodelling of the spiral arteries. The prevailing hypothesis as to the cause of abnormal placentation is that it is immunologically mediated with prior exposure to paternal antigen protective. This theory is supported by the finding that pre-eclampsia is common with first pregnancies, that risk is increased with new paternity and reduced with increased duration of sexual activity antedating pregnancy. Traditionally epidemiologic studies have demonstrated that pre-eclampsia is mainly a disease of first pregnancies, however, recent data suggests that the inter-pregnancy interval should also be taken into consideration. A large Danish study found that a long inter-pregnancy interval was associated with a higher risk of pre-eclampsia in women with no previous pre-eclampsia when the father was the same (Basso et al 2001). Similarly a Norwegian study reported that women

with no prior history were at higher risk of pre-eclampsia in a second pregnancy if there was an increased inter-pregnancy interval (Trogstad et al 2001). Pre-existing medical disorders known to increase the risk of pre-eclampsia such as hypertension, diabetes and collagen vascular disease are all characterised by microvascular disease. It has been proposed that abnormal microvasculature reduces placental perfusion.

The diverse features characterising pre-eclampsia could arise from circulatory disturbances secondary to diffuse maternal endothelial cell dysfunction (Roberts et al 1989). Some women are more sensitive to endothelial dysfunction or have pre-existing endothelial dysfunction associated with a future tendancy to such diseases as hypertension or diabetes (Ness & Roberts 1996). This would explain the long term association between these medical conditions and pre-eclampsia.

Endothelial dysfunction is one aspect of a generalised systemic maternal inflammatory response which also affects circulating leukocytes (Sacks et al 1998, Redman et al 1999). This inflammatory response is detected in normal pregnancy when it is not intrinsically different from that in pre-eclampsia except that it is milder. Pre-eclampsia may develop when there is an excessive systemic inflammatory response. In other words the disorder is not a separate condition but simply the extreme end of a range of maternal systemic inflammatory responses engendered by pregnancy itself. Certain women have increased sensitivities to the inflammatory stimulus of a normal pregnancy. Ness and Roberts (1996) proposed that maternal and placental susceptibilities converge at the point of the endothelium. Endothelial activation is intrinsic to the inflammatory response therefore the point of convergence is the maternal systemic inflammatory system.

Placental ischaemia and increased syncytiotrophoblast deportation may represent an end stage disease phenomenon very similar to that predicted by the conflict hypothesis which suggests that endothelial cell damage may have evolved as a high risk fetal strategy to increase nonplacental resistance when the uteroplacental blood supply to a fetus is inadequate (Dekker & Sibai 1998).

A rational strategy in the search for biologic mediation of pre-eclampsia would be to focus on the interaction between maternal and placental origins in causing endothelial damage by examining for example the risk factors predisposing to pre-eclampsia which also predispose to cardiovascular disease in later life. The association between plasma homocysteine and coronary artery disease has been well established (Boushey et al 1995, Hankey & Eikelboom 1999) and in this thesis the role of an elevated plasma homocysteine in early pregnancy as a risk factor for the development of pre-eclampsia has been investigated.

Recent studies on maternal-fetal cell trafficking for prenatal diagnosis of aneuploidies and even preeclampsia have been hopeful, hence the possibility of a novel molecular biological screening test for the prediction of preeclampsia was investigated as a second but unrelated objective of this thesis. It is possible that an influx of fetal DNA into the maternal circulation could provoke the inflammatory response of pregnancy and that this response is exaggerated if the burden of debris is abnormally high or if the woman responds excessively to the process thus resulting in the development of pre-eclampsia.

As pre-eclampsia is unlikely to have a single cause, it is reasonable to assume that a single screening test will not be found. Efficient tests may be based on detection of for example inflammatory changes. However because of variations in maternal responses, there may be no useful single test. The primary objectives of this thesis intend to further understanding of the pathophysiology of pre-eclampsia. As there are many routes to the final common pathway of pre-eclampsia, it is also unlikely that a single treatment or prophylactic measure will be universally effective. Different measures may apply to different subgroups (Redman et al 1999).

1.2 OBJECTIVES

The objectives of this thesis are:

1. To investigate if there is increased fetal DNA in the maternal circulation in early pregnancy prior to the development of pre-eclampsia

2. To investigate if there is increased fetal Rh "D" gene in the maternal circulation in early pregnancy prior to the development of pre-eclampsia

3. To investigate if plasma homocysteine is elevated in early pregnancy prior to the development of pre-eclampsia with a view to determine if homocysteine plays a role in the aetiology.

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CHAPTER 2.

MATERNAL – FETAL CELL TRAFFICKING AND PRE-ECLAMPSIA

2.1 FETAL CELLS IN THE MATERNAL CIRCULATION

During pregnancy, the fetal and maternal circulations are separated by placental membranes. However a variety of evidence has pointed towards the incompleteness of this barrier to cellular trafficking. Fetal nucleated cells have been demonstrated in the maternal circulation and have been widely pursued as potential substrates for non- invasive prenatal diagnosis. However the rarity of such fetal cells in maternal blood has been a major obstacle to the routine application of this concept.

The debate over whether fetal cells exist in the circulation of pregnant women has concluded that they are detectable but rare in most situations. An exception seems to be complications of pregnancy including fetal trisomy and pre-eclampsia, in which increased fetomaternal transfusion may be associated with abnormal placental development (Bianchi 2000).

The fact that fetal cells pass into the maternal circulation during pregnancy has been known for more than a century (Schmorl 1893). The association between passage of fetal red blood cells to the mother and development of antibodies against fetal red cell specific antibodies is also long recognised. More recently, isolation of fetal cells from maternal blood has stimulated much interest as a method of non-invasive screening for genetic disease.

Different cell types of fetal origin can be found in the maternal circulation. The deportation of trophoblast cells during the development of the placenta appears to be the most physiologic explanation for a mechanism by which fetal cells enter the maternal circulation. The trafficking period of these cells appears to be between 5 and 10 weeks gestation. After this time these cells have differentiated and their invasive properties and potential for deportation has decreased (Pertl & Bianchi 2001).

2.2 MATERNAL FETAL CELL TRAFFICKING AND COMPLICATIONS OF PREGNANCY

The increased recognition of fetal cells in the maternal circulation and the ability to quantitate them has stimulated much interest in the potential diseases of pregnancy that could be related to fetal cells. There appears to be a relationship between abnormal placentation and maternal fetal cell trafficking. The relationship between fetal cell microchimerism and maternal disease is currently under investigation. Aractingi studied women with polymorphic eruption of pregnancy (PEP) and detected male DNA in skin lesions of 6 out of 10 women with PEP and found none in 26 controls (Aractingi et al 1998). This suggests that fetal cells can migrate to the maternal skin during pregnancy and play a role in this cutaneous disease. After delivery, more male DNA presumed to be of fetal origin is present in the blood and skin of women with scleroderma when compared with healthy controls (Artlett et al 1998). Scleroderma is of particular interest because it demonstrates a female predilection and it is an autoimmune disease with clinical similarities to graft versus host disease. Therefore maternal fetal cell trafficking provides a possible explanation

for the increased prevalence of autoimmune disorders in adult women following their childbearing years (Bianchi 2000).

2.3 FETAL CELLS IN MATERNAL PLASMA

Although early work was carried out on whole blood, there has been much interest recently in the use of the non-cellular portion of blood for molecular analysis. This interest has been stimulated mainly by the finding of tumour derived DNA in the serum and plasma of cancer patients. These findings suggested that circulating DNA may be found in other clinical settings (Mulcahy et al 1996). There are some similarities between the placenta and a malignant tumour hence just as tumour DNA can be found in the plasma of cancer patients, fetal DNA may be found in maternal plasma.

This line of reasoning led Lo to look for fetal DNA in maternal plasma and serum (Lo et al 1997). This study produced the first demonstration of fetal DNA in maternal plasma and serum and the realisation that maternal plasma DNA is a chimeric mixture of fetal and maternal DNA. Lo used a rapid boiling method to extract DNA from the plasma and serum of 43 pregnant women and conventional "hot start" PCR for detection. Fetal derived Y sequences were detected in 24/30 maternal plasma samples (80%) and in 21/30 (70%) serum samples from women bearing male fetuses. When DNA from nucleated blood cells extracted from the same volume (200µl) of blood was used, only 5/30 gave a positive Y signal. This was the first time that fetal DNA was detected in maternal plasma and serum thus suggesting that use of maternal plasma

and serum for detection of fetal DNA for non-invasive prenatal diagnosis may be possible. These results were obtained using just 10μl of boiled plasma and serum.

However a number of important questions needed to be answered before clinical application of this new approach could be contemplated. Firstly, fetal DNA in maternal plasma and serum would need to be shown to be present in sufficient quantities for reliable molecular diagnosis to be carried out. Secondly, data on the variation of fetal DNA concentrations in maternal plasma and serum in relation to gestational age would be required to determine the possibility of applying this technology to early prenatal diagnosis.

Lo addressed both these issues by developing a real time quantitative TaqMan PCR assay to measure the copy number of DNA molecules in maternal plasma and serum (Lo et al 1998). This technique permits continuous optical monitoring of the progress of an amplification reaction, giving accurate target quantitation over a wide concentration range. Using both commercial column-based DNA extraction methods and a sensitive real time PCR technique, they were able to push the sensitivity to close to 100% for the detection of a fetal derived Y chromosomal sequence from maternal plasma and serum. There were 25 early pregnancy samples (11-17 weeks), 13 carrying male fetuses and 25 late pregnancy samples (37-43 weeks), 14 carrying male fetuses. Lo demonstrated that fetal DNA is present in high concentrations in maternal plasma, reaching a mean of 25.4 genome equivalents/ml (range 3.3-69.4) in early pregnancy and 292.2 genome equivalents/ml (range 76.9-769) in late pregnancy. These concentrations correspond to 3.4% (range 0.39%-11.9%) and 6.2% (2.33%-11.4%) of the total plasma DNA in early and late pregnancy respectively. The corresponding values for maternal serum were 0.13% and 1%

respectively, presumably reflecting the liberation of background maternal DNA during the clotting process. A positive signal was obtained for each of the 27 women bearing male fetuses and no signal was detected for each of the 23 women bearing female fetuses.

This system using a 5' nuclease and fluorescent primers is sensitive enough to detect the DNA in the equivalent of one male fetal cell against a background of 12,800 female genome equivalents. Lo used SRY, a single copy Y chromosome specific sequence to quantify the number of genome equivalents per millilitre of blood when the pregnant woman carries a male fetus. SRY was chosen because of the obvious lack of this sequence in the mother.

Lo also investigated the changes in fetal DNA concentrations in maternal serum at different gestational ages. Twenty women who conceived by IVF were followed up preconceptually and at multiple time points during pregnancy. Sequential follow up showed that fetal DNA can be detected in maternal serum as early as seven weeks. This data demonstrated a sharp increase in the amount of fetal DNA detected during the last eight weeks of pregnancy. This sharp increase could correlate with the peak phase of fetal growth in the third trimester or at a microscopic level, the placenta could possibly be breaking down in anticipation of delivery (Bianchi 1998).

Compared to their earlier report (Lo et al 1997), they were now able to detect fetal SRY genes in maternal plasma or serum from every subject carrying a male fetus. This detection rate was obtained with DNA extracted from just 40-80μl of maternal plasma or serum. This volume represents a 4-8 fold increase over the 10μl of boiled maternal plasma or serum and results in a significant improvement in sensitivity. The specificity was preserved since they did not observe

amplification signals from samples obtained prior to conception or from subjects carrying a female fetus (Lo et al 1998).

How do Lo's results in 1998 compare with those reported in previous studies? Almost all previous investigations have focussed on complete and intact fetal cells in the maternal circulation, so that their nuclei are available for either cell culture or interphase cytogenetic analysis using chromosome specific probes. Different methods of cell preparation have also been used. Most PCR based non-invasive prenatal diagnostic studies have also used DNA prepared from intact fetal and maternal cells. In the past plasma has been discarded. This was the approach used in a previous study in which a radioactive quantitative PCR was employed in an assay to measure the number of fetal cell DNA equivalents in 16mls of maternal blood samples. The mean number of male DNA equivalents in samples from women carrying karyotypically normal male pregnancies was 19 or 1.2/ml of whole blood (Bianchi et al 1997). Lo et al expressed their results as copies of SRY per millilitre and demonstrated a mean of 25.4 copies in the maternal plasma samples obtained early in pregnancy. However it is important to note that all of this work from Lo is based on very small numbers.

Bianchi has suggested that maternal whole blood and plasma can both be used as sources of cellular and free fetal DNA. If maternal blood is used to prepare cellular DNA, assays will detect all fetal cell types as well as previous transfusions and potentially cells from previous pregnancies. Plasma fetal DNA in contrast is limited to the current pregnancy and there is no evidence that this free fetal DNA persists after delivery (Bianchi 2000). Compared with the analysis of fetal nucleated cells that have entered into the maternal circulation which in many

cases requires the use of fetal cell enrichment procedures, plasma DNA analysis has the advantage of being rapid, reliable and easily carried out for a large number of samples.

Analysis of DNA in blood is conventionally performed on cells while plasma and serum are discarded. Free DNA has been demonstrated in serum in cancer and autoimmune disorders as well as in pregnancy. This group determined fetal gender by PCR on DNA from maternal venous blood, serum and plasma samples of 65 women by boiling with or without phenol/chloroform extraction. They found extracted plasma amplified better than extracted serum (89 vs 46%) and fetal gender could not be reliably determined from DNA extracted from maternal nucleated blood cells (Houfflin Debarge et al 2000).

Zhong investigated if concentrations of free extracellular fetal circulating DNA in maternal plasma are stable or fluctuate. They drew consecutive blood samples from 13 healthy non-pregnant volunteers and 16 healthy pregnant women over 3 days. In the non- pregnant controls, the total amount of cell free DNA fluctuated by an average of 13.5 fold. In samples obtained from pregnant women, the amount of maternal cell free DNA varied by an average of 21.5 fold. The mean variation in free fetal DNA levels in male fetuses was 2.2 fold. This degree of variation in free fetal DNA concentration implies that care should be exercised when considering quantitation of this fetal material for potential diagnostic or screening purposes (Zhong et al 2000).

2.4 CLEARANCE OF FETAL CELLS

The clearance of free fetal DNA from maternal plasma has been established by a study of women just before and after vaginal delivery and cesarean section. This study demonstrated that free fetal DNA is cleared rapidly from maternal plasma with a half- life of minutes. They analysed plasma samples from 12 women 1-42 days after delivery of male infants and found that circulating fetal DNA was undetectable after the first day postpartum. They then performed serial sampling of 8 women and found that 7 out of 8 had undetectable levels of circulating fetal DNA by two hours postpartum. The mean half-life for circulating fetal DNA was 16.3 minutes (range 4-30 minutes) (Lo et al 1999). The conclusion from this study parallels that from earlier work involving the injection of exogenous DNA into the circulation of laboratory animals which showed rapid elimination kinetics (Tsumita & Iwanaga 1963).

The rapid clearance kinetics of plasma fetal DNA demonstrates important differences from the clearance of fetal nucleated cells from maternal blood. In the latter scenario, although most of the cells are cleared within weeks from the maternal circulation, a cell subpopulation has been shown to persist for up to decades after delivery. Recent studies have even demonstrated a potential pathogenic effect of such fetal cell persistence on the development of autoimmune diseases such as systemic sclerosis (Artlett et al 1998). The notable differences between fetal cell and cell free DNA clearance suggest that the predominant cell populations may be distinct. It is possible that the trophoblasts may be the predominant cell population involved in the liberation

of fetal DNA into the cell free fractions. Fetal erythroblasts on the other hand have been postulated to be the predominant fetal cell population found in maternal blood (Bianchi 1999).

The organ systems involved in fetal DNA clearance from maternal plasma remain to be elucidated. Recent data shows that the kidneys may play a role in the clearance of plasma DNA because fetal DNA was found in the urine of pregnant women carrying male fetuses (Botezatu et al 2000). They used unfractionated urine and these women were in the first trimester. These results therefore not only demonstrate important biological pathways involved in the clearance of plasma DNA but also opens up a highly non-invasive method for prenatal diagnosis by molecular analysis of urine.

2.5 MATERNAL FETAL CELL TRAFFICKING AND PRE-ECLAMPSIA

The observation made by Schmorl more than a century ago that trophoblastic cells could be detected in the pulmonary capillaries of 14 of 17 patients who had succumbed to eclampsia indicates that the occurrence of maternal fetal cell trafficking in pre-eclampsia is not a new idea (Schmorl 1893). Similarily the presence of trophoblast in pulmonary tissue examined after maternal death was confirmed in 96 of 220 cases (Attwood & Park 1961).

Holzgreve noted by chance that the number of erythroblasts was increased in pregnancies complicated by pre-eclampsia (Holzgreve et al 1998). To ascertain whether these cells were fetal or maternal in origin, they performed a case- control study examining only pregnancies bearing male offspring using X and Y chromosome fluorescence in situ hybridisation to determine the number of male cells. This study was small with only 8 cases and 8 controls. Using cytospin preparations, they counted 200 nucleated cells and found a marked difference in the number of morphologically identified erythroblasts in pre-eclampsia patients compared to controls. FISH analysis for X and Y chromosomes was used to identify whether the erythroblasts were of fetal or maternal origin. They found a significantly higher number of fetal nucleated cells in pre-eclampsia subjects when compared to control patients thus demonstrating an increase in cross-placental traffic. These initial results raised hope for a new mode of diagnosis but this approach could not be implemented for routine use because the enrichment procedure and subsequent analysis would not permit the rapid evaluation of a huge population of pregnant women, many of whom would not be at risk for developing pre-eclampsia.

Increased transfer of fetal derived cells other than erythroblasts has been reported previously. Increased levels of trophoblast deportation have been observed (Douglas et al 1959), trophoblast deportation was found to be increased in patients with pre-eclampsia with both cytotrophoblast and syncytiotrophoblast present in uterine vein blood but there was no correlation with the severity of disease or gestational age (Chua et al 1991). Similarily Knight reported the release of syncytiotrophoblast microvilli in increased amounts in women with pre-eclampsia disrupting normal endothelial cell behaviour (Knight et al 1998).

Lo set out to investigate if in addition to disturbed cellular transfer, nucleic acid traffic would also be affected in pre-eclampsia. They studied 20 women with pre-eclampsia and 20 controls of similar gestational age (32 and 33 weeks respectively). Free male fetal DNA in maternal serum was measured using real time quantitative PCR for the SRY gene on the Y chromosome. The median circulating free fetal DNA was increased five fold in the 20 women with pre-eclampsia compared to the control group (381 vs 76 genome equivalents/ml, $p<0.001$). This was the first description of a quantitative abnormality involving circulating non-host DNA (Lo et al 1999)

There are a number of mechanisms which could explain the findings of Lo (Lo et al 1998). One possibility is that there is continuous leakage or transfer of fetal cells across the placenta but the maternal immune system rapidly destroys the material leaving free DNA remaining in the plasma. Therefore, isolation of fetal cells from maternal blood results in finding only a limited fraction of what was once there. There may be active remodelling of the placenta at the fetal-maternal interface with continuous cell lysis and release of fetal DNA into the maternal circulation. A third possibility is that developmentally associated apoptosis of fetal cells may be

occurring (Bianchi 1998).The normal processes of repair and renewal of syncytiotrophoblast which depend on apoptosis release a continuous stream of placental debris into the maternal circulation (Redman & Sargent 2000). This provokes a systemic inflammatory response in all women which is exaggerated if the burden of the debris is abnormally high or if the woman responds excessively to the process. Apoptosis rates are significantly increased in the syncytiotrophoblast in pre-eclampsia (Leung et al 1999) and fetal DNA in the maternal circulation could be a marker of syncytial debris. An increased influx of fetal DNA into the maternal circulation as a result of placental oxidative stress and increased apoptosis could trigger an excessive maternal inflammatory response as a result of the vast increase in the number of haplo-identical fetal cells.

Zhong and colleagues measured circulating DNA by real time quantitative PCR in plasma samples obtained from 44 women with pre-eclampsia and a matched cohort of 53 normotensive pregnant women, each carrying a male fetus. The mean gestational age of the control group was 37 weeks, mild-moderate pre-eclampsia 37.8 weeks and severe pre-eclampsia 33.7 weeks. They demonstrated that circulating fetal DNA levels were significantly elevated in pregnancies complicated by pre-eclampsia (3194.6 vs 332.8 copies/ml, $p<0.001$). They also showed for the first time that circulating maternal DNA levels are also elevated (219,023.9 vs 20,235.8 copies/ml, $p<0.001$). The increase in free DNA levels corresponded to the severity of the disorder and values correlated with each other in pregnancies complicated by pre-eclampsia but not in normotensive pregnancies (Zhong et al 2001).

The relationship between increased cellular and cell free DNA transfer in pre-eclampsia remains to be elucidated. The question of whether increased fetal DNA may be detected before the onset of pre-eclampsia is an important question which needs to be answered. It is not certain if the increase in maternal fetal cell trafficking precedes or is the consequence of placental damage associated with this complication of pregnancy.

2.6 MATERNAL FETAL CELL TRAFFICKING PRIOR TO THE ONSET OF PRE-ECLAMPSIA

Recent data has demonstrated that increased fetal erythroblast trafficking can be observed a few weeks before the onset of pre-eclampsia. Doppler ultrasound studies have shown that impaired placental perfusion at 24 weeks gestation is associated with increased impedance to flow in the uterine arteries and this abnormal waveform identifies pregnancies at risk of development of pre-eclampsia and/or intrauterine growth restriction (IUGR). Al-Mufti obtained maternal blood from 18 pregnancies with abnormal Doppler results at 22-24 weeks gestation and from 10 normal controls. Fetal erythroblasts were enriched from maternal blood and the percentage of erythroblasts determined. The median proportion of fetal erythroblasts in the group with abnormal Doppler results was 4.5%, significantly higher than the control group 1%. The median proportion of fetal erythroblasts was higher in the 10 cases which subsequently developed pre-eclampsia and/or IUGR (5.5%) than in those with a normal pregnancy outcome (2%). These findings suggest that impaired placental perfusion is associated with an increase in maternal fetal

cell traffic which precedes the onset of pre-eclampsia and/or IUGR by several weeks (Al-Mufti et al 2000).

To investigate if an elevation in maternal and fetal erythroblast counts occurs before the clinical onset of pre-eclampsia, Holzgreve enriched erythroblasts in 97 maternal blood samples obtained at a gestational age of 21 +/- 3 weeks. Significantly higher numbers of erythroblasts (mean 6041.7 vs 928.9, p=0.008) were detected in the blood samples of the 15 women who later developed pre-eclampsia (Holzgreve et al 2001).

Leung attempted to answer the question whether the abnormal increase in circulating fetal DNA concentrations can be detected in susceptible patients before onset of the clinical disease. The study group comprised 18 women who subsequently developed pre-eclampsia and 33 controls sampled at a mean gestational age of 17.2 and 18.0 weeks respectively. DNA was extracted from plasma samples. Fetal DNA concentrations were significantly higher in pre-eclampsia than control pregnancies (p=0.001), the median fetal DNA concentrations in pre-eclampsia and control pregnancies were 41.9 and 22.0 genome equivalents/ml respectively. Circulating fetal DNA was detected in all subjects carrying male fetuses in both the pre-eclampsia and control groups. There was no significant difference in the plasma concentrations of the ß-globin gene between the two groups suggesting that circulating maternal DNA levels are not elevated prior to the onset of pre-eclampsia. Addressing the question as to whether or not maternal plasma fetal DNA might be useful as a marker for predicting pre-eclampsia, the authors found that there was overlap in the fetal DNA concentrations between the pre-eclampsia and control groups. Therefore a low sensitivity and specificity would result if maternal plasma fetal DNA was used

as the sole predictor for pre-eclampsia. They found that on ROC curve analysis the best discrimination between the pre-eclampsia and control groups was obtained at a fetal DNA concentration of 33.5 genome equivalents/ml. The sensitivity at this cutoff was 67% and the specificity was 82%. The area under the ROC curve was 0.778. They did not correlate their results with severity of disease (Leung et al 2001).

Other questions such as the optimal fetal target sequence to be examined remains to be resolved as obviously the Y chromosome is rather restricted. Ultimately the key question of what role this deluge of fetal material plays in the development or progression of the disorder hopefully can be answered. Findings from the work to date have resulted in an interesting hypothesis regarding the aetiology and pathophysiological sequence of pre-eclampsia. It is possible that the primary hit may be impaired trophoblast invasion caused by genetic factors such as mutations in genes coding for cadherin and other proteins which are involved in trophoblast invasion. This is followed by an increased influx of fetal cells into the maternal circulation (Holzgreve et al 2001). The vast increase in the number of haplo-identical fetal cells could lead to a maternal inflammatory immune response, an immunological feature which has been proposed to be associated with pre-eclampsia (Redman et al 1999). In this way the surplus of allogenic material from the fetus can cause immunological problems in the mother and widespread endothelial damage leading to the symptoms of pre-eclampsia. The interesting question now however is whether the increased number of fetal cells in the maternal circulation is predictive early in pregnancy for later development of pre-eclampsia.

One of the most urgent tasks will be to find out whether the detection of fetal DNA in women in early pregnancy could be predictive of pre-eclampsia in asymptomatic women. Such an early screening test would identify women at risk before the disease sets in. Whether we are now closer to this long awaited goal needs to be shown by appropriate screening studies for the prediction of pre-eclampsia, raising hope that in the near future we will be better able to deal with this dangerous pregnancy related disease.

2.7 MATERNAL FETAL CELL TRAFFICKING OF THE Rh "D" GENE

The discovery of fetal DNA in the maternal circulation and the demonstration of the relative ease and reliability with which it can be detected have opened up new possibilities for non-invasive prenatal diagnosis. The detection of fetal derived Y chromosomal sequences in maternal plasma has potential application for the prenatal diagnosis of sex-linked disorders. Outside of the Y chromosome, the first genetic locus to which investigators have turned their attention has been the RhD gene, the presence of which produces the RhD positive phenotype.

The Rh blood group system is one of the most polymorphic and immunogenic systems known in humans. The D antigen is highly immunogenic and induces an immune response in 80% of RhD negative people when transfused with 200mls of RhD positive blood. The Rh blood group system is involved in haemolytic disease of the newborn, transfusion reactions and autoimmune haemolytic anaemia. Despite the widespread use of Rh immune globulin prophylaxis in RhD negative pregnant women, Rh isoimmunisation still occurs.

The RhD antigen is highly immunogenic and is responsible for the majority of cases of Rh alloimmunisation. Approximately 55% of the RhD positive white population are heterozygous for the presence of the RhD gene. The fetus of a RhD negative woman with a RhD positive heterozygous partner has a 50% chance of being RhD negative and a 50% chance of being heterozygous D positive. In the latter situation, if the mother has been sensitised from a previous RhD positive pregnancy, the fetus may be affected with severe haemolytic disease which can lead to fetal hydrops and intrauterine death. Therefore prenatal determination of fetal RhD status early in pregnancy is important in the management of pregnancies involving sensitised RhD negative women.

The Rh blood group has been mapped to chromosome 1 (1p34-36) and contains two homologous genes (Avent & Reid 2000). One gene encodes for the D antigen, which is present only on the erythrocyte membrane of Rh positive individuals. The other gene encodes for the C/c and E/e polypeptides present on the red blood cells of all individuals. Antisera to 5 Rh antigens (D, C, c, E, e) are available and are used to type individuals serologically. In general, the RhD gene is deleted and the D polypeptide is absent in people serologically typed as RhD negative. To date prenatal testing of fetal RhD status is achieved through amniocentesis, an invasive procedure which carries with it a 0.5-1% risk of fetal loss.

The detection of fetal derived RhD sequence in the plasma or serum of a RhD negative pregnant woman indicates the presence of a RhD positive fetus. This approach has been taken by several groups using different regions of the RhD gene as targets. Most reports have demonstrated that this method is highly reliable especially from the second trimester onwards.

Zhong obtained blood from 22 RhD negative women just prior to having an invasive procedure. DNA was extracted from maternal plasma and analysed by multiplex nested PCR. Positive signals of RhD were detected down to 6pg genomic DNA and at the single cell level. The 22 women were between 10 and 21 weeks gestation. The fetal genotype was determined in almost 100% of the cases, there was only one false positive result which on subsequent analysis was negative (Zhong et al 2000).

A detection efficiency of 70% for fetal RhD genotyping was achieved by Bischoff et al who used both conventional and fluorescent PCR on plasma samples. The authors explained their lower detection rate by degradation of DNA in their retrospectively collected and stored serum samples (Bischoff et al 1999).

Faas obtained blood samples from 31 RhD negative women at 16-17 weeks gestation prior to invasive fetal testing. DNA was extracted from plasma and amplified with PCR for RhD exon 7. All 13 fetuses who were RhD negative on plasma PCR were all confirmed negative by genotyping amniotic cell or chorionic villus DNA (Faas et al 1998).

Lo extracted DNA from the maternal plasma of 57 RhD negative women and analysed for the presence of the RhD gene with fluorescence based PCR. Fetal RhD status was determined directly by serologic analysis of cord blood or PCR analysis of amniotic fluid. There were 12 women analysed in the first trimester, 30 in the second trimester and 15 in the third trimester. They found that 18 were RhD negative and 39 RhD positive. Samples were completely

concordant with the results of serologic analysis in those patients analysed in the second and third trimester. Among the maternal plasma samples collected in the first trimester, 2 contained no RhD DNA but the fetuses were RhD positive, the other 10 samples were concordant. Therefore non-invasive fetal RhD genotyping can be performed reliably and rapidly using maternal plasma beginning in the second trimester (Lo et al 1998).

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CHAPTER 3

METHODOLOGY - PART I

3.1 SRY SOLUTION PHASE PCR

Solution phase PCR was performed to generate standards and to check for the insert.

A reaction volume of 25µl was used with the following constituents:

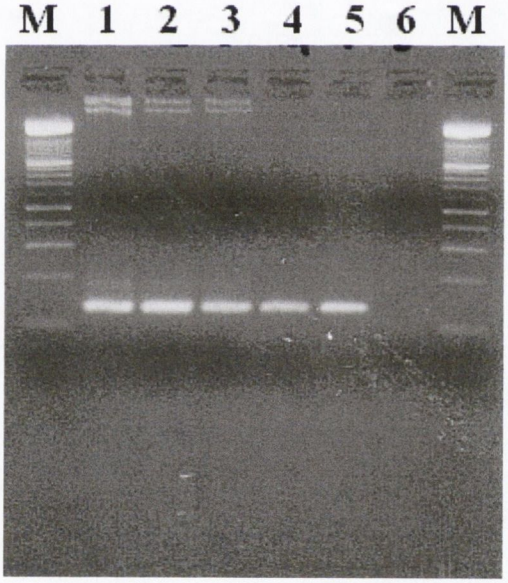
- 0.4mM dNTPs
- 1X PCR Buffer II (Applied Biosystems)
- 3mM Magnesium chloride
- 4µM Forward primer
- 4µM Reverse primer
- 2.5u /100µl Amplitaq Gold (Applied Biosystems)
- 0.1u AmpErase (Applied Biosystems)
- 50ng DNA template

The following cycling conditions were used on the 9700 thermal cycler

- 50^0C for 2mins
- 95^0C for 10mins
- 94^0C for 30secs/ 60^0C for 30secs/ 72^0C for 30secs for 40 cycles
- 72^0C for 10mins
- 4^0C for ∞

10µl of product was run on a 2% agarose gel (2g of agarose in 100ml of 1xTBE buffer) stained with 0.5µg/ml of ethidium bromide (see fig 1)

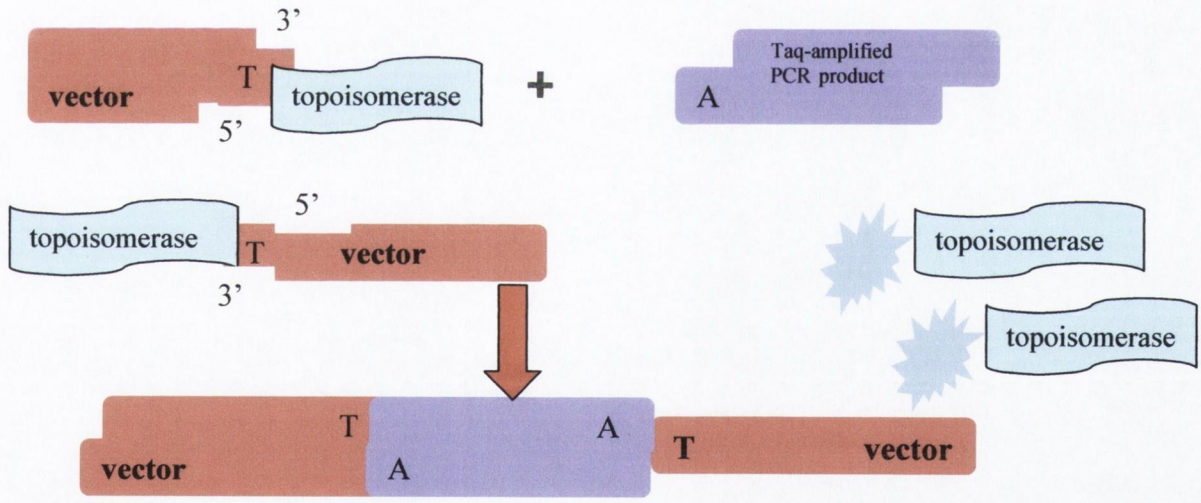
Figure 1: Agarose gel electrophoresis of SRY PCR amplicons generated from SRY cloned plasmid DNA and male DNA: M respresents 100 bp molecular weight marker, Lanes 1-3 represent amplicons generated from cloned SRY plasmids, lanes 4, 5 represent PCR products generated from male DNA, lane 6 is no template control.



3.2 CLONING

TOPOTM TA Cloning provides a highly efficient, fast, one-step cloning strategy for the direct insertion of Taq polymerase-amplified PCR products into a plasmid vector. The plasmid vector (pCR 2.1-TOPOTM) is supplied linearised with single 3' thymidine (T) overhangs for TA cloning, and Topoisomerase covalently bound to the vector ('activated' vector).

Figure 2. TOPO^{TM} TA Cloning Reaction



Taq polymerase has a non-template dependent terminal transferase activity which adds a single deoxyadenosine (A) on to the 3' ends of PCR products. The linearised vector has single, overhanging 3' deoxythymidine (T) residues. This allows the PCR inserts to ligate efficiently with the vector.

TOPOTM Cloning exploits the ligation activity of topoisomerase by providing an 'activated', linearised TA vector. Ligation of the vector with a PCR product containing 3' A overhangs is very efficient and occurs spontaneously within 5 minutes at RToC. The TOPOTM Cloning reaction can be transformed into chemically competent cells.

Cloning and preparation of DNA Plasmids for TaqMan PCR.

Due to the sensitivity of the molecular biological techniques in particular TaqMan PCR and the associated risk of contamination, work areas and practices have been organised to physically separate stages to control for PCR contamination. Each area is equipped with dedicated pipettes, aerosol barrier tips and protective clothing.

For generation of TaqMan standards the Invitrogen TOPO TA Cloning kit and TOP 10 F' One shot™ kit were used.

Producing PCR Products.

PCR products must be fresh. The product needs to have a single 3' Adenine overhang, this is achieved by using a proof reading Taq polymerase to amplify the product. PCR products to be cloned should be a discrete single band, it is possible to gel-purify the product before cloning using the Qiagen QiaQuick Gel Extraction Kit (Cat no. 28704).

Cloning Reaction and Transformation.

1. Before starting cloning reactions it was necessary to prepare the following:
   - A water bath at 42 ºC
   - Selective LB agar plates spread with 40 µl of 40 mg/ml X gal and 40 µl of 100 mM IPTG (depending on cells used).
   - Selective LB agar plates warmed to 37 ºC until ready for use.
   - SOC medium warmed to room temperature.
   - One vial of competent cells per transformation thawed on ice.
2. Fresh PCR product 0.5-4.0 µl

   Salt solution 1 µl (optional depending on cells used)

   Sterile Water to a final vol of 5 µl.

   TOPO Vector 1 µl
3. The reaction was mixed gently and incubated at room temperature for 5 minutes.
4. The reactions were placed on ice.
5. For chemical transformation, 2 µl of the cloning reaction were added into a vial of chemically competent *E. coli* and mixed gently.
6. This was then incubated on ice for 20 minutes.
7. The cells were heat shocked for 30 seconds at 42 ºC without shaking.
8. The tube was placed immediately on ice.
9. 250 µl of room temperature SOC medium were added and tubes incubated shaking at 37ºC for 1 hour.

10. 10 and 50 µl aliquots from each transformation were spread onto pre-warmed selective plates.
11. The plates were incubated overnight at 37 ºC.

Analysis of Positive Clones.

At least 5- 10 white colonies per transformation were analyzed. Potentially positive colonies were analyzed by two different methods.

(A) PCR directly from colonies.

(B) Isolation of plasmid, PCR and /or restriction digestion.

(A) PCR directly from colonies

1. 20 µl PCR reactions containing PCR buffer, dNTP's, primers (M13 Fwd and M13 Rev) taq polymerase and water were prepared.
2. Positive transformants were picked using a sterile toothpick patch onto a separate LB plate for future use and the colony resuspended in the PCR cocktail.
3. The reaction was incubated for 10 minutes at 94 C.
4. Amplification was performed using the following cycling conditions, 94 ° C for 1 min, 55 ºC for 1 min, 72 ° C for 1 min for 30 cycles, followed by a final extension at 72 ° C for 10 minutes.
5. Visualization was performed by gel electrophoresis.
6. Plasmid stocks were prepared from positive clones.

(B) Isolation of plasmid and analysis.

1. 5- 10 white colonies were selected and grown overnight in 10 ml of selective LB.
2. The cultures were spun at 4,000 rpm for 10 mins, and plasmid extracted using QiaAmp Miniprep DNA kit (Qiagen, Cat. No. 27104) as outlined in the manual.
3. The plasmids were analyzed by PCR and sequencing.

Glycerol Stocks.

Once cloning had been successful and colonies of interest selected and analysed, a glycerol stock of the transformed cells was made for long-term storage.

1. The original colony was streaked on selective LB plates.
2. A single colony was isolated and inoculated into 2ml of selective LB broth.
3. Then incubated at 37 ° C overnight.
4. 0.85 ml of culture was mixed with 0.15 ml of sterile glycerol, and transferred to a cryovial.
5. Stored at – 80 ° C.

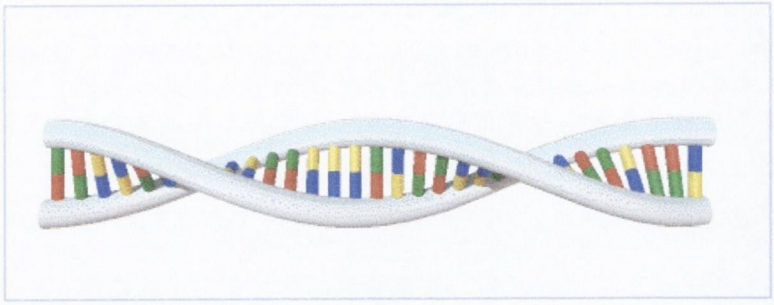
3.3 TAQMAN PCR

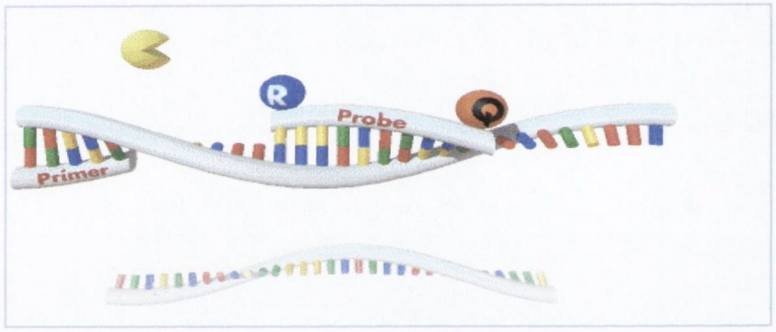
DNA was extracted from 200µl of whole blood using commercially available kits according to the QUIAGEN protocol using the QIAamp Blood Kit (Qiagen Ltd, Crawley, West Sussex, RH10 2AX, UK) following manufacturer's instructions for the "blood and body fluid protocol". All DNA samples were eluted in 200 µl of elution buffer (Tris-HCL). DNA concentrations were measured using a Beckmann UV vis spectrophotometer and absorbances read at A_{260}. Standard dosages of DNA were given to each PCR reaction. Feto-maternal trafficking was assessed using TaqMan primers and probes directed against SRY gene sequences and all reactions were gene dosage corrected by β-actin TaqMan PCR. In addition to feto-maternal trafficking, Rh "D"gene immunization was assessed using the feto-maternal traffic model employing TaqMan primers and probes specific for Rh "D" sequences. Results were expressed as copy number per millilitre of whole maternal blood.

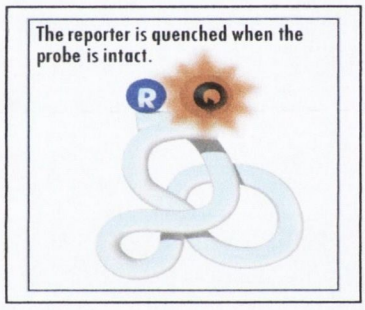
Theory of Operation

TaqMan is a technique which utilises a characteristic of Taq DNA polymerases, namely their Y-shaped structure dependent, polymerase associated, 5'-3' endonucleolytic activity. It allows for the direct detection of PCR product by the specific release of a fluorescent reporter molecule during the PCR reaction. Figure 3 demonstrates this schematically. TaqMan PCR utilises a primer pair and an internal oligonucleotide probe, called a TaqMan probe. Quantitative data is generated based on the PCR during early cycles, when the PCR fidelity is at its highest. Increase in fluorescence is detected by a 7700 DNA sequence detector (PE Biosystems). The 7700 has a linear dynamic range of at least 5 orders of magnitude, obviating the need for serial dilutions.

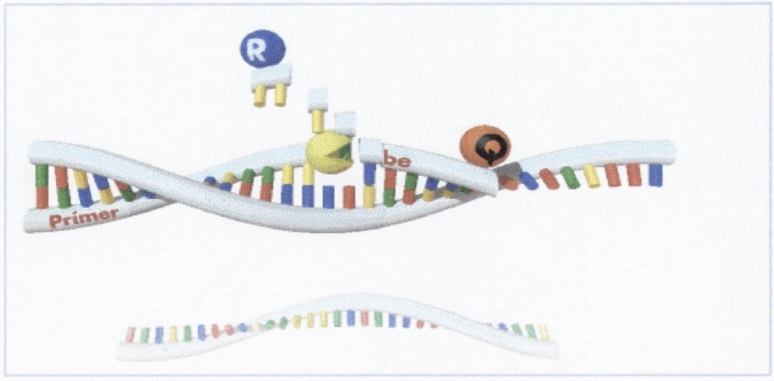
Figure 3. Schematic diagram of TaqMan PCR







Primer and TaqMan probe annealing



TaqMan probe cleavage during the extension phase of PCR

TaqMan Probe Behaviour During PCR

The TaqMan probe comprises an oligonucleotide 20-30 bases in length with a 5' reporter dye, a 3' quencher dye, and a 3' blocking phosphate. The most commonly used fluorescent reporter dye is FAM which is covalently linked to the 5' end of the oligonucleotide probe. Alternative reporter dyes are TET and HEX. Each of these reporters is quenched by TAMRA which is attached by a LAN (linker-arm-modified nucleotide) to the 3' end of the probe. The probe is chemically phosphorylated at its 3' end, which prevents probe extension during PCR cycling. When the probe is intact the proximity of the reporter dye to the quencher dye results in direct suppression of fluorescence from the reporter dye by Förster-type energy transfer.

During PCR, if the target of interest is present, the probe will specifically anneal between the forward and reverse primers (Figure 3). The probe will be cleaved due to the nucleolytic activity of AmpliTaq DNA polymerase if the probe is hybridised to its target. The subsequent release of the fluorescence reporter will only occur if target specific amplification occurs, obviating the need to confirm the amplicon following amplification. Importantly Taq DNA polymerase does not digest free probe. After cleavage, the shortened probe dissociates from the target and strand polymerisation continues. This process occurs every cycle and does not interfere with the exponential accumulation of product. The specificity of the 5' nuclease assay results from the requirement of the Taq DNA polymerase enzyme for sequence complementarity between the probe and the template, in order that specific cleavage of the probe occurs.

Fluorescence Detection on the ABI Prism 7700

During PCR, the multiplexer directs an argon ion laser through fiber optic cables that terminate above each position on the 96 well plate. The light passes through the MicroAmp Optical Caps and, for a default time of 25 milliseconds, the laser excites the fluorescent dyes present in each well. The fiber optic cables then collect the fluorescence emission between 500nm and 660nm from each of the wells, with a complete collection of data from all wells approximately once every 7 seconds. A system of lenses, filters and a dichroic mirror focus the fluorescence emission into a spectrograph. The spectrograph separates the light (based on wavelength) into a predictably spaced pattern across a charge-coupled device (CCD) camera. The Sequence Detection application collects the fluorescent signals from the CCD camera and applies data analysis algorithms.

Figure 4. Graphic representation of fluorescence detection system

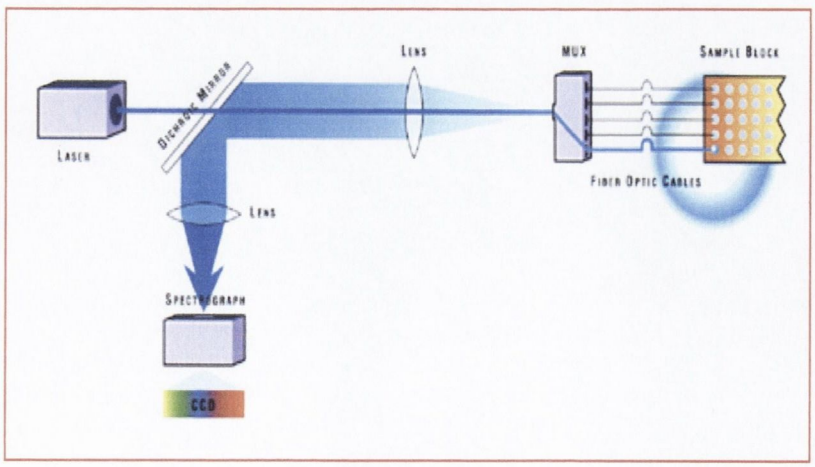
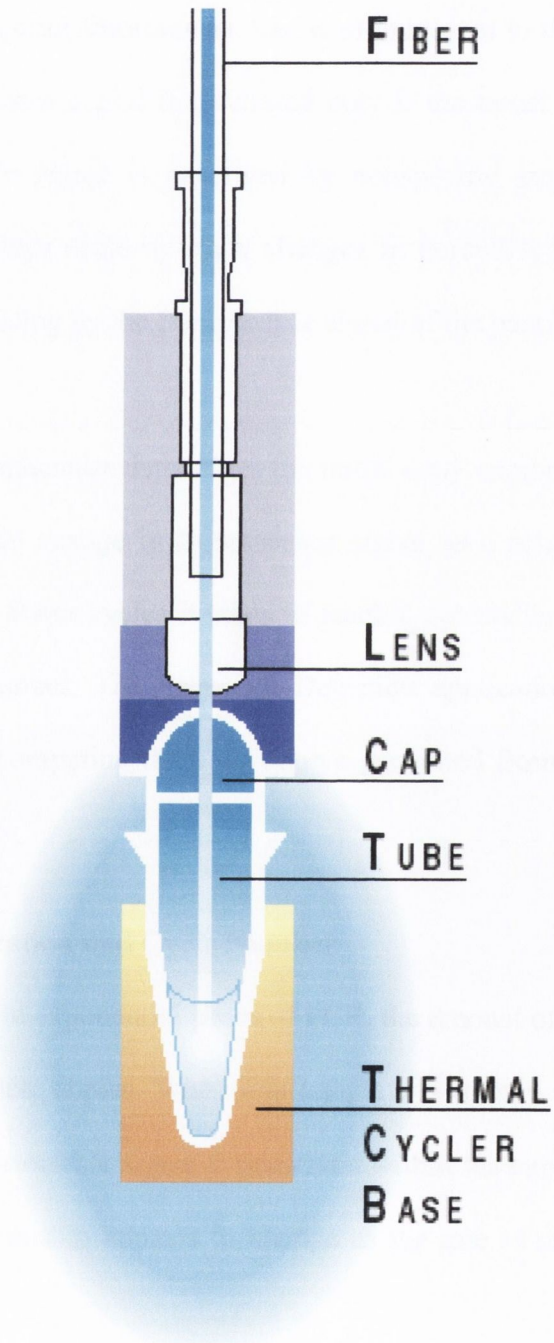


Figure 5. Schematic diagram of laser



Fluorescence Analysis on the Sequence Detection Application

Cleavage of the TaqMan fluorogenic probe between the reporter and quencher dyes during PCR results in an increase of reporter fluorescence that is proportional to the amount of PCR product accumulated. The fluorescence signal is generated only if the target sequence for the probe is amplified by the PCR. No signal is generated by nonspecific amplification. The Sequence Detection application displays cycle-by-cycle changes in normalised reporter signal. Reporter signal is normalised by dividing by the fluorescence signal of the passive reference.

The Sequence Detection application determines the initial copy number of the target template by analysing the cycle-to cycle change in fluorescence signal as a result of the amplification of template during PCR. The fewer cycles it takes to reach a detectable level of fluorescence, the greater the initial copy number. The Sequence Detection application determines initial copy numbers of unknowns by comparing them to a curve generated from samples of known initial copy number.

Initial Template Concentration and Cycle Number

At any given cycle within the exponential phase of PCR, the amount of product is proportional to the initial number of template copies. When one template is diluted several times, the ratio of template concentration to detectable signal is preserved within the exponential and linear phases for all dilutions. This relationship appears to change as the rate of amplification approaches a plateau.

Threshold Cycle (C_T)

The threshold cycle occurs where the Sequence Detection application begins to detect the increase in signal associated with exponential growth of PCR product.

To determine C_T, the Sequence Detection application collects data from the first few PCR cycles and calculates the average R_n and the standard deviation of the R_n of these cycles. In most PCR systems, the first 15 cycles represent the baseline. Next the algorithm multiplies the standard deviation of the background R_n by a default factor of 10 to define a threshold. Finally the algorithm searches the data for a point that exceeds the baseline by the value of the threshold. The cycle at which this point occurs is defined as C_T.

C_T represents a detection threshold for the Sequence Detector. C_T is dependent on the starting template copy number and the efficiency of both the DNA amplification, the PCR system and the cleavage of the TaqMan fluorogenic probe. When the starting concentration of a template remains constant as the concentration of other PCR components varies, the most efficient PCR system has the lowest C_T (Applied Biosystems 1996).

3.4 SRY TAQMAN PCR

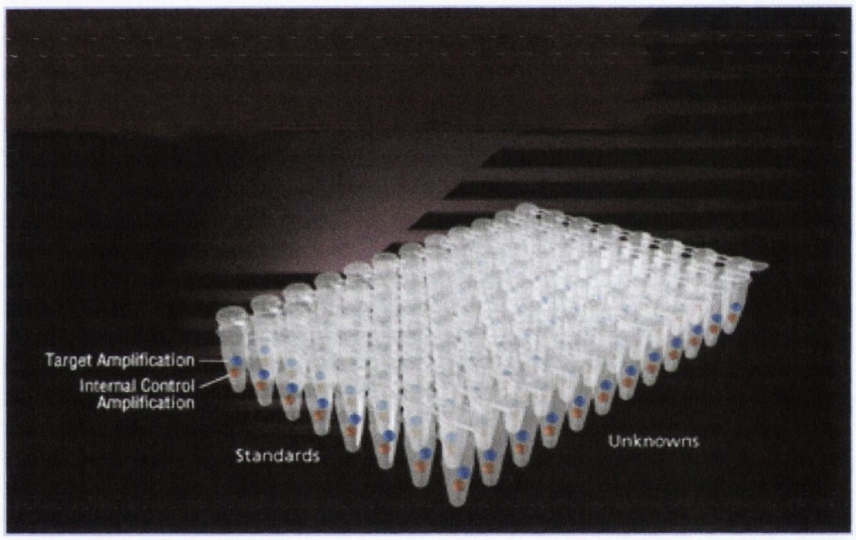
Real time quantitative PCR based on a 5' nuclease assay was performed using the ABI prism 7700 sequence detection system (PE Biosystems, Foster City, CA). The SRY TaqMan system

has been described by Lo (Lo 1998). The primer pair used was SRY-109F, 5' TGG CGA TTA AGT CAA ATT CGC-3' and SRY-254R, 5' CCC CCT AGT ACC CTG ACA ATG TAT T-3' (PE Biosystems, Warrington, Cheshire, UK). The dual labelled fluorescent TaqMan probe was SRY-142T, 5' (FAM) AGC AGT AGA GCA GTC AGG GAG GCA GA (TAMRA) (PE Biosystems, Warrington, Cheshire, UK). A second control PCR system using human endogenous control β-actin primer and probe sequence was used to calculate the total number of DNA copies per millilitre (PE Biosystems, Warrington, Cheshire, UK). The β-actin TaqMan system consisted of the amplification primers β-actin Forward Primer, 5'TCA CCC ACA CTG TGC CCA TCT ACGA, β-actin Reverse Primer, 5' CAG CGG AAC CGC TCA TTG CCA ATG G-3' and a dual labelled fluorescent TaqMan probe β-actin, 5'- (FAM) ATG CCC-X(TAMRA)-CCC CCA TGC CAT CCT GCG Tp-3'.

TaqMan amplification reactions were set up in a reaction volume of 25µl. We used 10µl of the extracted whole blood DNA for amplification for the SRY reactions and 5µl for the β-actin reactions. The exact amount was recorded for subsequent concentration calculations. DNA amplification was carried out in 96 well reaction plates that were frosted to prevent light reflection and closed by the use of caps designed to prevent light scattering. Each sample was analysed in duplicate. A standard curve was run in duplicate with each analysis. Identical thermal profiles were used for both the SRY and β-actin TaqMan systems except for the number of cycles. Thermal cycling was initiated with a 2 minute incubation at 50^0C to allow the uracil N-glycosylase to act followed by a first denaturation step of 10 minutes at 95^0C, then 40 cycles for β-actin and 45 cycles for SRY at 95^0C for 15 seconds and 60^0C for 1 minute. Amplification data

collected by the 7700 sequence detector and stored in the Macintosh computer were then analysed by use of the Sequence Detection system software developed by PE Applied Biosystems. Mean quantities for each duplicate of SRY and β-actin were recorded.

Figure 6. 96 well reaction plate



Strict precautions against PCR contamination were taken. Aerosol resistant pipette tips were used to handle all liquids. Separate areas were used for extraction of DNA, preparation of amplification reactions, addition of the DNA template, amplification and detection. The ABI 7700 system offers an extra level of protection in that its optical detection system does not need to reopen reaction tubes, thus minimising the possibility of carryover detection. The TaqMan assay also includes a preamplification treatment by use of uracil N-glycosylase which destroys uracil containing PCR products.

Figure 7. SRY amplification plot

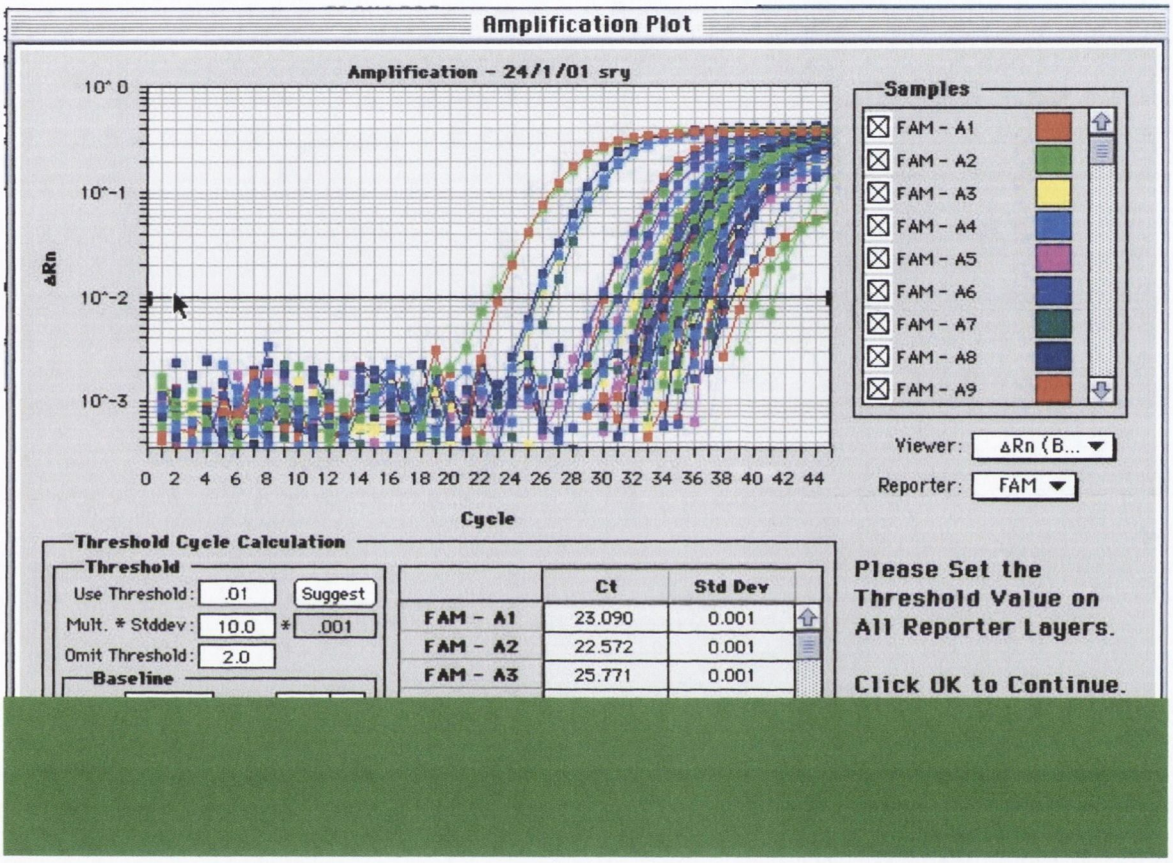
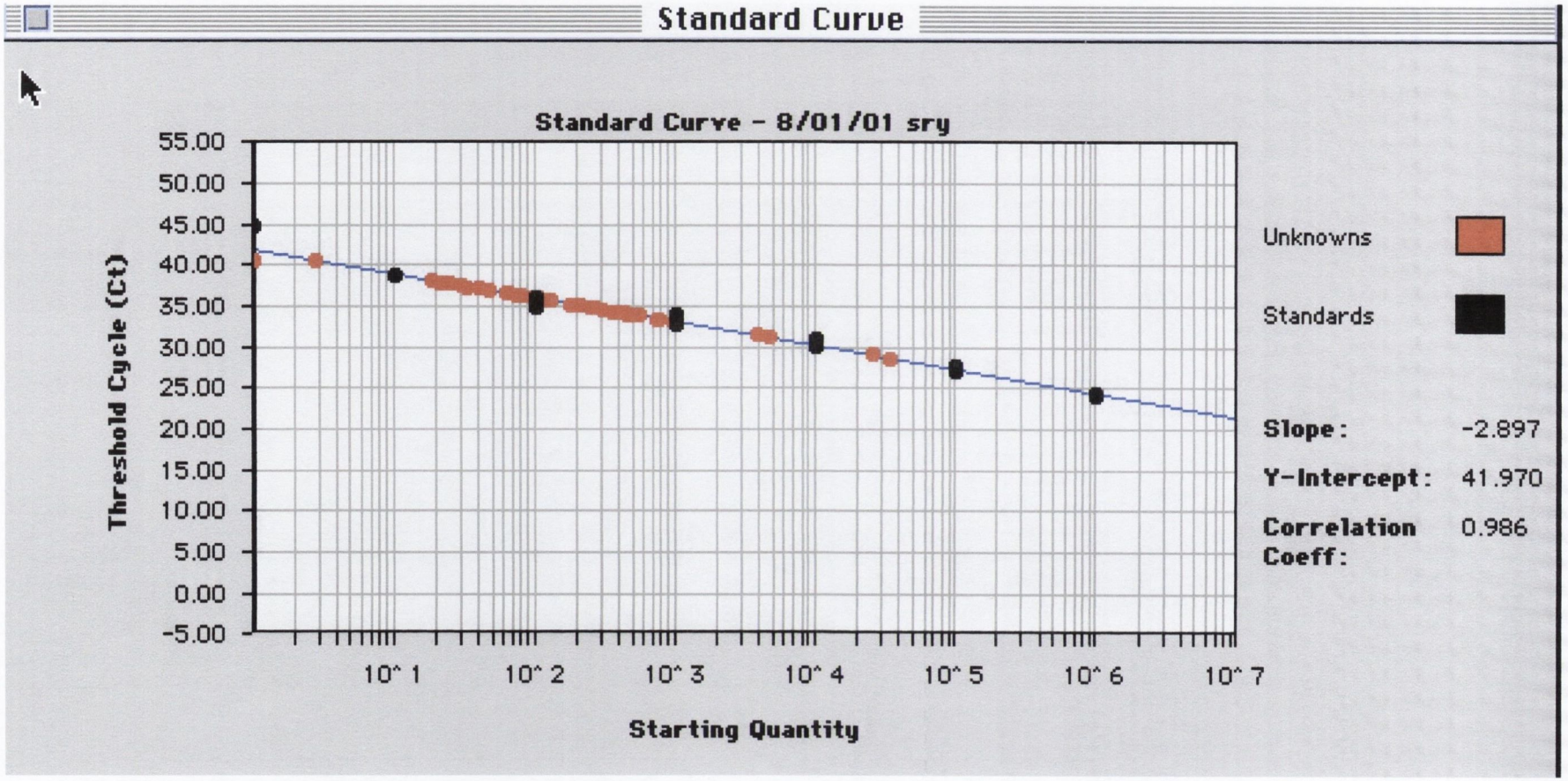


Figure 8. **SRY standard curve**



3.5 EXPERIMENT : OPTIMIZATION OF SRY ASSAY

OBJECTIVE

1. To determine the sensitivity of the SRY assay
2. To determine if amplification of SRY could be inhibited by the presence of female DNA
3. To investigate if false positive results could occur

METHODS

SRY TaqMan PCR

1x2 No Template Control

6x2 Standards (10^6-10^1 copies/µl)

26x2 Unknowns

66 Reactions x 25µl (+2 extra) = Total volume of 1700µl

COMPONENT	VOLUME (µl)
Universal Mastermix 2X	850
Forward Primer 200 nmol	34
Reverse Primer 200 nmol	34
Probe 100 nmol	34
Template (5µl / Rn)	340
Water	410

UNKNOWNS

1. To assess the sensitivity of the TaqMan PCR assay, serial dilutions of male DNA from 10ng/µl to 10Ag/µl were diluted in water and 5µl added per TaqMan PCR reaction to give a final concentration of 50ng to 50Ag.

2. To determine if PCR inhibition affects the sensitivity of the TaqMan PCR assay newborn female DNA was spiked with serially diluted male DNA from 10ng –10Ag/µl and 5µl was added per TaqMan PCR reaction to give a final concentration of 50ng to 50Ag of template DNA. Dilutions were subjected to analysis by the SRY TaqMan system.

3. To determine the specificity of the SRY TaqMan PCR assay female DNA (obtained from a woman at 14 weeks gestation in her first pregnancy who subsequently delivered a female fetus) was spiked with serial dilutions of male DNA as above and 5µl per reaction was added to a final concentration of 50ng to 50Ag. Dilutions were subjected to analysis by the SRY TaqMan system.

4. To detect the possibility of false positive results occurring the last 2 wells contained known female DNA.

Thermal cycling conditions on 7700 Sequence Detector (Applied Biosystems)

50 ˚C for 2 mins

95 ˚C for 10 mins

followed by 40 cycles of 95 ˚C for 15 secs and 60 ˚C for 1 min.

RESULTS

1. The assay was sensitive to the detection of 50pg of male DNA.
2. The assay was sensitive to the detection of 50pg of male DNA, which had been spiked into newborn female DNA.
3. The assay was sensitive to the detection of 5pg of male DNA, which had been spiked into female DNA of a woman in early pregnancy carrying a female fetus.
4. The known female DNA did not amplify.

CONCLUSION

Consistently a limit of detection of 5-50pg of DNA was achieved using the SRY TaqMan PCR assay, which is equivalent to 3.3 copies of male DNA.

The specificity of the PCR assay was confirmed and no false positive results were obtained when female DNA was used as template for the PCR.

Amplification of the SRY gene is not inhibited in the presence of female DNA and in fact the addition of female DNA appears to be acting as a carrier for male DNA.

3.6 RHESUS " D" TAQMAN PCR

DNA Extraction.

DNA was extracted from 200µl samples of whole blood using the QIAamp Blood Kit (Qiagen Ltd, Crawley, West Sussex, RH10 2AX, UK) following manufacturer's instructions for the "blood and body fluid protocol". All DNA samples were eluted in 200 µl of elution buffer (Tris-HCL)

Real Time Quantitative PCR.

Real time quantitative PCR based on the 5' nuclease assay was performed on an ABI 7700 Sequence detector (Applied Biosystems, Foster City, CA, USA). The RhD TaqMan assay has been described previously (Lo 1998). The amplification primers are as follows: RD1 5' CCT CTC ACT GTT GCC TGC ATT 3' and RD2 5' AGT GCC TGC GCG AAC ATT 3' and the dual labeled TaqMan probe : RD T 5' TAC GTG AGA AAC GCT CAT GAC AGC AAA GTC T 3' labeled at the 5'end with the fluorescent reporter FAM (6 carboxyflourescein) and at the 3' end with the quencher TAMRA (6 carboxytetramethylrhodamine). The primers and probe are targeted towards the 3' untranslated region (exon 10) of the Rhesus D gene (Lo 1998).

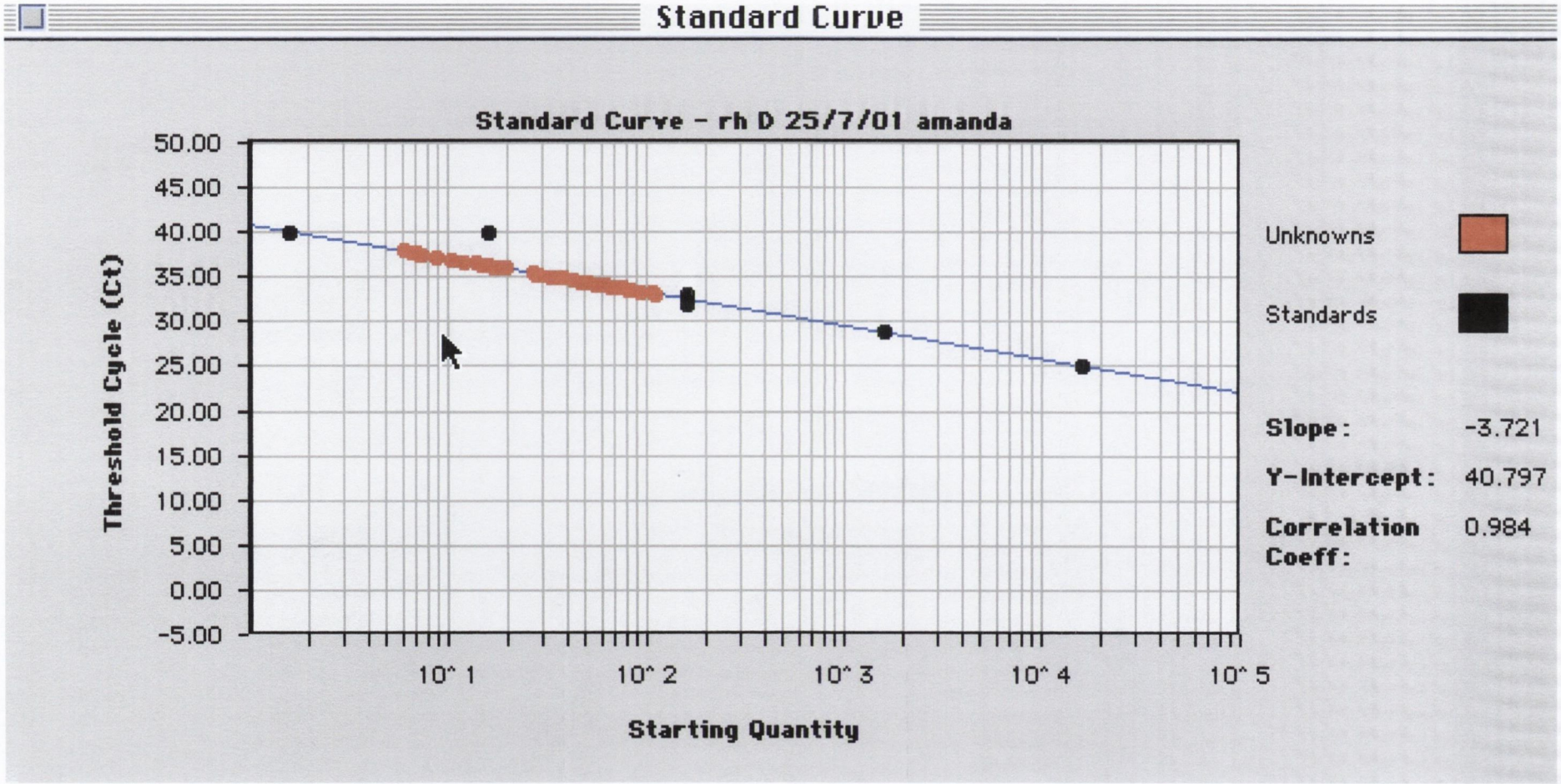
TaqMan PCR reactions were performed using the Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA). Reactions were performed in duplicate in a final volume of 25µl using 1x Universal mastermix, 200 nmol of each primer and 100 nmol of TaqMan probe with 5 µl of template DNA. The thermal cycling conditions on the 7700 were as follows 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 1 min.

Gene dosage correction was carried out using β-actin as an internal control. Quantitative TaqMan PCR was performed by generating standard curves for the Rhesus D amplicon using serial dilutions of Rhesus positive DNA from 50 ng-5 pg of DNA.

Anticontamination Measures

Strict precautions against contamination of the PCR assay were used. PCR mastermixes were prepared in a class 2 laminar flow using dedicated pipettors and aerosol resistant pipette tips. Template DNA was prepared and added to the mastermix in a separate dedicated area. No template controls were included on all PCR plates. Uracil N Glycosylase, which destroys uracil containing PCR products, prior to amplification is included in the Universal Mastermix.

Figure 9 Rh "D" standard curve



3.7 REFERENCES

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- Lo YMD, Hjelm NM, Fidler C, Sargent IL, Murphy MF, Chamberlain PF et al. Prenatal diagnosis of fetal RhD status by molecular analysis of maternal plasma. *N Engl J Med* 1998; 339: 1734-8.

- PE Applied Biosystems. Theory of operation. April 1996; 7.

CHAPTER 4.

INCREASED FETAL DNA IN THE MATERNAL CIRCULATION IN EARLY PREGNANCY IS ASSOCIATED WITH AN INCREASED RISK OF PRE-ECLAMPSIA

4.1 INTRODUCTION

Pre-eclampsia, an enigmatic condition specific to pregnancy, is one of the leading causes of maternal and perinatal morbidity and mortality. There is no test which reliably indicates who will develop this polymorphic disease (Dekker & Sibai 1991). Treatment is restricted to symptomatic management, and expedited delivery is the only way to resolve the disease. This may result in preterm delivery of a compromised growth restricted fetus leading to expensive prolonged treatment in the neonatal intensive care unit. Central to the management of any disorder is an understanding of the pathophysiology and the pathophysiology of pre-eclampsia remains poorly understood.

Endothelial cell dysfunction is a central feature of pre-eclampsia (Ness & Roberts 1996) and defective placentation appears to be a key factor (Roberts & Redman 1993). Failure of trophoblastic invasion of the spiral arteries leads to placental ischemia resulting in damage to the maternal vascular endothelium (Roberts & Redman 1993). The normal process of trophoblastic invasion is complete by 20 weeks gestation so that the initiating placental pathology which leads to pre-eclampsia most likely occurs long before development of the clinical syndrome. The goal is therefore to find a test to predict in early pregnancy who is at risk of developing pre-eclampsia.

Maternal fetal cell trafficking is significantly disturbed in pregnancies complicated by pre-eclampsia with elevated numbers of fetal cells detected in the maternal circulation during these pregnancies (Holzgreve et al 1998). Increased free fetal DNA in the plasma and serum of pregnant women with pre-eclampsia has also been reported (Lo et al 1999). Zhong et al demonstrated not only that maternal and fetal free DNA were elevated in pregnancies complicated by pre-eclampsia but also found that increments in free DNA levels corresponded to the degree of disease severity (Zhong et al 2001). The demonstration of increased fetal erythroblasts in the circulation of women at 22-24 weeks gestation who subsequently developed pre-eclampsia (Al-Mufti et al 2000) led investigators to wonder if fetal DNA could be present in the maternal circulation in early pregnancy. In a small study of women sampled at 17.2 weeks gestation who subsequently developed pre-eclampsia, a significantly higher concentration of fetal DNA was detected prior to the onset of pre-eclampsia (Leung et al 2001). In another small study of women sampled later in gestation, fetal DNA in plasma increased with severity of the hypertensive disorder (Swinkels et al 2002). With this evidence, our objective was to investigate if the presence of fetal DNA in the maternal circulation in early pregnancy might be a marker for the prediction of pre-eclampsia.

4.2 METHODS

From March 1986 until March 1990 at the Coombe Women's Hospital in Dublin, women attending for routine antenatal care consented to provide a blood sample at the first antenatal visit. Blood was collected into K EDTA and stored at 4^0C overnight.These samples were processed the following day. An aliquot of 2mls of mixed whole blood was transferred to one polypropylene tube, the remaining blood was centrifuged and plasma transferred to another tube. Haemolysed samples were discarded. The tubes were stored at -20^0C and not thawed for any reason in the period of time between processing and analysis. All freezers were alarmed for electrical cut off or an increase in temperature above -15^0C. These blood samples have been used for other studies and sample processing is described in detail elsewhere (Kirke et al 1993, Daly et al 1995).

Women who developed pre-eclampsia were identified from a computer database operational since January 1986. Individual chart reviews were then performed to confirm the diagnosis of pre-eclampsia. The criteria used for the diagnosis of pre-eclampsia were in accordance with the guidelines of the American College of Obstetricians and Gynecologists (ACOG). Severe pre-eclampsia was defined as a systolic BP≥160-180mmHg or a diastolic≥110mmHg while proteinuria was defined as ≥5g protein in a 24 hour urine collection or +3/+4 proteinuria on dipstick urinalysis. Non-severe pre-eclampsia was diagnosed based on a systolic BP≥140mmHg and a diastolic≥ 90mmHg while proteinuria was defined as ≥300mg in a 24 hour urine collection or +1/+2 proteinuria on dipstick urinalysis.

The date of the first antenatal visit for these subjects was used to search for an early pregnancy blood sample in the bank of stored bloods. Two controls were selected for each case and matched for parity and gestational age. The women in the control group had uncomplicated pregnancies and remained normotensive and without proteinuria throughout pregnancy. Both groups carried a single male fetus. There were no aneuploid fetuses in either group. Once the cases and controls were identified, the blood samples were relabelled to ensure the investigators were blinded.

DNA was extracted from 200µl samples of whole blood using the QIAamp Blood Kit (Qiagen Ltd, Crawley, West Sussex, RH10 2AX, UK) following manufacturer's instructions for the "blood and body fluid protocol". All DNA samples were eluted in 200µl of elution buffer (Tris-HCL). DNA concentrations were measured using a Beckmann UV spectrophotometer and absorbances at A_{260} read. Real time quantitative PCR based on a 5' nuclease assay was performed using the ABI prism 7700 sequence detection system (PE Biosystems, Foster City, CA). The SRY TaqMan system has previously been described by Lo (Lo et al 1999). To test the quality of the DNA extracted and to quantify the total volume of circulating DNA, the ß-actin housekeeping gene was amplified by TaqMan PCR in all samples. TaqMan amplification reactions were performed in duplicate with 10µl of template DNA and a standard curve was run in duplicate with each analysis. Thermal cycling conditions and the use of uracil N-glycosylase were as previously described (Lo et al 1999). Strict precautions against PCR contamination were taken. Amplification data was collected by the 7700 sequence detector and analysed using the Sequence Detection System software V 1.6.3 (Applied Biosystems, Foster City, CA, USA). Mean quantities for each duplicate were recorded.

Optimal PCR efficiency was considered to be achieved when the slope of the curve (-1/ log (1+E)) was ≥97%. Results were expressed as copy number per millilitre of whole blood and values were log transformed before analysis. Data analysis was performed using Stata version 7.0 with additional graphics from Datadesk version 6.1. Statistical significance was accepted for $p>0.05$.

4.3 RESULTS

There were whole blood samples available for 88 cases of pre-eclampsia and 176 controls, both sampled at a mean gestation (±SD) of 15.7±3.6 weeks. Among the 88 cases, fifty-three were primigravidas, 35 multigravidas and 20 fitted the criteria for severe pre-eclampsia. Controls were matched for parity and gestational age (Table I). Any detectable fetal DNA in the maternal circulation was associated with an eight-fold risk of developing pre-eclampsia (OR 8.3, 95%CI 2.9-23.4). Correcting for parity left the odds ratio unchanged. The median fetal DNA concentrations in pre-eclamptic and control pregnancies were 280.0 copies/ml (range 0-180,000) and 28.0 copies/ml (range 0-6,300) respectively (Figure 1). Fetal DNA was not detected in 5 cases nor in 57 controls, however positive amplification signals from the ß-actin gene were detected in all samples thus confirming the quality of the samples (Table II). There was no significant difference in copy number of ß-actin between cases and controls.

When quantifying fetal DNA by SRY copies/ml and dividing copies/ml into quartiles (Table III), an increase per quartile was associated with a 3 fold increase in risk (OR 3.1, 95%CI 2.1-4.5).

Table III also demonstrates the risk in each category relative to a baseline category. The area under the ROC curve was 0.77 (SE = 0.29, 95%CI 0.71-0.83). Sensitivities, specificities and likelihood ratios are illustrated in Table IV.

Table I. Patient details

	Cases (n = 88)	Controls (n = 176)	Statistical Significance
Maternal age (years)	27.0 ± 5.7	25.7 ± 5.9	NS
Parity			
0	53	106	
≥ 1	35	70	
Gestational age at first visit (wks)	15.7 ± 3.6	15.7 ± 3.6	NS
Gestational age at delivery (wks)	39.2 ± 2.2	40.0 ± 2.0	NS
Birth weight (grams)	3335 ± 639	3465 ± 567	p = 0.004

(Data are mean ± SD)

Figure 1. **Boxplot of copies/ml (log10 scale) by outcome demonstrating significantly higher copies/ml in patients who subsequently developed pre-eclampsia (PET). Low whiskers indicate the 10th percentile, high whiskers the 90th percentile, bottom of boxes indicates the 25th percentile, top of boxes indicate the 75th percentile, bars within the boxes indicates the medians, asterisks indicate outliers.**

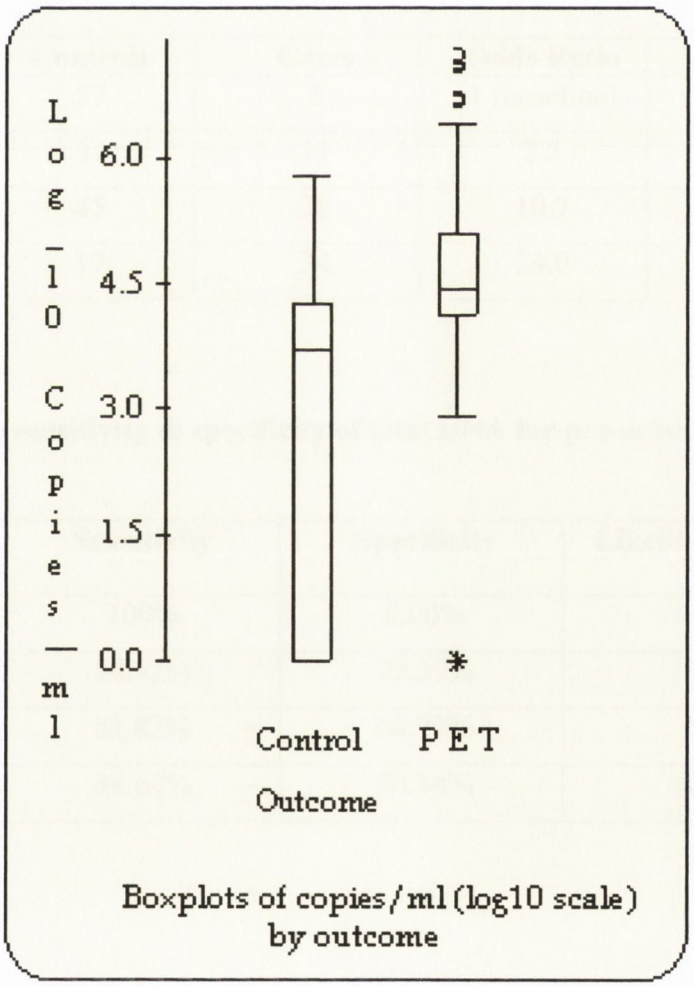


Table II. Any SRY copies / ml versus none detected

Any SRY Copies	Control	Pre-eclampsia
Negative	57	5
Positive	119	83
Total	176	88

Table III. The risk per quartile relative to the baseline.

SRY Copies/ml	Controls	Cases	Odds Ratio	95% CI
0	57	5	1 (baseline)	
<10,000	57	11	2.3	0.7-7.8
<50,000	45	38	10.7	3.4-33.9
>50,000	17	34	24.0	6.9-83.3

Table IV. Predictive sensitivity & specificity of fetal DNA for pre-eclampsia

SRY copies/ml	Sensitivity	Specificity	Likelihood Ratio
0	100%	0.00%	1.00
<10,000	94.32%	32.39%	1.39
<50,000	81.82%	64.77%	2.32
>50,000	38.64%	90.34%	4.00

4.4 DISCUSSION

Increased fetal DNA can be detected in early pregnancy in women who subsequently develop pre-eclampsia and it's presence is associated with an eight fold increased risk of developing pre-eclampsia. In addition there appears to be a graded response between the quantity of fetal DNA and the risk of developing pre-eclampsia. While the finding of fetal DNA in the maternal circulation is not new, we have now demonstrated that the effect is dose related and importantly that this fetal contamination of the maternal circulation occurs prior to pre-eclampsia becoming clinically evident. We believe that this is the first report of detectable fetal DNA in a large sample size of women at the early gestational age of 15.7± 3.6 weeks who subsequently developed pre-eclampsia. Although triple screening is not routinely performed in Ireland and so there are no results with which to compare, this would be the sample which could be used if fetal DNA detection for pre-eclampsia became a routine screening test.

Recent studies have focussed on the detection of free as well as cellular fetal DNA in maternal plasma (Pertl & Bianchi 2001). It has also been reported that significantly more fetal DNA is present in the plasma of pregnant women compared with fetal DNA extracted from the cellular fraction of maternal blood (Lo 2000). Bianchi has suggested that maternal whole blood and plasma can both be used as sources of cellular and free fetal DNA. If maternal blood is used to prepare cellular DNA, assays may detect all fetal cell types as well as previous transfusions and potentially cells from previous pregnancies (Bianchi et al 1997). Plasma fetal DNA in contrast is limited to the current pregnancy and there is no evidence that it persists after delivery (Bianchi

1999). In this study we investigated the total quantity of cellular and free DNA in maternal whole blood without attempting to isolate and enrich circulating fetal cells for the purposes of extracting cellular DNA. Since maternal whole blood was used, this assay detected DNA derived from all fetal nucleated cells in the sample including trophoblasts, erythroblasts, lymphocytes and granulocytes. The use of plasma would have been preferable but these were stored bloods and the plasma fraction had been used in a previous study. One of the limitations of using whole blood rather than plasma is that the technique may be more susceptible to false positive results caused by the persistence of fetal cells from a previous pregnancy (Bianchi 1999). We tested this and found no false positive results in our experiment. In view of this possibility of persistent fetal cells from a previous pregnancy, we controlled for parity but the odds ratio did not change.

A possible confounder in any analysis of pre-eclampsia is ethnicity but our population was almost 100% Irish and genetically very homogenous. Cases and controls were matched both for parity and gestational age. Although pre-eclampsia is considered a disease of first pregnancies, the odds ratio did not change when corrected for parity. Since the ultimate treatment of pre-eclampsia is delivery, it is not surprising that the patients who developed the disease were delivered earlier. Although gestational age at delivery was not significantly different, there was a significant difference in birthweight between cases and controls indicating the effect of pre-eclampsia on fetal growth.

Increased liberation of fetal DNA into the maternal circulation appears to be the cause of the increased fetal DNA concentrations in women who subsequently develop pre-eclampsia. This could be due to increased entry of fetal cells such as trophoblasts and erythroblasts into the

maternal circulation. Although the aetiology of pre-eclampsia is unknown, endothelial cell dysfunction and inflammation are important aspects of the overall puzzle (Ness & Roberts 1996, Roberts & Redman 1993). The hypothesis that endothelial dysfunction is a central feature of pre-eclampsia can be extended in two ways. Firstly some women are more sensitive to or have pre-existing endothelial dysfunction (Ness & Roberts 1996) and secondly endothelial activation is intrinsic to the inflammatory response (Sacks et al 1998). Endothelial dysfunction is one aspect of a generalised systemic maternal inflammatory response also affecting circulating leukocytes. This inflammatory response is detected in normal pregnancy and is not intrinsically different from that found in pre-eclampsia except that it is milder. Therefore pre-eclampsia may be an extreme end of a range of maternal systemic inflammatory responses engendered by pregnancy itself (Redman et al 1999). Any factor that increases the maternal systemic inflammatory response to pregnancy could predispose to the development of pre-eclampsia.

The normal processes of repair and renewal of syncytiotrophoblast which depend on apoptosis release a continuous stream of placental debris into the maternal circulation (Redman & Sargent 2000). This provokes a systemic inflammatory response in all women which is exaggerated if the burden of the debris is abnormally high or if the woman responds excessively to the process. Apoptosis rates are significantly increased in the syncytiotrophoblast in pre-eclampsia (Leung et al 1999) and fetal DNA in the maternal circulation could be a marker of syncytial debris. An increased influx of fetal DNA into the maternal circulation could trigger an excessive maternal inflammatory response as a result of the vast increase in the number of haplo-identical fetal cells. The interesting question to date has been whether the increased number of fetal cells in the maternal circulation is predictive early in pregnancy for later development of pre-eclampsia.

In this study we have demonstrated an increased quantity of fetal DNA in the maternal circulation in early pregnancy long before the clinical development of the disease. Any detectable fetal DNA in the maternal circulation in early pregnancy is associated with an eight fold increased risk of developing pre-eclampsia. In addition there is a dose response relationship between the copy number and development of disease. The majority (65%) of controls had less than 10,000 copies/ml in the maternal circulation. Although this is not a litmus paper test for the prediction of pre-eclampsia, levels between 10,000 and 50,000 copies of fetal DNA are associated with a likelihood ratio of 2.32 and a sensitivity of 82% and a specificity of 65%. An obvious limitation of the study was our ability to only investigate those women carrying a male fetus. The potential application of fetal DNA quantification in the future requires identification of fetal genes other than the Y chromosome.

The results of this study not only provide a window into the pathophysiology of pre-eclampsia but also suggest that quantifying fetal DNA in the maternal circulation in early pregnancy could be a screening test. This would allow possible interventions to be evaluated and may in the future permit disease modification strategies which could finally reduce the burden of this age old problem.

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CHAPTER 5.

INCREASED MATERNAL FETAL CELL TRAFFICKING AND Rh "D" GENE IMMUNIZATION IN PRE-ECLAMPSIA

5.1 INTRODUCTION

Although the exact aetiology of pre-eclampsia remains unknown, endothelial cell dysfunction and inflammation are certainly important aspects of the overall puzzle (Roberts & Redman 1993). There is evidence that normal pregnancy is characterised by a maternal inflammatory response involving intravascular leucocytes as well as the clotting and complement cascades. It has been proposed that pre-eclampsia is the consequence of an excessive maternal inflammatory response to pregnancy (Redman et al 1999). A trigger for this inflammatory response could be an antigenic substance produced by the placenta, the fetus or both.

There has been a large pregnancy experience of the antigenic RhD gene for many years (Landsteiner & Weiner 1940). The RhD gene is highly immunogenic and could be capable of activating such an inflammatory response. If impaired trophoblastic invasion is the primary insult in pre-eclampsia followed by an increased influx of fetal cells into the maternal circulation (Holzgreve & Hahn 2001), a vast increase in fetal RhD cells could provoke a maternal inflammatory response. The Rh blood group system is one of the most polymorphic and immunogenic systems known in humans resulting in haemolytic disease of the newborn, autoimmune haemolytic anaemia and transfusion reactions. The fetal "D" gene can be detected

in the maternal circulation as early as the second trimester (Zhong et al 2000, Lo et al 1998, Faas et al 1998, Bischoff et al 1999).

The objective of this study was to investigate if there is increased maternal fetal cell trafficking of the RhD gene in early pregnancy prior to the development of pre-eclampsia. The "D" gene could be capable of triggering an excessive inflammatory response resulting in the development of pre-eclampsia.

5.2 METHODS

From March 1986 until March 1990 at the Coombe Women's Hospital in Dublin, women attending for their first visit for routine antenatal care consented to provide a blood sample. The blood specimens were centrifuged, separated and stored at –20°C on the day of sample collection in chronological order. These blood samples have been used for other studies and sample processing is described in detail elsewhere (Kirke et al 1993, Daly et al 1995).

Women who developed pre-eclampsia were identified from a computer database in operation since January 1986. Individual chart reviews were performed to confirm the diagnosis of pre-eclampsia using the guidelines of the American College of Obstetricians and Gynecologists (ACOG). Severe pre-eclampsia was defined as a systolic BP≥160-180mmHg or a diastolic BP≥110mmHg while proteinuria was defined as ≥5g protein in a 24 hour urine collection or +3/+4 proteinuria on dipstick urinalysis. Mild–moderate pre-eclampsia was diagnosed based on a systolic BP≥140mmHg or a diastolic BP≥90mmHg while proteinuria was defined as ≥300mg protein in a 24 hour urine collection or +1/+2 proteinuria on dipstick urinalysis.

We identified both RhD negative women who subsequently developed pre-eclampsia as well as 23 RhD negative controls, all of whom had a whole blood sample available from the bank of early pregnancy samples. All pregnancies were singleton pregnancies and one control was selected for each case. The controls were matched for parity and gestational age. The women in the control group had uncomplicated pregnancies and remained normotensive and without

proteinuria throughout. Once the cases and controls were identified, the blood samples were relabelled to ensure the investigators were blinded.

DNA was extracted from 200µl samples of whole blood using the QIAamp Blood Kit (Qiagen Ltd, Crawley, West Sussex, RH10 2AX, UK) following manufacturer's instructions for the "blood and body fluid protocol". All DNA samples were eluted in 200 µl of elution buffer (Tris-HCL). DNA concentrations were measured using a Beckmann UV spectrophotometer and absorbances at A_{260} read. Real time quantitative PCR based on the 5' nuclease assay was performed on an ABI 7700 Sequence detector (Applied Biosystems, Foster City, CA, USA). The RhD TaqMan assay has been described previously (Lo et al 1998).

To test the quality of the DNA extracted, the β-actin housekeeping gene was amplified by TaqMan PCR in all samples. TaqMan amplification reactions were performed in duplicate with 10µl of template DNA and a standard curve was run in duplicate with each analysis. Quantitative TaqMan PCR was performed by generating standard curves for the Rhesus D amplicon using serial dilutions of Rhesus positive DNA from 50ng-5pg of DNA. Amplification data collected by the 7700 sequence detector and analysed using the Sequence Detection System software V 1.6.3 (Applied Biosystems, Foster City, CA, USA). Mean quantities for each duplicate were recorded. Strict precautions against contamination of the PCR assay were enforced.

Optimal PCR efficiency was considered to be achieved when the slope of the curve (-1/ log (1+E)) was ≥97%. Results were expressed as copy number per millilitre of whole blood and values were log transformed before analysis. Data analysis was performed using Stata version

7.0 with additional graphics from Datadesk version 6.1. Statistical significance was accepted for $p>0.05$.

5.3 RESULTS

Whole blood samples were available for 23 RhD negative women who subsequently developed pre-eclampsia and were matched to 23 RhD negative controls. Seven cases developed severe pre-eclampsia as defined by ACOG criteria. Of the babies born to pre-eclamptic women, 14 were female infants compared to 5 female infants born to the control population. Both groups were sampled at a similar gestational age, 16 patients in the first and 30 in the second trimester (range 9-22 weeks) (Table I). Forty-five infants were typed as RhD positive after delivery. The "D" gene amplified in all 23 cases and in 22 out of 23 controls, the one control with no amplifiable RhD gene detected was sampled at 12 weeks gestation and the infant was typed as RhD negative after delivery. The infants of these mothers were not isosensitized.

As the copy number of RhD gene/ml of whole blood increased, there was a significantly increased risk of developing pre-eclampsia ($p=0.05$) (Fig 1). For a 10-fold increase in RhD copies/ml, there was a fourfold increased risk of developing pre-eclampsia (OR 4.5, 95% CI 1-20). Correcting for parity left the odds ratio unchanged. There was a graded association between copy number of RhD gene in early pregnancy and severity of disease (Table II).

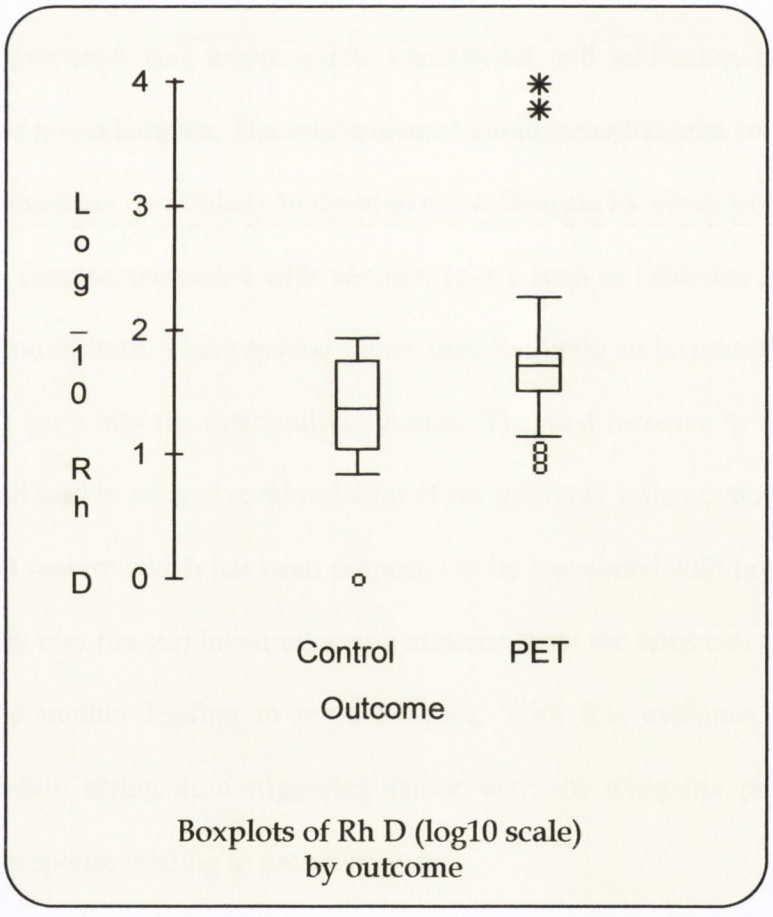
Table I. Patient details

	Cases (n = 23)	Controls (n = 23)	Statistical Significance
Mean maternal age (years ± SD)	28.6 ± 5.5	24.7 ± 5.4	p = 0.02
Parity			
0	13	13	NS
≥ 1	10	10	
Mean gestational age at first visit (wks ± SD)	15.3 ± 3.7	15.0 ± 3.2	NS
Mean gestational age at delivery (wks ± SD)	38.3 ± 3.2	39.9 ± 1.3	p = 0.02
Mean birth weight (grams ± SD)	3014 ± 800	3430 ± 540	p = 0.04

Table II. RhD gene copy number related to severity of pre-eclampsia (PET)

	Rh D copies/ml	**Log scale**
Controls	6,942	3.6
Mild-moderate PET	83,273	4.0
Severe PET	285,793	4.5

Figure 1. **Boxplot of RhD (log10 scale) by outcome demonstrating significantly higher copies/ml in patients who subsequently developed pre-eclampsia (PET). Low whiskers indicate the 10th percentile, high whiskers the 90th percentile, bottom of boxes indicates the 25th percentile, top of boxes indicate the 75th percentile, bars within the boxes indicates the medians, asterisks and circles indicate outliers.**



5.4 DISCUSSION

This case control study demonstrates increased maternal fetal cell trafficking of the RhD gene in early pregnancy among women who subsequently develop pre-eclampsia. These results suggest a possible role for "D" gene isoimmunization in the pathophysiology of pre-eclampsia.

Shallow endovascular cytotrophoblastic invasion in the spiral arteries, an exaggerated inflammatory response and inappropriate endothelial cell activation are key features in the pathogenesis of pre-eclampsia. The mechanisms behind these features are unknown. It is thought that certain women are more likely to develop pre-eclampsia by virtue of a genetic predisposition or an acquired disease associated with vascular injury such as Diabetes Mellitus (Chesley 1978) or a combination of both. This vascular injury may facilitate an increased influx of copies of the antigenic RhD gene into the maternal circulation. The vast increase in the number of antigenic fetal cells could lead to excessive stimulation of the maternal inflammatory immune response, an immunological feature which has been proposed to be associated with pre-eclampsia (Redman et al 1999). In this way the surplus of allogenic material from the fetus can cause an immunological reaction in the mother leading to pre-eclampsia. With this evidence, the RhD gene is one possible candidate acting as a triggering factor with the antigenic potential to stimulate an inflammatory response leading to pre-eclampsia.

Although fetal cells exist in the circulation of pregnant women, they have been reported to be rare with the exception of pre-eclampsia and fetal trisomies in which increased fetomaternal transfusion may be associated with abnormal placental development (Bianchi 2000). Studies demonstrating increased fetal cells in the circulation of women with established pre-eclampsia

have focussed on quantifying copies of the SRY gene in women carrying a male fetus in the third trimester (Holzgreve et al 1998, Lo et al 1999). In an attempt to investigate the events preceding pre-eclampsia, Holzgreve recently reported increased maternal fetal trafficking of fetal erythroblasts in the second trimester in women who subsequently developed the disease (Holzgeve et al 2001). A limitation to date of these studies has been the ability to study only pregnancies carrying a male fetus. This is the first study to demonstrate increased maternal fetal cell trafficking at 15 weeks gestation in women carrying female as well as male fetuses who subsequently developed pre-eclampsia.

A possible limitation of this study is the small sample size and therefore a larger study is needed to confirm our findings, this will be difficult as screening tests for pre-eclampsia are poor and we could only generate samples for 23 cases from 16,000 deliveries over a four year interval. A possible confounder in any analysis of pre-eclampsia is the presence of different ethnic groups. In our study the population was entirely of Irish background and therefore genetically very homogenous. The cases and controls were matched both for parity and gestational age. Although pre-eclampsia is traditionally considered a disease of first pregnancies, we found that the odds ratio did not change when we corrected for parity. Since the ultimate treatment of pre-eclampsia is delivery, it is not surprising that the patients who developed the disease were delivered at an earlier gestational age thus resulting in lower birthweights.

To date the belief has been that maternal fetal cell trafficking is rare in normal pregnancy and occurs only when there is placental pathophysiology. However we found the fetal D gene present in all of our controls with the exception of one fetus which was subsequently typed as RhD

negative after delivery. If we accept that it is usual then we must investigate quantity and our study suggests a dose dependent outcome. The graded association between trafficking of the "D" gene and severity of pre-eclampsia suggests that there may be a critical load of circulating fetal genetic material and any increase above this critical level may result in a triggering of the inflammatory response which leads to pre-eclampsia. The higher the fetal DNA load, the larger the subsequent inflammatory response thereby resulting in more severe disease.

Lo demonstrated that non-invasive fetal RhD genotyping could be performed using maternal plasma in the second trimester (Lo et al 1998). With a 100% successful detection rate we have also confirmed that the RhD gene can easily and reliably be detected as early as the first trimester.

While the underlying aetiology of pre-eclampsia is most likely multifactorial, women who develop this complex disease may have an excessive response to the inflammatory stimuli of normal pregnancy because they have underlying variations which render them susceptible. We suggest that a candidate factor in some women may be "D" gene chimerism.

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CHAPTER 6

FETAL CELLS IN MATERNAL PLASMA

6.1 INTRODUCTION

Although early work was carried out on whole blood, there has been much interest recently in the use of the non cellular portion of blood for molecular analysis. This interest has been stimulated mainly by the finding of tumour derived DNA in the serum and plasma of cancer patients. These findings suggested that circulating free DNA may be found in other clinical settings (Mulcahy et al 1996). There are some similarities between the placenta and a malignant tumour hence just as tumour DNA can be found in the plasma of cancer patients, fetal DNA may be found in maternal plasma.

Lo and colleagues were the first to demonstrate fetal DNA in maternal plasma and serum and to realize that maternal plasma DNA is a chimeric mixture of fetal and maternal DNA using a rapid boiling method to extract DNA from the plasma and serum of 43 pregnant women and conventional "hot start" PCR for detection (Lo et al 1997). Fetal derived Y sequences were detected in 80% of maternal plasma samples and in 70% of serum samples from women bearing male fetuses. These results were obtained using just 10µl of boiled plasma and serum.

Using commercial column-based DNA extraction methods and sensitive real time PCR techniques, this group were able to push the sensitivity close to 100% for the detection of a fetal derived Y chromosome sequence from maternal plasma and serum. In addition to improving the

sensitivity of detection, the real time PCR system can also be used to measure the concentration of fetal DNA in maternal plasma and serum. Lo and associates reported mean fractional DNA concentrations of 3.4% and 6.2% in maternal plasma during early and late pregnancy. The corresponding values for maternal serum were 0.13% and 1% respectively presumably reflecting the liberation of background maternal DNA during the clotting process. Furthermore by serially following pregnant women throughout gestation, they found that the absolute concentration of fetal DNA in maternal serum increases with gestational age with a sharp rise near the end of pregnancy and demonstrated that fetal DNA can be detected in maternal serum as early as seven weeks. This detection rate was obtained with DNA extracted from just 40-80μl of maternal plasma or serum. This volume represents a 4 - 8 fold increase over the 10μl of boiled maternal plasma or serum and results in a significant improvement in sensitivity (Lo 1998).

Bianchi has suggested that maternal whole blood and plasma can both be used as sources of cellular and free fetal DNA. If maternal blood is used to prepare cellular DNA, assays will detect all fetal cell types as well as previous transfusions and potentially cells from previous pregnancies. Plasma fetal DNA in contrast is limited to the current pregnancy and there is no evidence that this free fetal DNA persists after delivery (Bianchi 2000).

Having demonstrated in this thesis that increased fetal DNA trafficking in early pregnancy is associated with an increased risk of pre-eclampsia using whole blood, the objective of this study was to investigate if there was significantly increased maternal fetal cell trafficking occurring in plasma in early pregnancy prior to the onset of pre-eclampsia.

6.2 METHODS

From the bank of early pregnancy samples described previously, we identified 20 women for whom there was both a whole blood sample and a plasma sample available and who subsequently developed pre-eclampsia. We then identified 20 controls who also had whole blood and plasma samples available. One control was selected for each case and matched for parity and gestational age. The women in the control group had uncomplicated pregnancies and remained normotensive and without proteinuria throughout. The study and control groups carried a single male fetus. Once the cases and controls were identified, the blood samples were relabelled to ensure the investigators were blinded.

DNA was extracted from 200µl samples of whole blood and from 400µl of plasma using the QIAamp Blood Kit (Qiagen Ltd, Crawley, West Sussex, RH10 2AX, UK) following manufacturer's instructions for the "blood and body fluid protocol". All DNA samples were eluted in 200µl of elution buffer (Tris-HCL). DNA concentrations were measured using a Beckmann UV spectrophotometer and absorbances at A_{260} read. Real time quantitative PCR based on a 5' nuclease assay was performed using the ABI prism 7700 sequence detection system (PE Biosystems, Foster City, CA). The SRY TaqMan system has previously been described (Lo et al 1998). A second control PCR system using human endogenous control ß-actin primer and probe sequence was used to calculate the total number of DNA copies per millilitre and to confirm the quality of the DNA samples (PE Biosystems, Warrington, Cheshire, UK).

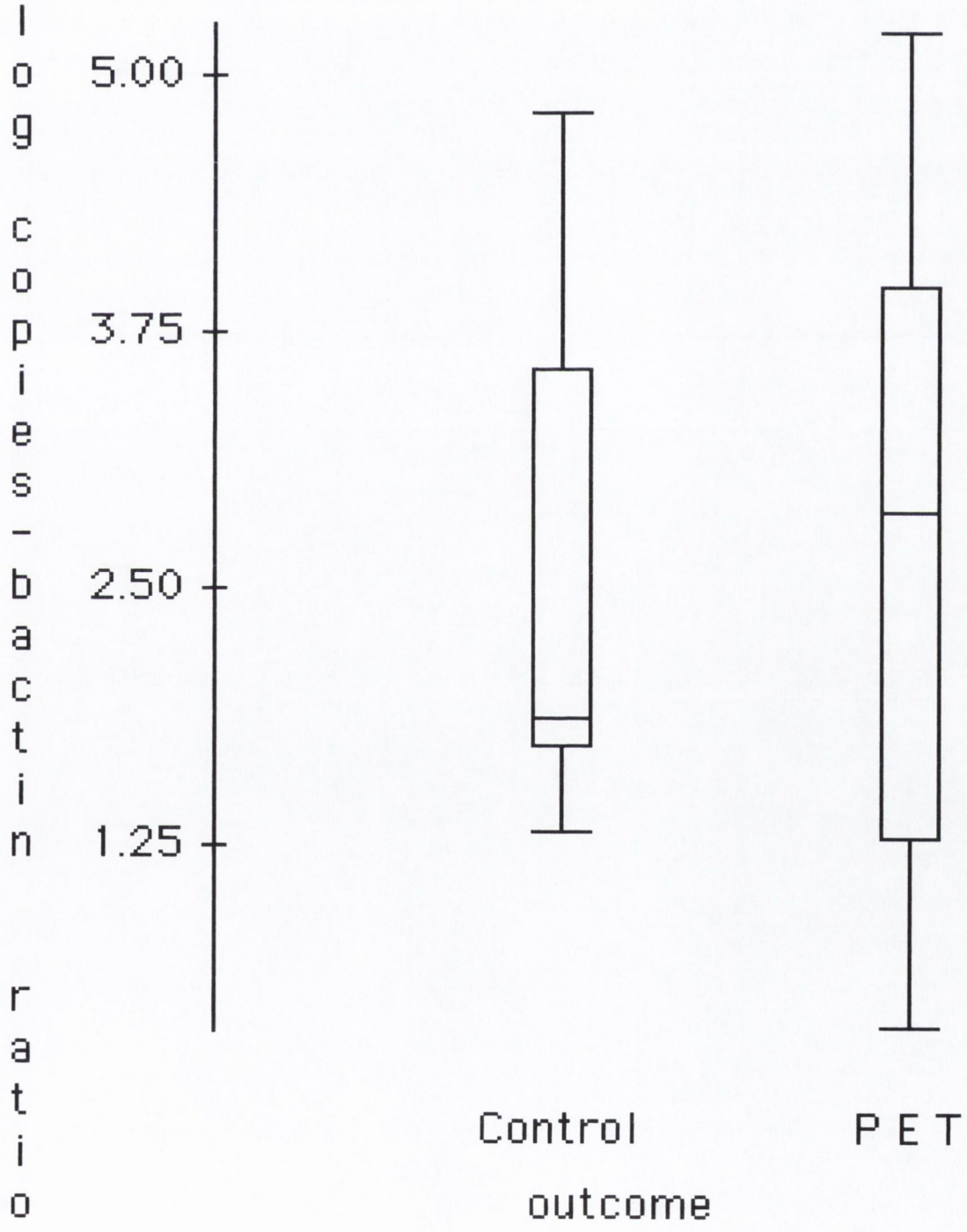
TaqMan amplification reactions were performed in duplicate in a reaction volume of 25µl using 1X Universal MasterMix (Applied Biosystems, Foster City, Ca), 200nmol of each primer and 100nmol of TaqMan probe, with 10µl of template DNA. DNA amplification was carried out in 96 well reaction plates that were frosted to prevent light reflection and closed by the use of caps designed to prevent light scattering. Each sample was analysed in duplicate. A standard curve was run in duplicate with each analysis. Strict precautions against PCR contamination were taken. Thermal cycling was initiated with a two minute incubation at 50^0C to allow the uracil N-glycosylase to act followed by a first denaturation step of 10 minutes at 95^0C, then 45 cycles at 95^0C for 15 seconds and 60^0C for 1 minute. Amplification data collected by the 7700 sequence detector and stored in the Macintosh computer were then analysed by use of the Sequence Detection system software developed by PE Applied Biosystems. Mean quantities for each duplicate were recorded.

Optimal PCR efficiency was considered to be achieved when the slope of the curve (-1/ log (1+E)) was ≥97%. Results were expressed as copy number per millilitre of whole blood and values were log transformed before analysis. Data analysis was performed using Stata version 7.0 with additional graphics from Datadesk version 6.1. Statistical significance was accepted for $p>0.05$.

6.3 RESULTS

There were whole blood and plasma samples available for 20 cases of pre-eclampsia and 20 controls sampled at a mean gestation (±SD) of 14.7±3.8 weeks (NS). Of the 20 cases, 14 fitted the criteria for severe pre-eclampsia. Of the cases, 12 were primigravidas and 8 multigravidas while controls were matched for parity. When quantifying the mean copy number of SRY, there was a mean difference of 1.4 log units, 5.3 versus 3.9 in plasma versus whole blood (p=0.0006). When quantifying the overall DNA content of the samples, there was a mean difference in copy number of ß-actin of 2.4 log units, 5.0 versus 2.6 in whole blood versus plasma (p=0.00001). There was no predictable relationship between the copy numbers of SRY or ß-actin found in whole blood or plasma. This prediction could be improved by adding a constant value but the regression coefficient was not significant. We examined the ratios of SRY to ß-actin measured in plasma. The boxplots in figure 1 show this ratio for both cases and controls, using a $\log_{10}$ scale. There is no evidence of a systematic difference ($t = 0.2$, $p = 0.822$).

Figure 1 **Boxplot of ratios of SRY : ß- actin in cases versus controls. Low whiskers indicate the 10th percentile, high whiskers the 90th percentile, bottom of boxes indicates the 25th percentile, top of boxes indicate the 75th percentile, bars within the boxes indicates the medians.**



6.4 DISCUSSION

This case control study demonstrates that increased maternal fetal cell trafficking was not evident in early pregnancy in women who subsequently develop pre-eclampsia when using plasma samples and there was no relationship between the results obtained for the plasma samples and those obtained for the whole blood samples. We were unable to reproduce the findings of Lo and his coworkers when using plasma samples obtained in early pregnancy. This may be attributed to the small number of samples.

Until recently most investigators considered only the presence of intact fetal cells in maternal blood. In 1997, Lo described a sensitive technique of real time PCR analysis that can detect minute quantities of fetal DNA in maternal plasma. It is difficult to know what correlation exists between the intact fetal cells and the degraded fetal DNA present in maternal plasma and serum. When Lo studied fetal DNA sequentially during pregnancy, he demonstrated that it accumulated in maternal plasma and serum as the pregnancy advanced. The marked increase during the last eight weeks of pregnancy could represent a gradual breakdown of the fetomaternal placental interface (Bianchi 1998). It is therefore possible that this breakdown had not yet occurred in our study group who were sampled early in pregnancy.

However a more likely explanation for our results is the fact that these stored samples had been thawed and frozen repeatedly. Lee and colleagues have recently investigated if fetal DNA could be quantified using archived serum. They found that while archived serum appears to be a useful source of clinical material for retrospective analysis, controlling for duration of sample storage

needs to be considered. They found an inverse correlation approaching statistical significance between the median concentrations of fetal DNA in the serum control samples at 16 weeks gestation and the duration of sample storage in months (Lee et al 2002). This suggests that duration of freezer storage affects the amount of extractable fetal DNA.

Ideally all studies would be carried out using plasma samples but these were not available here. More evidence from additional experiments indicates preferential compartmentalization of fetal DNA in the plasma fraction of maternal blood which appears to be a non random and active process (Crowley & O'Leary, in press). The process bears similarities to the transport of tumour DNA in the circulation of patients with solid tumours.

In order to find an explanation for our findings, the following experiments were conducted:

6.5 EXPERIMENT 1: TO COMPARE THE YIELD OF DNA EXTRACTED FROM WHOLE BLOOD VERSUS PLASMA USING DIFFERENT VOLUMES

Objective

To investigate if the yield of DNA extracted from whole blood was comparable to DNA extracted from plasma using different volumes.

Methods

DNA was extracted from volumes 200µl, 400µl and 600µl of whole blood and plasma with a QIAamp Blood Kit according to the "blood and body fluid protocol" recommended by the manufacturer. An elution volume of 200µl Tris-HCL was used.

Real Time Quantitative PCR.

Real time quantitative PCR based on the 5' nuclease assay was performed on an ABI 7700 Sequence detector (Applied Biosystems, Foster City, CA, USA). The β-actin TaqMan system consisted of the amplification primers β-actin Forward Primer, 5'TCA CCC ACA CTG TGC CCA TCT ACGA, β-actin Reverse Primer, 5' CAG CGG AAC CGC TCA TTG CCA ATG G-3' and a dual labelled fluorescent TaqMan probe β-actin, 5'- (FAM) ATG CCC-X (TAMRA)-CCC CCA TGC CAT CCT GCG Tp-3'.

TaqMan PCR reactions were performed using the Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA). Reactions were performed in duplicate in a final volume of 25µl using 1x Universal mastermix, 200 nmol of each primer and 100 nmol of TaqMan probe

with 5 µl of template DNA. The thermal cycling conditions on the 7700 were as follows 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 1 min.

RESULTS

1. DNA extracted from 200µl of whole blood yielded 2.4 x 10^6 ß-actin copies
2. DNA extracted from 400µl of whole blood yielded 4.6 x 10^6 ß-actin copies
3. DNA extracted from 600µl of whole blood yielded 2.8 x 10^6 ß-actin copies
4. DNA extracted from 200µl of plasma yielded 8.9 x 10^3 ß-actin copies
5. DNA extracted from 400µl of plasma yielded 1.5 x 10^4 ß-actin copies
6. DNA extracted from 600µl of plasma yielded 1.8 x 10^4 ß-actin copies

CONCLUSION

The yield of DNA extracted from whole blood is greater than DNA extracted from plasma. The yield of DNA extracted from whole blood is similar regardless of the initial volume.

6.6 EXPERIMENT 2: TO DETECT PCR INHIBITION IN DNA EXTRACTED FROM PLASMA VERSUS DNA EXTRACTED FROM WHOLE BLOOD

Based on the experiment described above (section 6.3) SRY yield was higher in plasma than in whole blood while ß-actin was lower. To eliminate the possibility of PCR inhibition in whole blood extracts, the following experiments were performed. Dilutions of measles DNA plasmid were spiked into DNA extracted from whole blood and plasma both before and after separation of whole blood and before and after extraction.

Real Time Quantitative PCR.

Real time quantitative PCR based on the 5' nuclease assay was performed on an ABI 7700 Sequence detector (Applied Biosystems, Foster City, CA, USA). The measles amplification primers are as follows: F1 Fwd 5' TGA CTC GTT CCA GCC ATC AA 3', F1 Rev 5' TGG GTC ATT GCA TTA AGT GCA 3' and the dual labeled TaqMan probe : 5' CTG CAC GAG GGT AGA GAT CGC AGA ATA CAG 3' labeled at the 5'end with the fluorescent reporter FAM (6 carboxyflourescein) and at the 3' end with the quencher TAMRA (6 carboxytetramethylrhodamine).

TaqMan PCR reactions were performed using the Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA). Reactions were performed in duplicate in a final volume of 25µl using 1x Universal mastermix, 200 nmol of each primer and 100 nmol of TaqMan probe with 5 µl of template DNA. The thermal cycling conditions on the 7700 were as follows 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 60 °C for 1 min.

The quality of the samples was confirmed using β-actin as an internal control. Quantitative TaqMan PCR was performed by generating standard curves for the measles virus amplicon using serial dilutions of measles virus plasmid diluted in water (from 10^6- 10^1 copies/μl)

6.7 EXPERIMENT 2A: TAQMAN PCR FOR MEASLES VIRUS F GENE IN DNA EXTRACTED FROM WHOLE BLOOD VS PLASMA SPIKED WITH MEASLES VIRUS PLASMID.

METHODS

1 X 2 No Template Controls

6 X 2 Standards – measles DNA plasmid (10^6 – 10^1 copies /μl)

6 X 2 Unknowns

25μl x 26 reactions (+2 extra) = 700μl

Measles Plasmid: Measles virus F gene cloned into PCR 2.1 vector (Invitrogen) (concentration 10^{10} copies / μl)

Reporter: FAM

Unknowns:

1. DNA extracted from plasma + 100,000 copies / μl of measles plasmid.
2. DNA extracted from plasma + 1,000 copies / μl of measles plasmid.
3. DNA extracted from plasma + 100 copies / μl of measles plasmid

4. DNA extracted from whole blood + 100,000 copies / µl of measles plasmid
5. DNA extracted from whole blood + 1,000 copies / µl of measles plasmid.
6. DNA extracted from whole blood + 100 copies / µl of measles plasmid.

RESULTS

UNKNOWN	QUANTITY
1	1.4×10^5 copies
2	3.9×10^3 copies
3	2.2×10^2 copies
4	1.4×10^5 copies
5	4.0×10^3 copies
6	2.5×10^2 copies

CONCLUSION

Measles virus DNA plasmid was amplified at a similar level in both whole blood and plasma thus indicating that there was no inhibition of PCR detected between the whole blood or plasma samples.

6.8 EXPERIMENT 2B: TAQMAN PCR FOR MEASLES VIRUS F GENE IN DNA EXTRACTED FROM WHOLE BLOOD VS PLASMA SPIKED WITH MEASLES VIRUS PLASMID PRIOR TO DNA EXTRACTION.

METHODS

PREPARATION OF SAMPLES

1. 3mls of whole blood was separated into aliquots of 1ml and vortexed.
2. From each of the 1ml aliquots, DNA was extracted from 200µl of whole blood with a QIAamp Blood Kit according to the "blood and body fluid protocol". An elution volume of 200µl was used.
3. Each sample was then spiked with 10µl of the measles plasmid at concentrations of 10^6, 10^3 and 10^2 respectively.
4. 3mls of whole blood was separated into aliquots of 1ml. Each aliquot was spiked with 10µl of the measles plasmid at concentrations of 10^6, 10^3 and 10^2 respectively and vortexed. Each spiked aliquot was then centrifuged at 3000xg and the buffy coat removed and the plasma samples centrifuged at 3000xg. DNA was then extracted from 400µl of plasma.
5. 3mls of whole blood were centrifuged at 3000xg and the plasma transferred into a plain polypropylene tubes with care taken not to disturb the buffy coat. The buffy coat was then removed. The plasma sample was centrifuged at 3000xg and the plasma was aliquoted into three 1ml tubes. Each aliquot was spiked with 10µl of the measles plasmid at concentrations of 10^6, 10^3 and 10^2 respectively. DNA was extracted from 400µl of plasma as before.

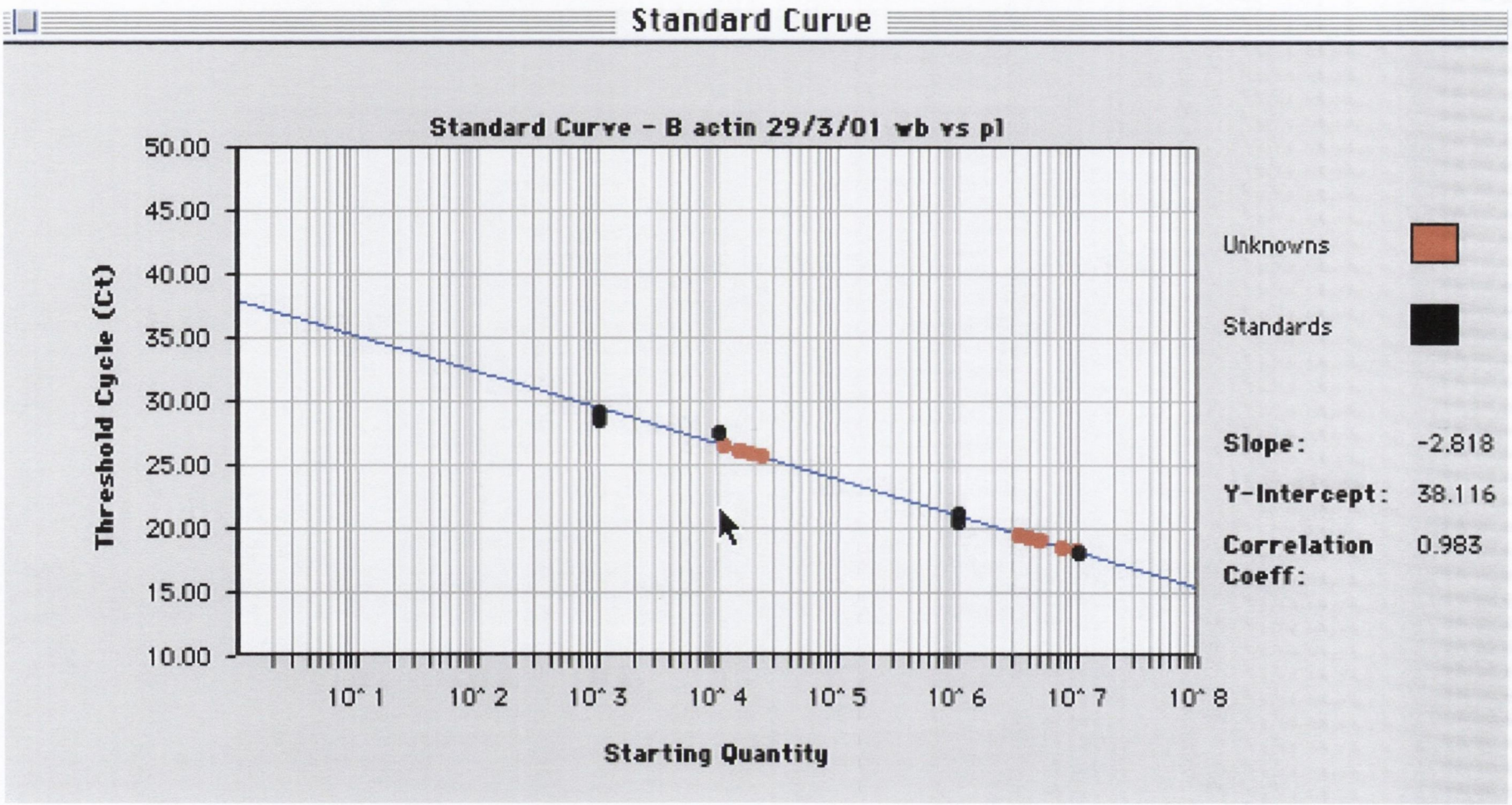
RESULTS

SAMPLE	ß-ACTIN COPY NUMBER	MEASLES COPY NUMBER
Whole blood spiked with 10^6 measles	2.7×10^5	1.2×10^6
Whole blood spiked with 10^3 measles	3.1×10^5	1.9×10^3
Whole blood spiked with 10^2 measles	2.6×10^5	1.0×10^2
Plasma from whole blood spiked with 10^6 measles	1.1×10^3	1.9×10^6
Plasma from whole blood spiked with 10^3 measles	6.2×10^2	2.0×10^3
Plasma from whole blood spiked with 10^2 measles	6.0×10^2	4.0×10^2
Plasma spiked with 10^6 measles	1.7×10^3	1.2×10^6
Plasma spiked with 10^3 measles	2.0×10^5	1.2×10^3
Plasma spiked with 10^2 measles	4.7×10^2	6.0×10^2

CONCLUSION

There is no evidence of PCR inhibition however the yield of DNA is significantly greater from the whole blood samples.

Figure 1 **Standard curve of ß-actin yield from whole blood versus plasma**



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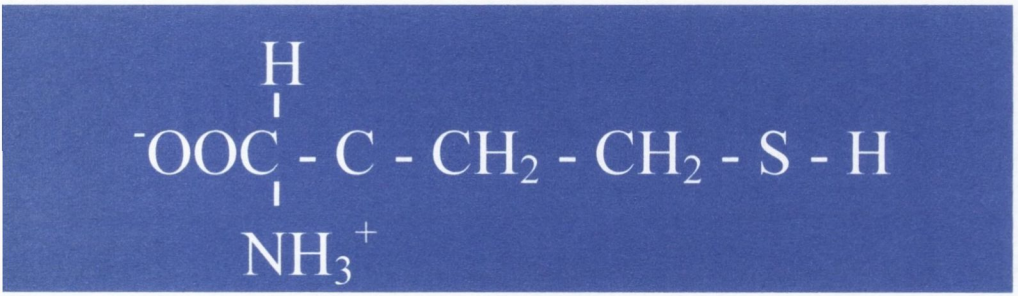
CHAPTER 7

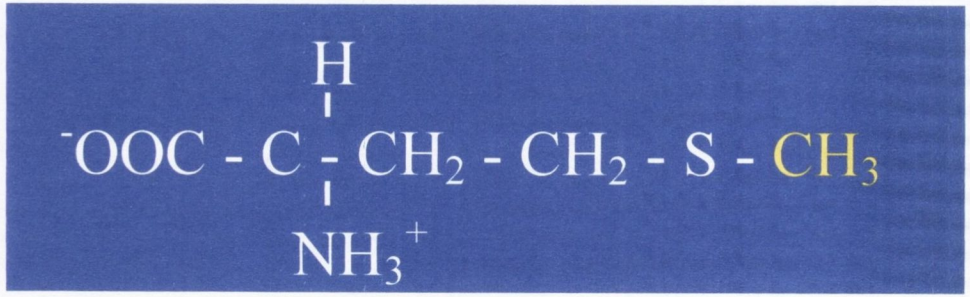
HOMOCYSTEINE : A RISK FACTOR FOR PRE-ECLAMPSIA

7.1 STRUCTURE AND PROPERTIES OF HOMOCYSTEINE

Homocysteine is the demethylated derivative of the essential amino acid methionine and undergoes metabolism by two main pathways. It is either degraded via the transsulphuration pathway or it is remethylated to methionine. There are three enzymes involved in these pathways and each enzyme requires the presence of a vitamin for its normal function. Cystathionine β synthase requires the presence of vitamin B6 and methionine synthase requires vitamin B12 as cofactors while methylene tetrahydrofolate reductase (MTHFR) requires folate.

Figure 1. Structure of Homocysteine





HOMOCYSTEINE ← **METHIONINE**

HOMOCYSTEINE - SH

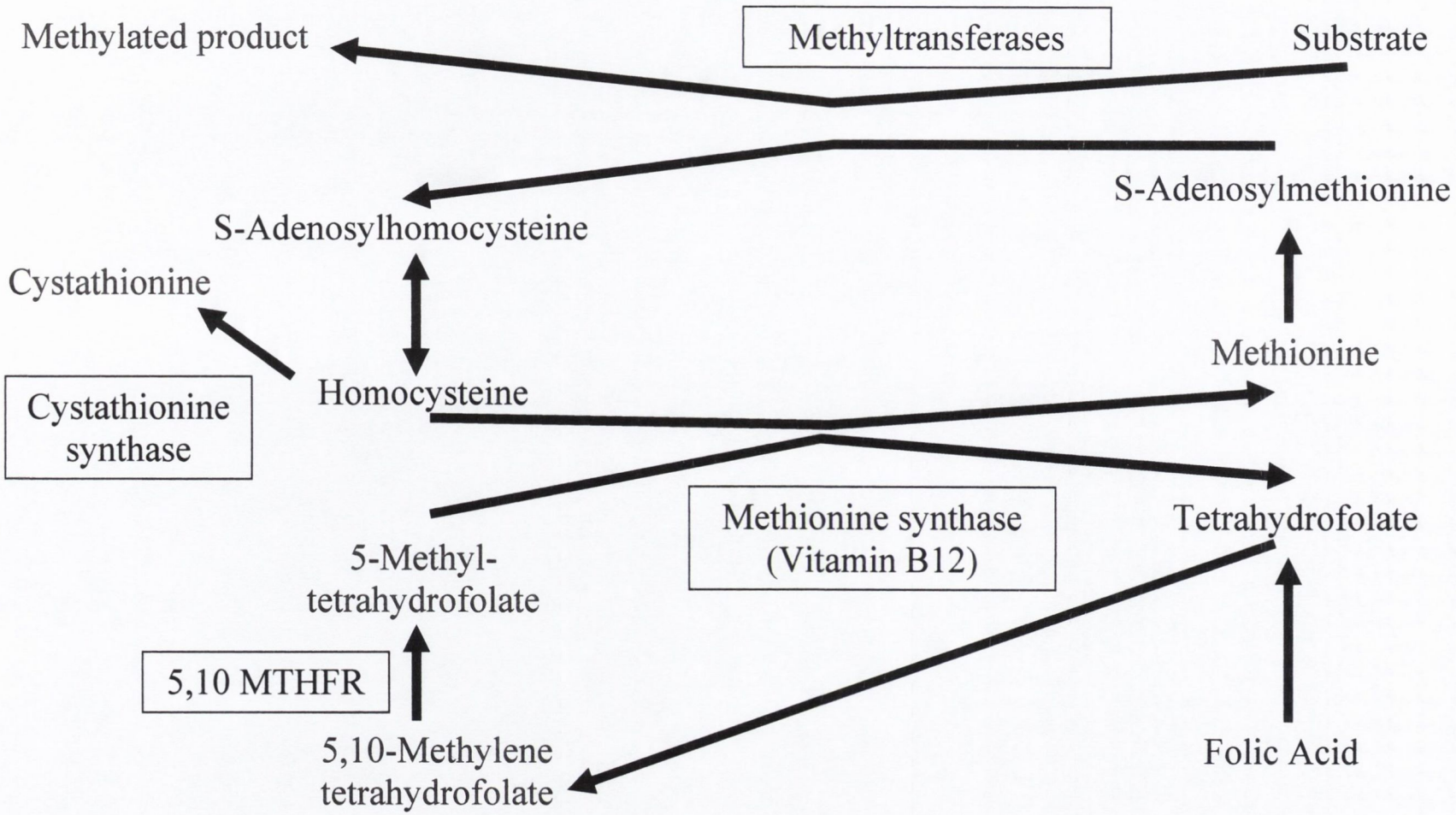
HOMOCYSTEINE – S – S - HOMOCYSTEINE

HOMOCYSTEINE – S – S - CYSTEINE

HOMOCYSTEINE – S – S - GLUTATHIONE

HOMOCYSTEINE – S – S – PROTEINS

Figure 2. The Methylation Cycle



5,10 MTHFR catalyses the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. This reaction commits tetrahydrofolate bound one-carbon units to the pathways of S-adenosylmethionine dependent biological methylations. It is effectively irreversible making the activity of the enzyme a possible control point in the distribution of one-carbon units between DNA synthesis and methylations dependent on S-adenosylmethionine. In 1995 Frosst described a point mutation in the gene which codes for the enzyme MTHFR (Frosst et al 1995). This missense mutation, a C to T substitution at nucleotide 677 resulting in an alanine to valine amino acid substitution has been associated with increased circulating homocysteine as well as an increased risk for premature cardiovascular disease. The mutation results in a thermolabile variant of the enzyme which has a significant reduction in specific activity. Homozygosity for this mutation results in decreased synthesis of 5-methyltetrahydrofolate, the primary methyl donor in the conversion of homocysteine to methionine and the resulting increase in plasma homocysteine concentration is a risk factor for venous and arterial thrombosis. Homozygosity for the variant has been described in 5-15% of the normal population and is associated with higher than normal homocysteine levels and low folate status (Molloy et al 1997).

Homocysteine is present in plasma in four forms: approximately 1% circulates as the free thiol, 70-80% is disulphide bound to plasma proteins chiefly albumen and the remaining 20-30% combines with itself to form the dimer homocysteine or with other thiols including cystein with which it forms the homocysteine-cysteine mixed disulphide (Ueland et al 1995). The term total plasma homocysteine refers to the combined pool of all four forms of homocysteine. At the first

international meeting on homocysteine, it was agreed that total plasma homocysteine concentrations would be denoted by the symbol tHcy.

There is some variation between laboratories but the normal range in our laboratory is 5-12 μmol/l in women and 8-15μmol/l in men. Ingestion of a high loading dose of methionine in normal subjects leads to a temporary rise in tHcy which returns rapidly to the baseline level. The methionine loading test reflects vitamin B6 status and is a measure of the transsulphuration pathway. The fasting test reflects folate and vitamin B12 status and remethylation capacity. Blood specimens should ideally be centrifuged immediately because delay leads to a time and temperature dependent release of homocysteine from blood cells (Hankey & Eikelboom 1999).

Plasma homocysteine levels are influenced by both environmental and genetic factors. Nutritional deficiency of any of the vitamins which are necessary cofactors for the normal function of the enzymes can result in an elevated tHcy. From a genetic viewpoint, mutations in the gene encoding MTHFR resulting in an unstable thermolabile variant form of this enzyme can result in an elevated tHcy level. tHcy levels are higher in men than in women, tHcy rises with age and are higher in postmenopausal than premenopausal women. Diseases such as pernicious anaemia and renal disease can cause elevations in tHcy levels, clinical nutrient deficiency is rare (<5%) but inadequate nutrient status is the most common cause of increased tHcy (10-50%) (Weir & Scott 1998).

7.2 EVIDENCE FOR A PATHOGENIC ASSOCIATION BETWEEN HOMOCYSTEINE AND VASCULAR DISEASE

The association between tHcy and vascular disease is well established. There is evidence available from:

- Inborn errors
- Retrospective studies
- Prospective studies
- In vitro studies
- In vivo studies

Inborn errors of the three enzymes that control the level of tHcy all cause premature cardiovascular disease. The most common genetic cause of severe hyperhomocysteinaemia and classic homocystinuria is a homozygous deficiency of cystathionine β synthase. It occurs in only 1 in 100,000 live births and results in an increase of up to 40 fold in fasting tHcy. It is inherited as an autosomal recessive trait. A vascular event occurs before 30 years in 50% of untreated homozygotes.

Boushey performed a quantitative meta-analysis using mostly retrospective case control studies and found that a raised tHcy (above the 90-95th percentile) is associated with an increased risk of fatal and non fatal atherosclerotic vascular disease in the coronary (OR 1.7, 95%CI 1.5-1.9), cerebral (OR 2.5, 95%CI 2.0-3.0) and peripheral circulation (OR 6.8, 95%CI 2.9-15.8). She found that 10% of all coronary artery disease risk in the population can be attributed to tHcy.

This evidence resulted from 27 studies relating tHcy to arteriosclerotic vascular disease and 11 studies of folic acid effects on tHcy levels. Assuming a graded response, a 5μmol/l increment in tHcy elevated the risk of coronary artery disease by one-third, similar to cholesterol increases of 0.5mmol/l (Boushey et al 1995).

In a meta-analysis of 8 previous studies of the relationship between tHcy and ischaemic heart disease (IHD) carried out by Wald, for each 5μmol/l rise in tHcy, the rate of IHD risk increase was 84% (95%CI 52-123). The strongest evidence is from prospective studies which show that subjects who develop IHD previously had higher levels of tHcy and the risk associated with tHcy is continuous right down to the lowest levels encountered in plasma. In the BUPA prospective study of 21,520 men aged 35-64 years, where 229 subsequently died of IHD, tHcy was higher in cases than controls. For each 5μmol/l rise in tHcy there was a continuous dose response relationship which increased the risk by 41% (95%CI 22-59) (Wald et al 1998).

In vivo studies demonstrated a clear association between tHcy and the extent of arteriosclerotic thickening of carotid, brachial and aortic arteries. In vitro studies have demonstrated that homocysteine associated vascular disease is related to vascular endothelial dysfunction, smooth muscle proliferation and abnormalities of coagulation (Weir & Scott 1998).

How then is homocysteine related to pre-eclampsia? It has been established that pre-eclampsia is an endothelial disorder. Endothelial cell dysfunction appears to be a central feature of the pathophysiology leading to altered vascular reactivity, activation of the coagulation cascade and loss of vascular integrity (Roberts & Redman 1993). Indeed Powers demonstrated not only an

elevated tHcy in patients with pre-eclampsia but also showed a positive correlation between tHcy and cellular fibronectin levels thus providing more evidence for endothelial activation (Powers et al 1998). Endothelial dysfunction is demonstrable within the myometrial arteries of women with preeclampsia and the incubation of healthy vessels with plasma obtained from women with preeclampsia induces similar endothelial changes (Ashworth et al 1998).

7.3 HOMOCYSTEINE LEVELS IN PREGNANCY

There is a change in homocysteine levels during normal pregnancy. Walker demonstrated that the mean tHcy concentration was 5.6 (3.9-7.3) at 8-16 weeks, 4.3 (3.5-5.3) at 20-28 weeks, 5.5 (3.3-7.5) at 36-42 weeks gestation and 7.9 (6.2-9.6) in the non pregnant control group. Homocysteine levels were significantly lower in all three trimesters of pregnancy compared with non pregnant controls ($p<0.001$). Homocysteine levels correlated directly with albumen levels which decreased during pregnancy and homocysteine concentrations were decreased in subjects taking folic acid supplementation (Walker et al 1999).

As with other inherited thrombophilias, defects in homocysteine metabolism have been reported to be associated with a number of obstetric complications but for the purposes of this thesis, the focus will be on pre-eclampsia.

7.4 HOMOCYSTEINE AND PRE-ECLAMPSIA

In the Netherlands, a study of women with a history of severe early onset pre-eclampsia showed a 7 fold higher frequency of hyperhomocysteinaemia compared to normotensive controls. Dekker reported that of 79 patients with a history of severe early onset pre-eclampsia tested at least 10 weeks postpartum, 17.7% had a positive methionine loading test. (Dekker et al 1995)

Rajkovic et al measured plasma homocysteine levels in 20 primigravidas with pre-eclampsia at the time of their delivery at a mean gestational age of 35 ± 4 weeks and in 20 primigravidas without pre-eclampsia at a mean gestational age of 40 ± 1 weeks. They found that plasma homocysteine levels were significantly higher among women with pre-eclampsia than among normotensive women (8.66 ± 3.05 vs 4.99 ± 1.11 μmol/l, $p < 0.001$) (Rajkovic et al 1997).

Rajkovic and colleagues then went on to measure plasma homocysteine levels in African women with either pre-eclampsia or eclampsia using random samples collected immediately postpartum and used high performance liquid chromatography. The gestational age at delivery for the study group was 36.3 weeks while the controls were delivered at 38.4 weeks. They found that women with pre-eclampsia or eclampsia had significantly higher mean homocysteine levels than normotensive controls (12.54 μmol/l vs 9.93 μmol/l, $p<0.001$) (Rajkovic et al 1999).

Powers et al (1998) collected antenatal blood samples from 33 women with uncomplicated pregnancies and from 21 women with pre-eclampsia. These women were recruited on admission to the labour ward. The mean gestational age for the cases was 33.3 ± 4.2 weeks while the

control patients had a mean gestation of 37.5 ± 3.3 weeks. The concentration of total homocysteine was significantly higher in those women with pre-eclampsia than in healthy pregnant women ($p < 0.04$). Of 20 women with pre-eclampsia, six had a tHcy > 97.5th centile of the normal pregnant patients. They also found that cellular fibronectin concentrations were greater in women with pre-eclampsia than controls and positively correlated strongly with tHcy in women with pre-eclampsia (Powers et al 1998).

Sorensen et al are the only other group of investigators to measure homocysteine levels in pre-eclampsia before onset of the disease. This was a prospective nested case control study design to compare second trimester maternal homocysteine concentrations in 52 patients who developed pre-eclampsia compared with 56 women who remained normotensive throughout pregnancy. These blood samples were obtained at an average gestational age of 16 weeks. They found that second trimester tHcy concentrations were higher but not significantly so compared with controls (4.8 vs 4.4 μmol/l, $p = 0.08$). This result approaches but does not reach statistical significance and this could be in part due to the small sample size in this study (Sorensen et al 1999).

Wang et al measured tHcy in the maternal and fetal circulation in 26 normal pregnancies and 60 pregnancies with clinical evidence of placental vascular disease which they defined as the presence of pre-eclampsia or IUGR with an abnormal Doppler. There were 19 pregnancies complicated by pre-eclampsia, 17 with an abnormal Doppler and 24 with both pre-eclampsia and umbilical placental vascular disease. All the pregnancies studied were in the third trimester. They found that maternal plasma homocysteine was significantly higher in all pregnancies with clinical evidence of placental vascular disease while elevated fetal plasma homocysteine

concentrations were found only in pre-eclampsia without umbilical placental vascular disease. There was a correlation between maternal and fetal tHcy levels with fetal levels being significantly lower (Wang et al 2000). This finding is supported by work from Malinow which demonstrated a descending concentration gradient of tHcy from maternal vein to umbilical vein and to umbilical artery in normal healthy pregnant women at term (Malinow et al 1998).

7.5 PRE-ECLAMPSIA AND MTHFR POLYMORPHISM

Studies from independent groups in Japan, Italy and Israel have described significantly higher frequencies of the C677T genotype among women with pre-eclampsia than among control subjects. Sohda et al found the T677 allele and the genotype homozygous for the T677 allele significantly increased in patients with pre-eclampsia as compared to controls, 24% vs 11% ($p < 0.02$ and $p < 0.004$). This was an entirely Japanese population and the normotensive controls were mainly male. However the authors admit that the patient sample was small and association studies are susceptible to various biases including population stratification (Sohda et al 1997).

In Italy, Grandone performed a case control study using 96 women with pre-eclampsia and 129 controls. However only 45 of the cases actually had pre-eclampsia as defined by ACOG criteria and 51 had in fact pregnancy induced hypertension. MTHFR homozygosity was found in 28 (29.8%) of cases and in 24 (18.6%) of controls (OR 1.8, 95%CI 1.0-3.5). Based on these findings they suggested that the TT genotype was associated with pre-eclampsia (Grandone et al 1997). The Italian and Japanese studies used different definitions for pre-eclampsia. Multiparous women with pre-eclampsia were included in these studies so it is possible that women with this

mutation may be at higher risk of developing recurrent pre-eclampsia in successive pregnancies and therefore the inclusion of these women may affect the results.

Kupferminc et al (1999) reported a case control study comparing 110 consecutive women who presented to their hospital in Israel with pregnancies complicated by either severe pre-eclampsia, severe IUGR, stillbirth or placental abruption. They found that 22% of these women were homozygous for the thermolabile MTHFR defect compared with 8% of the control population ($p = 0.005$) (OR 3.7, 95%CI 1.5-9.0). When looking at the individual obstetric complications, there were 34 women with severe pre-eclampsia of which 7 were homozygous for the C677T mutation (Kupferminc et al 1999).

Chikosi et al (2000) attempted to verify these findings in a black African population with a high incidence of pre-eclampsia. Homozygosity for the MTHFR mutation is rare in the African population. No difference in frequency of the TT allele was observed in 105 women with pre-eclampsia compared with 110 healthy pregnant normotensive women. Only one woman with pre-eclampsia was homozygous for the MTHFR mutation suggesting that MTHFR polymorphism is not an important factor in the pathogenesis of pre-eclampsia in black South African women (Chikosi et al 2000).

Powers et al (1999) determined the MTHFR genotype for 114 control subjects and 99 pre-eclampsia patients and found no significant difference in prevalence of the variant among pre-eclampsia patients compared to controls. The incidence of the TT genotype among control subjects was 12% and there was no difference in the prevalence of the mutation in either mild or

severe pre-eclampsia as compared with controls. Similarily there was no significant difference in the incidence of the genotype in infants from pre-eclampsia pregnancies or control mothers (Powers et al 1999).

Rigo et al (2000) found the prevalence of homozygous MTHFR mutations among Hungarian pre-eclamptic women was 6.7%. This rate was similar to that found among healthy pregnant controls (5.9%) (Rigo et al 2000). Hence the potential role of MTHFR polymorphism in pre-eclamptic pathogenesis may be population specific.

Livingston et al (2001) performed a cross sectional study to compare the maternal and fetal genotype frequencies for MTHFR. Patients with severe preeclampsia (n = 110) were matched for gestational age to normotensive pregnancies (n = 97). Their results approached but did not reach statistical significance between the two groups, MTHFR C677T mutation 9.6% versus 6.3%, p = 0.54. They further divided their subjects into Caucasian and African American subsets thus weakening their study in terms of numbers. Their heterogenous population is more genetically diverse from some of the homogenous populations previously studied. We know that mutations such as MTHFR are less frequent in African Americans thus emphasising the fact that different populations may be predisposed to pre-eclampsia by different pathways. The authors also make the point that vitamin supplementation is common in an American pregnant population and folate supplementation may be a significant confounder here whereas vitamin supplementation would not affect the factor V Leiden or prothrombin mutations (Livingston et al 2001).

Lachmeijer et al (2001) studied the MTHFR mutation in 47 women with pre-eclampsia, 49 women with pre-eclampsia and hyperhomocysteinaemia, 127 women with familial pre-eclampsia and 120 control subjects. They found that 10.6% of women with pre-eclampsia had a tHcy > 97.5th centile but no excess of the TT genotype when compared to controls. Women with pre-eclampsia and mild hyperhomocysteinaemia (>75th centile) had a significant excess of the TT genotype relative to women with pre-eclampsia without elevated tHcy levels (OR 8.2, 95%CI 1.8-39). Therefore although an elevated tHcy level was more frequent among Dutch women with a history of pre-eclampsia, the mutation was not more frequent (Lachmeijer et al 2001).

In non pregnant populations there are several major intervention trials ongoing to assess the benefit of correcting raised homocysteine levels with folic acid supplements. Leeda reported that 27 women with a previous history of pre-eclampsia or IUGR were shown to have an elevated tHcy and were treated with folic acid. All 27 women had normal tHcy levels after supplementation (Leeda et al 1998). This obviously raises the question as to whether women with significant obstetric complications who are found to have an elevated tHcy will have improved obstetric outcomes if they are subsequently corrected with folic acid.

While the evidence from all these investigators must be considered, it is important to remember that the quality of this data is limited with insufficient matching of cases to controls, inaccurate definitions of pre-eclampsia and possible laboratory measurement bias relating to pregnancy. With the exception of Sorensen's study, all other tHcy measurements were taken late in pregnancy when the disease process of pre-eclampsia was fully established. We know that the glomerular filtration rate can be reduced by as much as 25% in true pre-eclampsia and that

plasma Hcy levels are inversely related to glomerular filtration (Ray & Laskin 1999), therefore it is important to measure tHcy levels early in pregnancy and prior to the onset of pre-eclampsia.

When considering the possible role of homocysteine in the pathophysiology of pre-eclampsia, it is interesting to note the findings of Smith et al who performed a retrospective cohort study of 129,290 births to investigate whether pregnancy complications associated with low birthweight are related to risk of subsequent ischaemic heart disease in the mother. They found that women with a history of pre-eclampsia had a two fold increased risk of developing ischaemic heart disease over the next 15-19 years (Smith et al 2001). If homocysteine plays a role in early pregnancy in the subsequent development of pre-eclampsia, this information would seem to provide further evidence to strenghten the argument and complete the circle in terms of the effects of homocysteine on endothelial dysfunction in all vascular systems.

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CHAPTER 8

METHODOLOGY - PART II

8.1 SAMPLE COLLECTION

From March 1986 to March 1990, at the three Dublin maternity hospitals in Ireland, which combined have in excess of 20,000 deliveries annually, women attending for their first antenatal visit consented to provide a blood sample. These women were attending for routine antenatal care. Irish women did not take prenatal vitamins at that time and this was prior to the introduction of periconceptual folic acid supplementation. The blood specimens were centrifuged, separated and stored at -20^0C on the day of sample collection in chronological order. This bank of bloods has been used for other studies and sample processing is described in detail elsewhere (Kirke et al 1993, Daly et al 1995).

For the purpose of this research, a decision was made to investigate only those women attending for a first antenatal visit, who subsequently delivered at the Coombe Women's Hospital during this four year interval. Women who developed pre-eclampsia were identified by a computer database which has been in operation since January 1986. Individual chart reviews were performed to identify those cases of severe and non-severe pre-eclampsia. Power calculations had indicated that a sample size of 440 would be required. As there were insufficient samples to generate this, two controls were selected for each case.

The criteria used for the diagnosis of pre-eclampsia were in accordance with ACOG guidelines.

Severe pre-eclampsia:

Hypertension was defined as a systolic BP ≥ 160-180 mmHg or a diastolic BP of 110mmHg while proteinuria was defined as ≥ 5g protein in a 24 hr urine collection or +3/+4 proteinuria on dipstick urinalysis.

Non-severe pre-eclampsia:

Hypertension was defined as a systolic BP ≥ 140 mmHg or a diastolic BP of 90mmHg while proteinuria was defined as ≥ 300mg protein in a 24 hr urine collection or +1/+2 proteinuria on dipstick urinalysis.

The date of the first antenatal visit for these subjects was then used to search for an early pregnancy blood sample in the bank of stored bloods. Controls were selected for each case. The women in the control group had uncomplicated pregnancies and remained normotensive throughout. Once the cases and controls were identified, the blood samples were relabelled to ensure the investigators were blinded.

8.2 MATERIALS

Microbiological assay:

Ascorbic acid, sodium ascorbate, manganese sulphate and Tween 80 were purchased from Sigma Chemical Co., Poole, UK. Chloramphenicol was obtained from Parke Davis & Co., Pontypool, Wales, UK. Folic acid standard and vitamin folic acid test broth were supplied by Merck, Darmstadt, Germany. Microtitre plates, plate sealers, disposable tubes, pipette tips, weighboats

and K3-EDTA 10 ml vacutainer blood sampling tubes were supplied by Sarstedt Co, Wexford, Ireland.

Microorganism:

The chloramphenicol resistant strain of Lactobacillus casei (NCIB 10463) was obtained initially as a freeze dried preparation from The National Collections of Industrial and Marine Bacteria Ltd., Torry Research Station, 135 Abbey Road, Aberdeen, Scotland, UK. Cryopreserved subcultures of this stock culture were used in all assays.

Homocysteine assay:

Abbott Germany provided all the consumables for the Homocysteine assay including two internal controls while the remaining external control was supplied by Bergen University.

Genotyping:

DNA extraction was performed using the QIAamp Blood Kit supplied by Quiagen Ltd, Crawley, West Sussex RH10 2AX, UK. Proteinase K, Taq DNA Polymerase, Primers, DTNPs, Hinf1 enzyme and DNA molecular weight markers were supplied by Boehringer Mannheim, Lewes UK. Ethidium bromide, buffers, agarose, and tween were purchased from Sigma Chemical Co., Poole, UK. Other general laboratory supplies were obtained locally.

8.3 THEORY OF OPERATION OF HOMOCYSTEINE ASSAY

The Imx homocysteine assay (Abbott Germany) is based on the Fluorescence Polarization Immunoassay (FPIA). Using this FPIA method, the concentration of the analyte is determined using two technologies: competitive protein binding and fluorescence polarisation (Leino 1999).

The FPIA technology is based on the fact that molecules rotate in liquid and the rate of rotation is related to the size of the molecule. Unbound analyte rotates more rapidly than the complex formed when the analyte binds to the antibody. Polarized light travels in a single plane and can be distinguished from non-polarized light by the Imx System. Polarized light can be used to produce a fluorescent response from the analyte tracer. The fluorescent emission is also polarized. If the tracer emits polarized light in a liquid, the intensity of the polarized light detected is related to the rotation of the molecule.

Each Homocysteine reagent kit contains 4 different reagents:

1. Dithiothreitol (DTT) which reduces homocysteine and protein bound forms of homocysteine to free homocysteine.
2. SAH hydrolyse and Adenosine which converts free homocysteine to S-adenosyl-L homocysteine (SAH).
3. Monoclonal antibody molecule. SAH and Fluorescein Tracer compete for sites on the antibody.

Analytes from the specimen and analyte tracer compete for the binding site on antibodies. If the specimen contains a high concentration of the analyte, then specimen analytes bind to the antibodies, leaving the analyte tracer unbound. If the specimen contains a low concentration or no concentration of the analyte, then few or no analyte molecules bind to the antibodies, leaving the antibodies open for the analyte-tracer to bind. FPIA optics measure the change in polarized light to determine the concentration of unbound analyte tracers and therefore the concentration of analytes in the specimen. The change in polarized light intensity is inversely proportional to the concentration of the analyte in the specimen. The FPIA optical assembly uses a tungsten halogen lamp to emit light of varying wavelengths. The emitted light is directed through an excitation filter which selects light with a nominal wavelength of 485nm (blue light). The blue excitation light excites the fluorophore in the cuvette. When the fluorophore returns to its ground state, it emits light with a wavelength between 525-550nm (green light). To measure the intensity of light emitted by the fluorophore, the green light is focussed on to the photomultiplier tube. Intensity signals for both blank and final readings are used to calculate polarization values. When known concentrations of analyte are measured, a calibration curve is calculated and stored in the memory. Unknown analyte concentrations are then derived from this stored curve.

8.4 MEASUREMENT OF PLASMA HOMOCYSTEINE

1. The carousel was filled with glass cuvettes and sample cartridge.
2. The sample tubes were inverted five times.
3. 50µl of specimen is the minimum volume required to perform the assay. To obtain this recommended volume, the 200µl pipette was set to 55µl and reverse pipetting was performed.
4. To reverse pipette, the thumb button on the pipette was depressed all the way to the second stop. The tip was placed just under the surface of the liquid and the thumb button smoothly released. The tip was withdrawn from the liquid touching against the edge to remove access. The present volume was delivered by smoothly depressing the thumb button to the first stop. The operating button was held at the first stop. The liquid that remained in the tip was not included in the delivery and was discarded with the tip.
5. There are two internal controls (Imx Homocysteine controls) which should be placed into position #1 and #20 with the external control (from Bergen) in position #19. The 17 samples should be placed in positions #2-#18. The run is activated by pressing assay 73 and takes one hour. The print out must be checked to ensure controls fall within the range.

8.5 PLASMA FOLATE MICROBIOLOGICAL ASSAYS

These assays were carried out by a research technician according to the method of Molloy and Scott (1997). The assay was performed using a microtitre plate adaptation of the chloramphenicol resistant Lactobacillus casei method. This is a growth based assay. The organism has specific requirements for the vitamin to be assayed. Growth can be very sensitively monitored by turbidity at an optical density of 592nm. The CV for the folate assay is 5-10%.

1. Duplicate 50µl aliquots of plasma were pipetted into polypropylene tubes and diluted to 1ml with 0.5% sodium ascorbate. In addition to unknowns, 3 quality control samples were included with each batch.
2. For each diluted sample, duplicate aliquots of 100µl and 50µl were transferred to 4 separate wells of a 96 well microtitre plate using a repetitive sampling pipette. 50µl of 0.5% sodium ascorbate was added to any well containing 50µl of analytical sample to bring the total volume in all wells to 100µl. This resulted in an overall use of 8 wells for each plasma specimen analysed.
3. Standards were aliquoted into a separate microtitre plate (12 columns with 8 replicates for each standard) using folic acid concentrations between 0 and 50pg per well. Appropriate additions of 0.5% sodium ascorbate were made to each well, such that the final volume in all wells was 100µl.
4. Medium was prepared using 5.7g of Merck folic acid broth per 100ml. The broth was weighed, transferred to a 500ml conical flask and 100ml of water added. Chloramphenicol (3mg) was added and the mixture was heated. When the solution was hot, 30µl of Tween 80

was added. The mixture was boiled until the broth powder had fully dissolved. It was then cooled to room temperature. Just before use, 75mg of ascorbic acid was dissolved into the medium and 200µl of thawed lactobacillus casei cryopreserved suspension was added. The medium was mixed thoroughly. A solution of manganese sulphate (15mg/ml) was added (1ml/100ml medium) to chelate excess EDTA.

5. 200µl of prepared innoculated folate medium was added to all wells using an 8 arm multichannel pipette.
6. Plates were covered with plastic plate sealers to avoid evaporation of medium during incubation.
7. Plates were incubated at 37 ^{0}C for 42 hours. After this time plates were inverted carefully to produce an even cell suspension and read in a Labsystems Multiscan Plus plate reader.

8.6 VITAMIN B12 ASSAY

The method used is similar to the microbiological assay described for folate (Molloy and Scott 1997) using a microtitre plate adaptation of the colistin resistant microorganism Lactobacillus Leichmannii after carrying out an initial extraction step. The method has been described and modified by Kelleher and O'Broin (1991). Like the folate assay, this is a growth based assay using a microorganism with specific requirements for vitamin B12. Growth can be sensitively monitored by turbidity at an optical density of 592nm. The CV for the assay is 5-10%.

1. Duplicate 75µl aliquots of plasma were pipetted into Micronic tubes and placed in a Micronic 96 tube plate holder suitable for autoclaving. In addition to unknowns, three quality control samples are included with each batch.

2. 675µl of extraction buffer pH 4.5 (consisting of 8.3mmol/l NaOH, 20.7 mmol/l acetic acid and 0.45mmol/l NaCN) were added to each tube.
3. The tubes were capped and autoclaved at 121^0C for 10 minutes.
4. After cooling, the precipitate was loosened from the walls of the tubes by vortexing and the tubes were then centrifuged at 2500 RPM for 15 minutes. The supernatant was decanted into clean plastic tubes.
5. For each tube, duplicate aliquots of 50µl and duplicate aliquots of 25µl were transferred to four separate labelled wells of a 96 well microtitre plate. 50µl of extraction buffer was added to all wells containing 50µl plasma extract and 75µl of extraction buffer added to all wells containing 25µl plasma extract to bring the total volume in all wells to 100µl. (This results in an overall use of eight wells for every plasma specimen analysed).
6. Cytamen solution (1mg/ml) available in vials suitable for intramuscular injection to patients with pernicious anaemia was used to prepare the vitamin B12 standard. Stock standard (Solution A; 500µg/l) was prepared by diluting 500µl of cytamen into 1 litre of distilled water. Aliquots (1.5ml) of this were stored at -80^0C and used to prepare the working standards. On the day of the assay, Solution B (5µg/l) was prepared by thawing an aliquot of Solution A and diluting 1ml to 100ml with distilled water in a volumetric flask. The working standard solution (50ng/l) was prepared by diluting 1ml of Solution B to 100ml with distilled water in a volumetric flask. Thus 100µl of this standard contained 5pg Vitamin B12. Standards were aliquoted into a separate microtitre plate (12 columns with 8 replicates for each standard) using vitamin B12 concentrations between 0 and 5 pg per well (0 and 100µl per well) and the volume in all wells was adjusted to 100µl with extraction buffer.

7. Medium was prepared using 6.2g of Merck vitamin B12 broth per 100ml. The broth was weighed, transferred to a 500ml conical flask and 100 ml of water added. When the solution was hot, 150µl of Tween 80 was added. The mixture was boiled until the broth powder had fully dissolved. It was then cooled to room temperature and colistin sulphate (11mg) added. Just before use, 200µl of thawed L.Leichmannii cryopreserved suspension was added. The medium was mixed thoroughly. A solution of manganese sulphate (15mg/ml) was added (1ml/100ml medium) to chelate excess EDTA in the plasma samples.
8. 200µl of prepared innoculated vitamin B12 medium was added to all wells using an 8-arm multichannel pipette.
9. Plates were covered with plastic plate sealers and a roller was used to ensure that all wells were individually sealed. This avoids evaporation of medium during incubation.
10. Plates were incubated at 37^0C for 42 hours. After this time plates were inverted carefully to produce an even cell suspension and read in a plate reader linked to an IBM 486 computer. The program was used to collect data, draw a standard curve and calculate the concentration of vitamin B12 in each well.

8.7 GENOTYPE ANALYSIS OF THE MTHFR C677T VARIANT

8.7.1 DNA Extraction

DNA was extracted according to the QIAGEN protocol.

1. 20µl of QIAGEN Protease was pipetted into the bottom of a 1.5ml microcentrifuge tube.
2. 200µl of whole blood was added to the microcentrifuge tube.

3. 200µl of Buffer AL was added to the sample and mixed by pulse vortexing for 15 seconds.
4. Incubated at 56^0C for 10 minutes.
5. The 1.5ml microcentrifuge tube was centrifuged briefly to remove drops from the inside of the lid.
6. 200µl 100% ethanol was added to the sample and mixed again by pulse vortexing for 15 seconds. After mixing, the 1.5ml microcentrifuge tube was centrifuged briefly to remove drops from the inside of the lid.
7. The mixture was carefully applied to the QIAamp spin column in a 2ml collection tube and centrifuged at 6000g for 1 minute. The QIAamp spin column was placed in a clean 2ml collection tube and the tube containing the filtrate discarded.
8. The QIAamp spin column was carefully opened and 500µl Buffer AW1 added, then centrifuged at 6000g for 1 minute. The QIAamp spin column was placed in a clean 2ml collection tube and the tube containing the filtrate discarded.
9. Then 500 µl Buffer AW2 was added and centrifuged at 20000g for 3 minutes.
10. The QIAamp spin column was placed in a new 2ml collection tube and the collection tube with the filtrate discarded, then centrifuged at full speed for 1 minute.
11. The QIAamp spin column was placed in a clean 1.5ml microcentrifuge tube and the collection tube containing the filtrate discarded. 200µl Buffer AE was added, then incubated at room temperature for 1 minute and centrifuged at 6000g for 1 minute.

8.7.2 PCR method for detecting MTHFR C677T genotype

Identification of the C to T substitution which converts an alanine to a valine residue was performed by a research technician according to the protocol outlined by Frosst et al (1995).

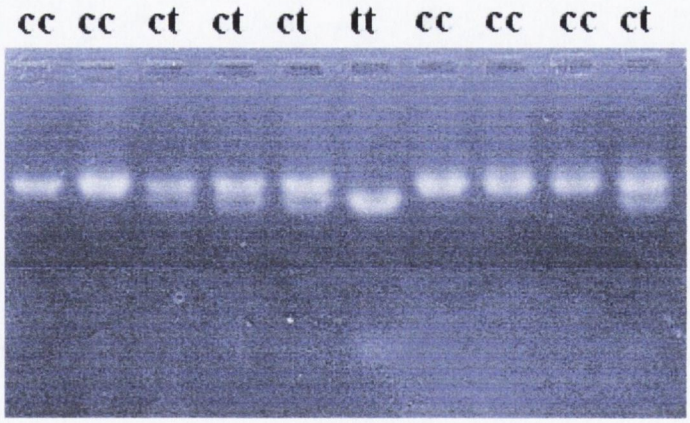
1. In a labelled autoclaved eppendorf tube, 2µl of extracted DNA was added to a PCR mix containing:
   - 5 µl 15mM Magnesium buffer
   - 8 µl dinucleotide triphosphates
   - 0.5 µl 10% Triton X-100
   - 0.5 µl Primer 1
   - 0.5 µl Primer 2
   - 1 µl Taqpolymerase
   - 32.5µl water

2. An overlay of 3 drops of mineral oil was added to the tube to prevent evaporation of the sample during the PCR cycles. A negative control with only the PCR mix was also included. The samples were placed in the PCR cycler and the cycler was programmed as follows: 94^0 for a 5 minute ramp, 94^0 for 1 minute, 62^0 for 1 minute and 72^0 for 1 minute. Steps 2-4 were repeated for 40 cycles.
3. After completion, a sample (10µl) of the PCR product was run out on a 2% agarose gel to ensure that amplification of the target DNA had occurred. If the reaction was successful, 26µl of the PCR product was digested overnight with 1µl of the enzyme hinf1 and 3µl of reaction buffer @ 37^0C.

4. After digestion the samples were run on a 2% agarose gel in 1X Tris/Borate/EDTA/Buffer and the products were visualised by ethidium bromide staining.

The presence of the mutation was determined by enzymatic digestion with Hinf 1 which in the presence of the mutation in both alleles of the gene results in a DNA fragment of 175 base pairs (bp) in length. Individuals who are heterozygous for this mutation show both the 198 and the 175 bp fragment, whereas individual who lack the mutation only have a band at 198 bp when visualised on 2% agarose gel (Figure 1).

Figure 1: Gel showing the 3 possible DNA banding patterns after Hinf1 digestion of the PCR product of the MTHFR gene containing the 677C-T missense mutation



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CHAPTER 9

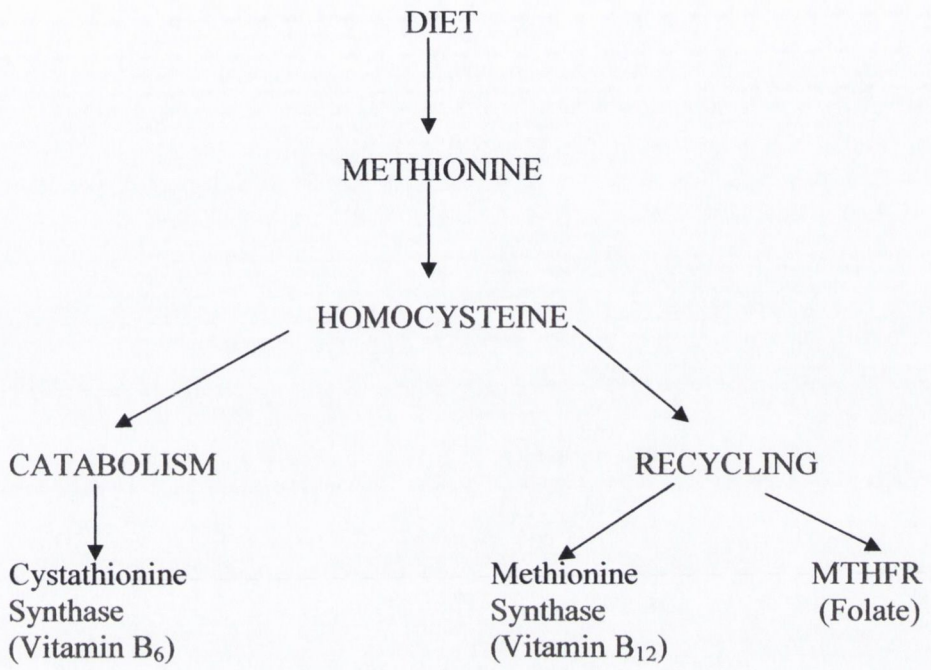
ELEVATED PLASMA HOMOCYSTEINE IN EARLY PREGNANCY: A RISK FACTOR FOR THE DEVELOPMENT OF PRE-ECLAMPSIA

9.1 INTRODUCTION

Homocysteine, the demethylated derivative of the essential amino acid methionine, undergoes metabolism by two pathways, as shown in Figure 1. Homocysteine (tHcy) is either catabolised via the transsulphuration route or is remethylated back to methionine. There are three enzymes involved in these pathways, each requiring the presence of a vitamin. Cystathionine ß-synthase requires the presence of vitamin B6, methionine synthase requires folate and vitamin B12 and methylenetetrahydrofolate reductase (MTHFR) requires folate for its normal function.

Plasma tHcy levels are influenced by both genetic and environmental factors. Elevated tHcy levels can occur due to a nutritional deficiency of the vitamin co-factors which are necessary for the normal function of the metabolic enzymes. From a genetic viewpoint, a variant form of MTHFR has been described which occurs in 10% of the normal population (Whitehead et al 1995). Homozygosity for this C677T mutation, expressed as the TT genotype, is associated with elevated tHcy levels especially when there is a low folate nutritional status (Molloy et al 1997).

Figure 1. Pathways of Homocysteine Metabolism



The association between homocysteine and vascular disease is well established. A quantitative meta-analysis of mainly retrospective case-control studies demonstrated a positive association between tHcy and coronary artery disease, cerebrovascular disease and peripheral vascular disease and calculated that 10% of all coronary artery disease risk in the population was due to high tHcy (Boushey et al 1995). The risk is seen to increase across a range of tHcy values. Similarily in a meta-analysis of 8 retrospective studies of the relationship between tHcy and ischaemic heart disease (IHD), for each 5μmol/l increase in tHcy, the rate of IHD risk increased by 84% (95% CI 52-123%) (Wald et al 1998).

In vitro studies suggest the pathogenesis of vascular disease associated with tHcy is related to endothelial dysfunction, smooth muscle proliferation and abnormalities of coagulation (Weir &

Scott 1998). Endothelial cell dysfunction appears to be a central feature of the pathophysiology of pre-eclampsia leading to altered vascular reactivity, activation of the coagulation cascade and loss of vascular integrity (Roberts & Redman 1993). Powers et al demonstrated not only that tHcy was elevated in pre-eclampsia but also showed a positive correlation with cellular fibronectin thus providing more evidence of endothelial activation (Powers et al 1998). Several studies have reported elevated plasma tHcy in patients with pre-eclampsia at the time of delivery (Rajkovic et al 1997) and in the postpartum period (Dekker et al 1995). The difficulty to date has been that there is no good predictor of pre-eclampsia and therefore longitudinal studies have been unable to include sufficient cases from which to draw meaningful conclusions. The objectives of the two studies reported below was to investigate if an elevated plasma tHcy in early pregnancy is associated with the development of severe and non-severe pre-eclampsia.

9.2 SEVERE PRE-ECLAMPSIA

9.2.1 METHODS

From March 1986 to March 1990, at the three Dublin maternity hospitals in Ireland, which combined have in excess of 20,000 deliveries annually, women attending for their first antenatal visit consented to provide a blood sample. These women were attending for routine antenatal care. Irish women did not take prenatal vitamins at that time and this was prior to the introduction of periconceptual folic acid supplementation. The blood specimens were centrifuged, separated and stored at -20^0C on the day of sample collection in chronological order. This bank of bloods has been used for other studies and sample processing is described in detail elsewhere (Kirke et al 1993, Daly et al 1995).

For the purpose of our study, a decision was made to investigate only those women attending for a first antenatal visit, who subsequently delivered at the Coombe Women's Hospital during this four year interval. Women who developed pre-eclampsia were identified by a computer database which has been in operation since January 1986. Individual chart reviews were then performed to identify those cases of severe pre-eclampsia. The criteria used for the diagnosis of severe pre-eclampsia were in accordance with ACOG guidelines. Hypertension was defined as a systolic BP $\geq$ 160-180 mmHg or a diastolic BP of 110mmHg while proteinuria was defined as $\geq$ 5g protein in a 24 hr urine collection or +3/+4 proteinuria on dipstick urinalysis.

The date of the first antenatal visit for these subjects was then used to search for an early pregnancy blood sample in the bank of stored bloods. Two controls were selected for each case and were matched for gestational age and for date of sample collection. This ensured that controls and case blood specimens were processed in the same batch and therefore treated identically. The women in the control group had uncomplicated pregnancies and remained normotensive throughout. Once the cases and controls were identified, the blood samples were relabelled to ensure the investigators were blinded.

Plasma tHcy was measured using fluoresence polarisation immunoassay (Leino 1999). The coefficient of variance for this assay was 5.15%. Plasma folate concentrations were determined by microbiological assay with lactobacillus casei (Molloy & Scott 1997). Vitamin B12 concentrations were determined by microbiological assay using lactobacillus leichmanii

(Kelleher & O'Broin 1991). Genotyping for the C677T variant of MTHFR was performed according to the protocol outlined by Frosst (Frosst et al 1995).

Power calculations indicated that a sample size of 437 would be required to achieve 80% power. However since this number of blood samples was not available, two controls were selected for each case. Blood vitamin measurements were not normally distributed therefore statistical analysis was performed using non parametric methods. Homocysteine data were analysed using the Wilcoxon rank sum test. Proportional data were compared with the Fisher exact test; $p < 0.05$ was considered to be significant. A logistic regression model was created to check for confounding variables, the dependent variable was the development of severe pre-eclampsia.

9.2.2 RESULTS

During the time period 1986-1990, blood samples were obtained from 54,000 women attending the three Dublin maternity hospitals. The base cohort population for our study consisted of 16,000 women who attended the Coombe Women's Hospital. The database yielded a total of 600 cases of pre-eclampsia, of which 76 fitted the criteria for severe pre-eclampsia (incidence 0.48%). Blood samples were available for 56 of these cases. Patient details are shown in Table I. Of the 56 cases and 112 controls, there was no significant difference in maternal age or gestational age at time of sample collection. A significantly higher number of primigravidas developed severe pre-eclampsia. As expected, the cases had a significantly shorter pregnancy and there was a significant difference in birthweight of 610g between the two groups.

Those patients who developed severe pre-eclampsia, had in early pregnancy a significantly higher plasma tHcy of 9.8 ± 3.3μmol/l than the control population where the plasma tHcy was 8.4 ± 1.9μmol/l, ($p \leq 0.0001$) (Table II). To assess whether a specific plasma tHcy value would be a useful predictor of severe pre-eclampsia, we divided our continuous data into quartiles. The cut-off for the upper quartile of tHcy was 10μmol/l and those patients who had a plasma tHcy >10μmol/l in early pregnancy had a significantly increased risk of developing severe pre-eclampsia (OR 2.84, 95%CI 1.37-5.88). Using a logistic regression model we found that tHcy is an independent risk factor for the development of severe pre-eclampsia (Table III). Neither plasma folate, vitamin B12, the TT genotype nor parity were significantly associated with the development of severe pre-eclampsia.

Table I. Patient details

	Cases (n = 56)	Controls (n = 112)	Statistical Significance
Mean maternal age (years ± SD)	26.5 ± 5.8	26.3 ± 5.5	p = 0.93
Parity (%)			
0	69	47	p ≤ 0.0001
≥ 1	31	53	
Mean gestational age at first visit (wks ± SD)	15.3 ± 4.0	14.9 ± 3.4	p = 0.61
Mean gestational age at delivery (wks ± SD)	37.5 ± 2.8	40.3 ± 1.1	p ≤ 0.0001
Mean birth weight (grams ± SD)	2876 ± 853	3489 ± 461	p ≤ 0.0001

Table II. Laboratory results of plasma tHcy, folate and vitamin B12 assays.

	Cases (n = 56)	Controls (n = 112)	Statistical Significance
Plasma tHcy (μmol/l ± SD)	9.8 ± 3.3	8.4 ± 1.9	$p \leq 0.0001$
Plasma Folate (nmol/l ± SD)	6.17 ± 6.01	6.13 ± 5.4	$p = 0.97$
Vitamin B_{12} (pmol/l ± SD)	337.9 ± 123.6	365.4 ± 169.0	$p = 0.26$

Figure 2. **Box and Whisker Plot of Plasma Homocysteine (HCY) related to Case (C) and Control (c) Pregnancies. Low whiskers mark the 10th percentile, high whiskers mark the 90th percentile, bottom of box marks the 25th percentile, top of box the 75th percentile. Bars within the boxes indicate the median values. Asterisks indicate extreme outliers, o marks outliers.**

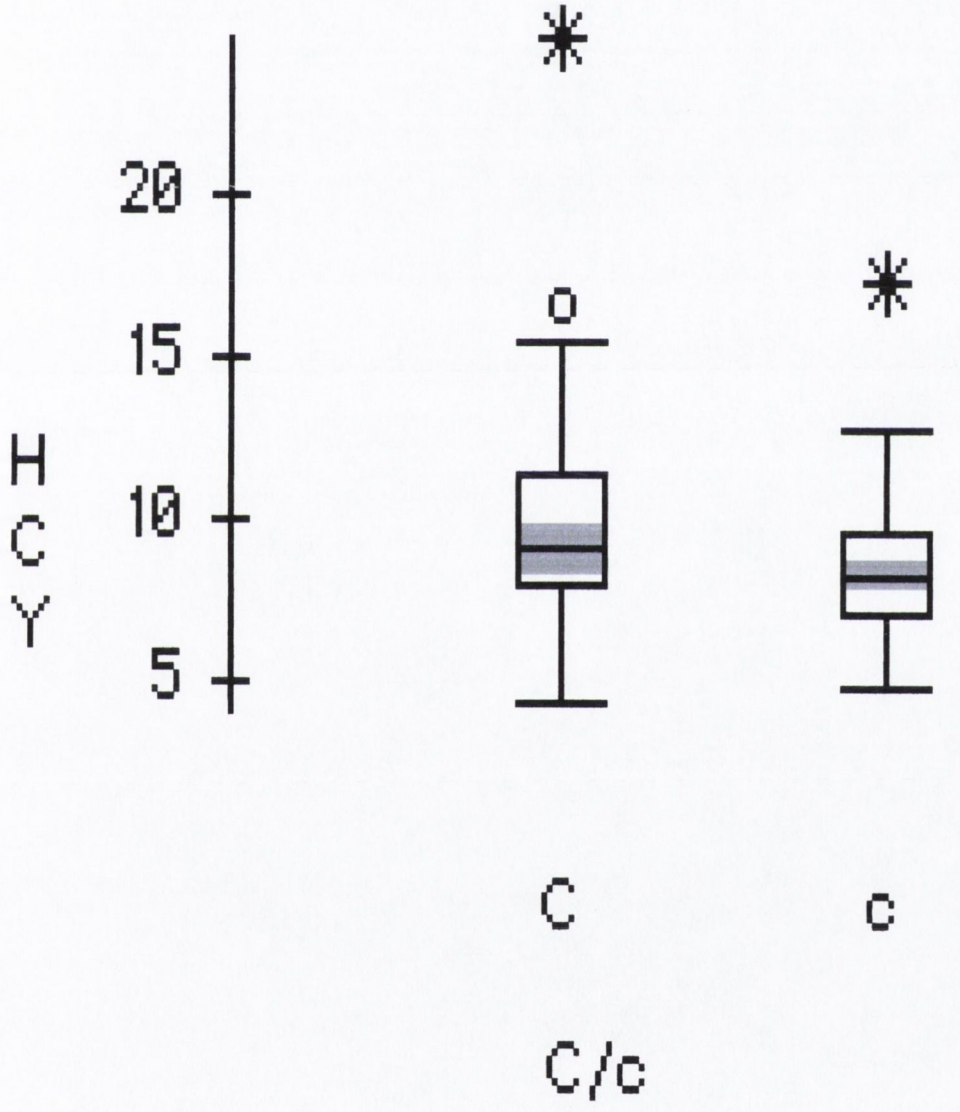


Table III. Logistic regression model for confounding variables

Variables	Statistical Significance
Plasma Homocysteine	p = 0.002
Plasma Folate	p = 0.32
Vitamin B12	p = 0.24
Parity	p = 0.32
TT genotype	p = 0.96

9.2.3 DISCUSSION

This case-control study demonstrates that women who develop severe pre-eclampsia have higher plasma tHcy levels in early pregnancy when compared to women who remain normotensive throughout pregnancy. Additionally, plasma tHcy in early pregnancy may predict a group of women at increased risk of developing severe pre-eclampsia.

A possible limitation of our study is that we had insufficient numbers to examine the influence of MTHFR polymorphism on the risk of developing severe pre-eclampsia, such an association has been previously described (Grandone et al 1997, Sohda et al 1997) but a recent study from Pittsburgh did not concur with these findings (Powers et al 1999). Whole blood samples were not available for many of our subjects thus we were only able to carry out MTHFR genotyping in 50% of our study population. Although this was not an objective of our study, we found distribution of the homozygote to be similar to what has been established for the Irish population and was not different between cases and controls, with 4 TT genotypes in each group.

The strengths of our study include the fact that our case and control populations were similar in age as we know that plasma tHcy increases with increasing age (Weir & Scott 1998). Plasma tHcy decreases throughout pregnancy (Walker et al 1999) but the study population provided blood samples at a similar gestational age. A possible confounder in any analysis of pre-eclampsia is the presence of different ethnic groups but in our study the population was of Irish background and genetically very homogenous.

The significantly lower birthweights can be attributed to the shorter pregnancies in those patients with severe pre-eclampsia, the mean birthweight for the cases plots on the 37th centile for gestational age and on the 50th centile for the control group. The significantly shorter pregnancies and associated lower birthweights are appropriate for the clinical management of severe pre-eclampsia and their significance is obviously not related to the plasma tHcy levels which were obtained in early pregnancy.

As tHcy was the primary factor under investigation in this study, we did not match the cases and controls for parity and instead focussed on matching the samples for batch, to ensure identical processing techniques as this may affect the blood homocysteine concentration. However the logistic regression model demonstrates that parity was not a significant risk factor for the development of severe pre-eclampsia. We recognise that there may be an increased risk of recurrent pre-eclampsia in patients with a pre-existing elevated plasma tHcy but we found no difference between those patients who developed pre-eclampsia in their first pregnancy when compared to multiparous patients who developed pre-eclampsia.

The logistic regression model demonstrates that homocysteine is an independent risk factor for the development of severe pre-eclampsia and the usual driving forces of elevated tHcy levels, namely folate and vitamin B_{12} are not significant here. This finding supports our hypothesis that homocysteine may even be elevated prior to pregnancy and exacerbates endothelial cell dysfunction as an ongoing process. Once pregnancy ensues, this homocysteine related endothelial injury predisposes the patient to some other secondary influence, the identity of which is unknown, thus leading to the development of severe pre-eclampsia.

There is strong evidence that tHcy correlates with an increased risk of cardiovascular disease, stroke and a range of other thromboembolic conditions, although it is not clear whether the association is causal or an effect of the disease process. It is clear from studies that the risk associated with homocysteine is continuous down to the lowest plasma levels (Wald et al 1998). Similarily, we found that a plasma tHcy in the upper quartile was associated with a significant risk of pre-eclampsia but we also found that a plasma tHcy in the lower quartiles was not entirely protective against the development of severe pre-eclampsia, thus suggesting that the risk associated with plasma tHcy may, as has been shown for other vascular pathology, be continuous.

Although pregnancy and childbirth have never been safer for the mother and child, pre-eclampsia is still a leading cause of maternal and perinatal morbidity and mortality. A serious shortcoming in studying the disease is that there is no reliable test to identify those women at risk for developing the disorder early enough in their pregnancies to permit preventative treatment or prospective investigations. With a plasma tHcy >10μmol/l in early pregnancy, the actual risk of developing severe pre-eclampsia in our study was 51.3%. It is unlikely that there will ever be a single universal predictive test for a multifactorial disease such as pre-eclampsia but the use of plasma tHcy as a screening test for severe pre-eclampsia warrants further investigation.

9.3 NON-SEVERE PRE-ECLAMPSIA

9.3.1 METHODS

Women who developed pre-eclampsia were identified by a computer database. Individual chart reviews were then performed to confirm cases of non-severe pre-eclampsia. Using the guidelines of the American College of Obstetricians and Gynecologists, non-severe pre-eclampsia was defined as a systolic BP $\geq$ 140 mm Hg or a diastolic BP of 90 mm Hg while proteinuria was defined as $\geq$ 300 mg protein in a 24 hour urine collection or +1/+2 proteinuria on dipstick urinalysis.

The date of the first antenatal visit for these subjects was used to search for an early pregnancy blood sample in the bank of stored bloods described in section 9.2.1. Two controls were selected for each case and matched for parity, gestational age and date of sample collection. This ensured that controls and case blood specimens were processed in the same batch and therefore treated identically. The women in the control group had uncomplicated pregnancies and remained normotensive throughout. Once the cases and controls were identified, the blood samples were relabelled to ensure the investigators were blinded. Assays and MTHFR genotyping were performed as described in section 9.2.1.

All statistical analyses were performed using SPSS for Windows version 10.0 (SPSS Inc, Chicago). The Student t-test was applied to compare cases with controls; $p<0.05$ was considered to be significant. A logistic regression model was created to check for confounding variables, the dependent variable was the development of non-severe pre-eclampsia.

9.3.2 RESULTS

Blood samples were available for 71 cases of pre-eclampsia and 142 controls, sampled at a mean gestation (± SD) of 15.9 ± 3.6 and 15.6 ± 3.4 weeks respectively (NS). Patient details are shown in Table IV. Of the 71 cases and 142 controls, there was no significant difference in maternal age or gestational age at time of sample collection. As expected, the cases had a significantly shorter pregnancy and there was a significant difference in birthweight of 188g between the two groups.

The women who developed pre-eclampsia, had in early pregnancy a significantly higher plasma homocysteine of 8.4 ± 2.4 μmol/l than the control population (Figure 3) where the plasma homocysteine was 7.07 ± 1.5 μmol/l, ($p \leq 0.0001$) (Table V). To assess whether a specific plasma homocysteine value would be a useful predictor of pre-eclampsia, we divided our continuous data into thirds. The cut-off for the upper third of homocysteine was 7.8 μmol/l. Those patients who had a plasma homocysteine ≥7.8 μmol/l in early pregnancy had a significantly increased risk of developing pre-eclampsia (OR 4.1, 95% CI 1.4 -12.6). They also had a lower plasma level of folate ($p=0.06$) and vitamin B12 ($p=0.06$) and subsequently delivered infants with a significantly lower birthweight ($p=0.004$).

Using a logistic regression model we found that homocysteine is an independent risk factor for the development of pre-eclampsia (Table VI). Neither plasma folate, vitamin B12, the TT genotype nor parity were significantly associated with the development of non-severe pre-eclampsia. Since whole blood samples were not available for many of our subjects we were only

able to carry out MTHFR genotyping in 36% of our study population. Although this was not an objective of our study, we found distribution of the homozygote to be similar to what has been established for the Irish population. There were 3 cases and 4 controls with the TT genotype.

Table IV. Patient details

	Cases (n = 71)	Controls (n = 142)	Statistical Significance
Mean maternal age (years ± SD)	27.14 ± 5.4	26.47 ± 5.8	p = 0.42
Parity (%)			
0	40	80	p = 0.14
≥ 1	31	62	
Mean gestational age at first visit (wks ± SD)	15.9 ± 3.6	15.6 ± 3.4	p = 0.54
Mean gestational age at delivery (wks ± SD)	39.4 ± 1.7	40.1 ± 1.8	p = 0.01
Mean birth weight (grams ± SD)	3289 ± 575	3477 ± 445	p = 0.01

Table V. Laboratory results of plasma tHcy, folate and vitamin B12 assays.

	Cases (n = 71)	Controls (n = 142)	Statistical Significance
Plasma tHcy (μmol/l ± SD)	8.4 ± 2.4	7.0 ± 1.5	$p \leq 0.0001$
Plasma Folate (nmol/l ± SD)	5.69 ± 4.3	5.88 ± 4.2	$p = 0.76$
Vitamin B_{12} (pmol/l ± SD)	333.7 ± 112.3	347.9 ± 123.7	$p = 0.44$

Figure 3. **Box and Whisker Plot of Plasma Homocysteine (HCY) related to Case(C) and Control(c) Pregnancies. Low whiskers mark the 10th percentile, high whiskers mark the 90th percentile, bottom of box marks the 25th percentile, top of box the 75th percentile. Bars within the boxes indicate the median values. Asterisks indicate extreme outliers, o marks outliers.**

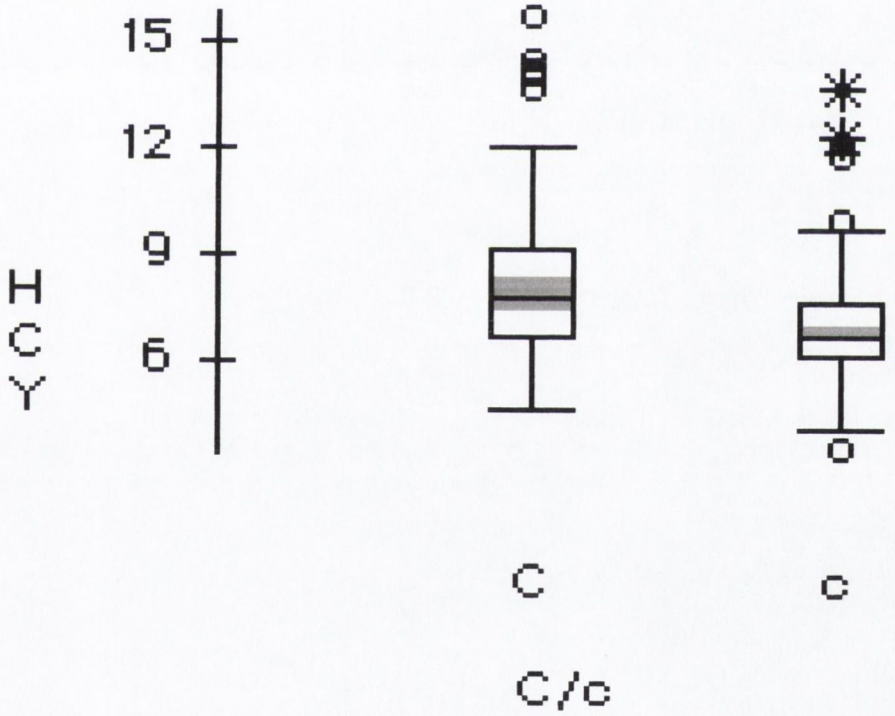


Table VI. Logistic regression model for confounding variables

Variables	Statistical Significance
Plasma homocysteine	p = 0.03
Plasma folate	p = 0.71
Vitamin B12	p = 0.47
Parity	p = 0.62
TT genotype	p = 0.77

9.3.3 DISCUSSION

This case-control study demonstrates that women who develop non-severe pre-eclampsia have higher plasma homocysteine levels in early pregnancy when compared to women who remain normotensive throughout pregnancy. This study supports the hypothesis that women who develop pre-eclampsia have a predisposition and that one predisposing factor is higher plasma homocysteine. These results provide further evidence to support our hypothesis that homocysteine may even be elevated prior to pregnancy causing endothelial cell dysfunction as an ongoing process and once pregnant serves as a predisposing factor to some other secondary influence leading to the development of pre-eclampsia.

The strengths of our study include the fact that our case and control populations were matched for several confounding variables including maternal age and gestation. Both of these parameters are known to affect plasma homocysteine levels (Weir & Scott 1998, Walker et al 1999). A possible confounder in any analysis of pre-eclampsia is the presence of different ethnic groups but in our study the population was of Irish background and genetically very homogenous. A limitation of our study is that we had insufficient numbers to examine the influence of MTHFR polymorphism on the risk of developing pre-eclampsia, such an association has been previously described (Grandone et al 1997, Sohda et al 1997). However recent studies from Pittsburgh and Germany do not concur with these findings (Powers et al 1999, Prasmusinto et al 2002).

The plasma homocysteine values obtained in this study were in general lower than those obtained in our previous report. We attribute this to a time lag of two years between analyses

coupled with instrument related changes. However the fact that we selected two matched controls per case allowed us to carry out a sub-analysis of cases against each of the two control groups. Both were significantly different (cases versus group 1 controls $p \leq 0.0001$, cases versus group 2 controls $p=0.0002$).

The significantly lower birthweights can be attributed to the shorter pregnancies in those patients with pre-eclampsia. The significantly shorter pregnancies and associated lower birthweights are appropriate for the clinical management of pre-eclampsia. However those women with a plasma homocysteine in the upper third delivered infants with a significantly lower birthweight suggesting a possible association between homocysteine induced endothelial dysfunction and fetal growth.

Although pre-eclampsia is traditionally a disease of first pregnancies, we included multigravida patients, matching for parity and included parity as a variable in the logistic regression analysis which demonstrated that parity was not a significant risk factor for the development of non-severe pre-eclampsia. We recognise that there may be an increased risk of recurrent pre-eclampsia in patients with a pre-existing elevated plasma homocysteine however we found no difference between those patients who developed pre-eclampsia in their first pregnancy compared to multiparous patients who developed pre-eclampsia. The logistic regression model demonstrates that homocysteine is an independent risk factor for the development of severe pre-eclampsia and the usual driving forces of elevated homocysteine levels, namely folate and vitamin B12 are not significant here.

A serious shortcoming in studying pre-eclampsia is the lack of a reliable test to identify women at risk for developing the disorder early enough in pregnancy to permit preventative treatment or prospective investigations. Markers consistent with underlying maternal disease may account for 20% to 40% of pre-eclampsia (Ness & Roberts 1996). The hypothesis that risk factors which predispose to pre-eclampsia can also predispose to cardiovascular disease in later life supports a possible role for homocysteine as an aetiological factor especially as women with a history of pre-eclampsia have a two fold increased risk of developing ischaemic heart disease (Smith et al 2002). While we recognise the limitations of a case control study, our results lend further strength to our original hypothesis. With a plasma tHcy $\geq$7.8μmol/l in early pregnancy, the risk of developing non severe pre-eclampsia in our study was 52%. Identification of different groups of women with more aetiologically homogenous disease groupings may result in earlier identification of women at risk for the development of pre-eclampsia.

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