GENOME DAMAGE AND FOLATE NUTRIGENOMICS IN UTEROPLACENTAL INSUFFICIENCY

Denise Lyndal Fleur Furness

GENOME DAMAGE AND FOLATE NUTRIGENOMICS IN UTEROPLACENTAL INSUFFICIENCY

Denise Lyndal Fleur Furness
BSc (Honours)

A thesis submitted for the degree of Doctor of Philosophy

University of Adelaide, School of Health Sciences, Discipline of Obstetrics and Gynaecology
AND
CSIRO Human Nutrition, Genome Health and Nutrigenomics Laboratory

October, 2007

This Thesis is
Dedicated to My Mother

Jann Cheryl Furness

All Religions, Arts And Sciences Are Branches Of The Same Tree. All These Aspirations Are Directed Toward Ennobling Man's Life, Lifting It From The Sphere Of Mere Physical Existence And Leading The Individual Towards Freedom.

ALBERT EINSTEIN

TABLE OF CONTENTS

1 ABSTRACT XII

2 DECLARATION XIII

3 ACKNOWLEDGEMENTS XIV

4 ABBREVIATIONS XVI

5 PUBLICATIONS XVIII

6 GENERAL INTRODUCTION 1

6.1 PREGNANCY AND UTEROPLACENTAL INSUFFICIENCY 1

6.1.1 Preeclampsia 3

6.1.2 Intrauterine growth restriction 4

6.1.3 Placental abruption 5

6.2 MATERNAL NUTRITION 6

6.2.1 Folate 6

6.2.2 Homocysteine 8

6.2.3 Vitamin-B_{12} 9

6.2.4 Vitamin-B_6 13

6.3 ONE-CARBON METABOLISM 14

6.3.1 One-carbon metabolism enzymes 16

6.4 GENOME DAMAGE AND NUTRIGENOMICS 18

6.4.1 Epigenetic modifications 20

6.5 GENOME DAMAGE, FOLATE NUTRIGENOMICS AND UTEROPLACENTAL INSUFFICIENCY 21

7 AIMS AND HYPOTHESES 25

7.1 AIM 25

7.2 HYPOTHESES 25

8 STUDY DESIGN AND GENERAL METHODOLOGY 26

8.1 STUDY DESIGN 26

8.1.1 Index pregnancies 26

8.1.2 Recruiting criteria 27

8.2 PATIENT CLASSIFICATION 27

8.2.1 High risk classification 27

8.2.2 Low risk classification *27*
8.2.3 Exclusion criteria *28*
8.3 CLINICAL DIAGNOSIS OF PREGNANCY OUTCOME 28
8.3.1 Primary outcomes *28*
8.3.2 Secondary outcomes *29*
8.4 PREGNANCY GROUPS 29
8.4.1 Demographic and clinical questionnaire *31*
8.4.2 Maternal blood collection *31*
8.4.3 Placental cord collection *31*
8.4.4 Placental cord DNA extraction *32*

9 MATERNAL GENOME DAMAGE AND UTEROPLACENTAL INSUFFICIENCY 33

9.1 AIM 33
9.2 HYPOTHESES 33
9.3 INTRODUCTION 33
9.4 METHODOLOGY 36
9.4.1 Study design and power calculations *36*
9.4.2 Rationale for DNA damage assessment in lymphocytes at 20 weeks gestation *37*
9.4.3 Whole blood 68 hour cytokinesis block micronucleus cytome assay *37*
9.4.4 Slide scoring criteria *39*
9.4.5 Statistics *45*
9.5 RESULTS 46
9.5.1 Age, BMI and smoking status in study groups *46*
9.5.2 Genome damage biomarker correlations with age, BMI and smoking *46*
9.5.3 Genome damage and cytotoxicity markers in low risk and an at risk population for pregnancy complications at 20 weeks gestation *47*
9.5.4 Genome damage and cytotoxicity markers in a low risk pregnancy population that developed normal and adverse outcomes *49*
9.5.5 Genome damage and cytotoxicity markers in a high risk pregnancy population that developed normal and adverse outcomes including UPI, PE and IUGR *50*
9.5.6 Genome damage and cytotoxicity markers in a in low risk healthy pregnancy (controls) and those who developed UPI including PE and IUGR *51*
9.5.7 Predictive values *54*
9.6 DISCUSSION 55
9.7 CONCLUSION 58

10 B-VITAMINS AND HOMOCYSTEINE IN UTEROPLACENTAL INSUFFICIENCY....59

10.1 AIM....59
10.2 HYPOTHESES....59
10.3 INTRODUCTION....59
10.4 METHODOLOGY....62
10.4.1 Blood, plasma and serum collection....62
10.4.2 Quantification of serum and red blood cell folate....62
10.4.3 Quantification of vitamin-B_{12} in serum....63
10.4.4 Quantification of vitamin-B_6 status in red blood cells via red cell aspartate amino-transferase (AST)....64
10.4.5 Quantification of total L-Homocysteine in plasma....64
10.4.6 Statistics....65
10.5 RESULTS....67
10.5.1 B-vitamin supplement results....67
10.5.2 Circulating B-vitamins and homocysteine results....71
10.5.3 Circulating B-vitamins in low risk pregnancies and those at high risk of developing UPI....76
10.5.4 Comparison of circulating micronutrient concentrations in low risk healthy pregnancies (controls) and women who developed uteroplacental insufficiency....78
10.5.5 Predictive values....81
10.6 DISCUSSION....82

11 POLYMORPHISMS IN ONE - CARBON METABOLISM GENES AND UTEROPLACENTAL INSUFFICIENCY....86

11.1 AIM....86
11.2 HYPOTHESES....86
11.3 INTRODUCTION....87
11.4 METHODOLOGY....90
11.4.1 DNA isolation from maternal granulocytes....90
11.4.2 DNA isolation from cord tissue....92
11.4.3 Polymorphism detection....92
11.4.4 Micronutrients and genome damage biomarker results....93
11.4.5 Statistics....93
11.5 RESULTS....94
11.5.1 Maternal polymorphisms....94

11.5.2 Fetal polymorphisms *97*
11.5.3 Index pregnancy analysis *101*
11.6 DISCUSSION 102

12 GLOBAL DNA METHYLATION AND UTEROPLACENTAL INSUFFICIENCY 104

12.1 AIM 104
12.2 HYPOTHESES 104
12.3 INTRODUCTION 104
12.4 METHODOLOGY 107
12.4.1 Whole blood lymphocyte isolation *107*
12.4.2 DNA isolation from frozen lymphocytes *108*
12.4.3 Global CpG methylation assay *108*
12.4.4 Statistics *109*
12.5 RESULTS 110
12.5.1 Descriptive statistics and Pearson's correlation of maternal and placental CpG methylation index *110*
12.5.2 Pearson's correlation of global CpG methylation with age, BMI and smoking 111
12.5.3 Pearson's correlation of global CpG methylation with vitamin supplement intake. *111*
12.5.4 Pearson's correlation of global CpG methylation with circulating maternal B-vitamins and plasma homocysteine *112*
12.5.5 Pearson's correlation of global CpG methylation with maternal genome damage 112
12.5.6 CpG methylation and maternal one-carbon metabolism genotypes *113*
12.5.7 Placental global CpG methylation and fetal/placental genotypes involved in one-carbon metabolism *114*
12.5.8 Global CpG methylation and the development of uteroplacental insufficiency .114
12.6 DISCUSSION 115

13 GENERAL DISCUSSION 118

13.1 FUTURE DIRECTIONS 120
13.2 CONCLUSION 124

14 REFERENCES 126

15 APPENDIX 166

Appendix 1 Pearsons correlation of genome damage markers age, BMI and smoking 166
Appendix 2: Alleleic discrimination output 167
Appendix 3 Distribution of various maternal alleles involved in one carbon metabolism genes in low risk pregnancies with normal and adverse outcomes 168
Appendix 4 Distribution of various maternal alleles involved in one carbon metabolism genes in high risk pregnancies with normal and adverse outcomes 169
Appendix 5 Distribution of various maternal alleles involved in one carbon metabolism genes in low risk healthy pregnancies and UPI 170
Appendix 6 Distribution of various maternal alleles involved in one carbon metabolism genes in low risk healthy pregnancies and PE 171
Appendix 7 Distribution of various maternal alleles involved in one carbon metabolism genes in low risk healthy pregnancies and IUGR 172
Appendix 8 Distribution of various maternal genotypes involved in one carbon metabolism genes in low risk pregnancies and pregnancies at high risk of developing UPI 173
Appendix 9: Distribution of various maternal genotypes involved in one carbon metabolism genes in low risk pregnancies with normal and adverse outcomes 174
Appendix 10: Distribution of various maternal genotypes involved in one carbon metabolism genes in high risk pregnancies with normal and adverse outcomes 175
Appendix 11: Distribution of various maternal genotypes involved in one carbon metabolism genes in low risk healthy pregnancies (controls) and uteroplacental insufficiency 176
Appendix 12: Distribution of various maternal genotypes involved in one carbon metabolism genes in low risk healthy pregnancies (controls) and those who develop preeclampsia 177
Appendix 13: Distribution of various fetal alleles involved in one carbon metabolism genes in low risk pregnancies with normal and adverse outcomes 178
Appendix 14: Distribution of various fetal alleles involved in one carbon metabolism genes in high risk pregnancies with normal and adverse outcomes 179

APPENDIX 15: DISTRIBUTION OF VARIOUS FETAL ALLELES INVOLVED IN ONE CARBON METABOLISM GENES IN LOW RISK HEALTHY PREGNANCIES AND UPI 180
APPENDIX 16: DISTRIBUTION OF VARIOUS FETAL ALLELES INVOLVED IN ONE CARBON METABOLISM GENES IN LOW RISK HEALTHY PREGNANCIES AND PE 181
APPENDIX 17: DISTRIBUTION OF VARIOUS FETAL ALLELES INVOLVED IN ONE CARBON METABOLISM GENES IN LOW RISK HEALTHY PREGNANCIES AND IUGR 182
APPENDIX 18: DISTRIBUTION OF VARIOUS FETAL GENOTYPES INVOLVED IN ONE CARBON METABOLISM GENES FROM HIGH RISK PREGNANCIES WITH NORMAL AND ADVERSE PREGNANCY OUTCOMES 183
APPENDIX 19: DISTRIBUTION OF VARIOUS FETAL GENOTYPES INVOLVED IN ONE CARBON METABOLISM GENES IN LOW RISK HEALTHY (CONTROL) PREGNANCIES AND THOSE WHO DEVELOP PREECLAMPSIA 184
APPENDIX 20: DISTRIBUTION OF VARIOUS FETAL GENOTYPES INVOLVED IN ONE CARBON METABOLISM GENES IN LOW RISK HEALTHY NORMAL (CONTROL) PREGNANCIES AND THOSE WHO DEVELOP IUGR 185
APPENDIX 21 DISTRIBUTION OF VARIOUS MATERNAL ALLELES INVOLVED IN ONE CARBON METABOLISM GENES IN LOW RISK HEALTHY NORMAL (CONTROL) PREGNANCIES AND THOSE WHO DEVELOP UPI IN THEIR INDEX PREGNANCY 186
APPENDIX 22 *CPG*LOBAL™ PROTOCOL IN DETAIL. 187

LIST OF TABLES

TABLE 1. COMPARISON OF AGE, BMI AND SMOKING STATUS 46
TABLE 2. THE DIFFERENCE IN GENOME DAMAGE MARKERS BETWEEN SMOKERS AND NON-SMOKERS 47
TABLE 3. DESCRIPTION OF MN-BN, NPB-BN, NBUD-BN, NDI, APOPTOSIS AND NECROSIS IN LOW RISK AND HIGH RISK PREGNANCY GROUPS AT 20 WEEKS GESTATION 47
TABLE 4. FREQUENCY OF MN-BN, MN-NPB, MN-NBUD, NDI, APOPTOSIS AND NECROSIS IN HIGH RISK AND LOW RISK PREGNANCY GROUPS 48
TABLE 5. FREQUENCY OF MN-BN, NPB-BN, NBUD-BN, NDI, APOPTOSIS AND NECROSIS IN A LOW RISK PREGNANCY GROUP....................49
TABLE 6. FREQUENCY OF MN-BN, NPB-BN, NBUD-BN, NDI, APOPTOSIS AND NECROSIS IN HIGH RISK PREGNANCIES THAT DEVELOPED ADVERSE OUTCOMES....................50
TABLE 7. FREQUENCY OF MNED BNS, NPBS, NBUDS, NDI, APOPTOSIS AND NECROSIS IN CONTROLS AND WOMEN WHO DEVELOPED UPI....................51
TABLE 8. POSITIVE PREDICTIVE VALUE (PPV), NEGATIVE PREDICTIVE VALUE (NPV), SENSITIVITY, SPECIFICITY, ODDS RATIO (OR) AND P VALUES AT DEFINED CUT OFFS OF MN-BN FREQUENCY FOR HIGH RISK WOMEN AND WOMEN WHO DEVELOPED UPI, PE AND IUGR 54
TABLE 9. PEARSONS CORRELATION OF B-VITAMIN SUPPLEMENT WITH AGE, BMI AND SMOKING 69
TABLE 10. B-VITAMIN SUPPLEMENTS AND CIRCULATING CONCENTRATIONS OF B-VITAMINS AND HOMOCYSTEINE 70
TABLE 11. PEARSONS CORRELATION OF CIRCULATING MICRONUTRIENTS WITH AGE AND BMI .71
TABLE 12. MEAN DIFFERENCE IN CIRCULATING B-VITAMINS AND HOMOCYSTEINE IN SMOKERS AND NON-SMOKERS....................71
TABLE 13. PEARSONS CORRELATION WITH CIRCULATING MICRONUTRIENTS AND HOMOCYSTEINE 72
TABLE 14. PEARSONS CORRELATION OF MATERNAL CIRCULATING B-VITAMINS AND HOMOCYSTEINE 73
TABLE 15. PEARSONS CORRELATION OF MATERNAL CIRCULATING B-VITAMINS AND HOMOCYSTEINE WITH GENOME DAMAGE MARKERS 74
TABLE 16. CIRCULATING B-VITAMIN AND HOMOCYSTEINE CONCENTRATIUONS AT 20 WEEKS GESTATION IN LOW RISK AND HIGH RISK PREGNANCIES 75

TABLE 17. CIRCULATING MICRONUTRIENT CONCENTRATIONS AT 20 WEEKS GESTATION IN HIGH RISK PREGNANCIES THAT DEVELOPED NORMAL OUTCOMES OR UPI, PE AND IUGR 76
TABLE 18. BLOOD MICRONUTRIENT CONCENTRATIONS AT 20 WEEKS GESTATION IN LOW RISK HEALTHY PREGNANCY CONTROLS AND WOMEN WHO DEVELOPED UTEROPLACENTAL INSUFFICIENCY 78
TABLE 19. POSITIVE PREDICTIVE VALUES (PPV), NEGATIVE PREDICTIVE VALUES (NPV), SENSITIVITY, SPECIFICITY, LIKELIHOOD RATIO (LR), ODDS RATIO (OR) AND P VALUES FOR HOMOCYSTEINE (HCY) WITH RESPECT TO HIGH AND LOW RISK PREGNANCIES, HIGH RISK PREGNANCIES WITH NORMAL OR ADVERSE OUTCOMES 82
TABLE 20. POSITIVE PREDICTIVE VALUES (PPV), NEGATIVE PREDICTIVE VALUES (NPV), SENSITIVITY, SPECIFICITY, LIKELIHOOD RATIO (LR), ODDS RATIO (OR) AND P VALUES FOR RED CELL FOLATE WITH RESPECT TO IUGR IN COMPARISON TO CONTROLS 82
TABLE 21. DISTRIBUTION OF MATERNAL POLYMORPHISMS IN LOW RISK HEALTHY PREGNANCIES (CONTROL) AND WOMEN WHO DEVELOPED IUGR..........95
TABLE 22. EFFECT OF MATERNAL POLYMORPHISMS ON CIRCULATING MICRONUTRIENT AND PLASMA HOMOCYSTEINE CONCENTRATIONS 96
TABLE 23. ASSOCIATION OF MATERNAL POLYMORPHISMS ON MATERNAL GENOME DAMAGE....97
TABLE 24. DISTRIBUTION OF VARIOUS FETAL POLYMORPHISMS IN ONE CARBON METABOLISM .98
TABLE 25. DISTRIBUTION OF VARIOUS FETAL POLYMORPHISMS IN ONE CARBON METABOLISM FROM LOW RISK HEALTHY (CONTROL) PREGNANCIES AND THOSE WHO DEVELOPED UTEROPLACENTAL INSUFFICIENCY 99
TABLE 26. ASSOCIATION OF FETAL POLYMORPHISMS WITH CIRCULATING MICRONUTRIENTS AND HOMOCYSTEINE CONCENTRATIONS IN THE MOTHER..........100
TABLE 27. DISTRIBUTION OF VARIOUS MATERNAL GENOTYPES INVOLVED IN ONE CARBON METABOLISM GENES IN LOW RISK HEALTHY NORMAL (CONTROL) PREGNANCIES AND THOSE WITH UPI IN THEIR INDEX PREGNANCY 101
TABLE 28 MATERNAL AND PLACENTAL CPG METHYLATION INDEX DESCRIPTIVE STATISTICS.110
TABLE 29 PEARSON'S CORRELATIONS OF CPG GLOBAL METHYLATION WITH AGE, BMI AND SMOKING STATUS IN MATERNAL LYMPHOCYTE AND PLACENTAL DNA..........111
TABLE 30 PEARSON'S CORRELATION OF CPG GLOBAL METHYLATION WITH B-VITAMIN SUPPLEMENT INTAKE IN MATERNAL AND PLACENTAL DNA..........111
TABLE 31 PEARSON'S CORRELATION OF GLOBAL CPG GLOBAL METHYLATION WITH CIRCULATING B-VITAMINS AND PLASMA HOMOCYSTEINE IN MATERNAL AND PLACENTAL DNA..........112
TABLE 32 PEARSON CORRELATION OF GLOBAL CPG METHYLATION IN MATERNAL LYMPHOCYTE AND PLACENTAL DNA WITH CBMN ASSAY BIOMARKERS IN MATERNAL LYMPHOCYTES 112

TABLE 33. MATERNAL AND PLACENTAL GLOBAL CPG METHYLATION IN RELATION TO POLYMORPHISMS 113
TABLE 34 PLACENTAL GLOBAL CPG METHYLATION IN RELATION TO POLYMORPHISMS 114
TABLE 35 MEAN CPG METHYLATION STATUS IN PREGNANCY GROUPS 115
TABLE 36. DISTRIBUTION OF FETAL POLYMORPHISMS IN CONTROL AND IUGR PREGNANCIES.185

LIST OF FIGURES

FIGURE 1. UTEROPLACENTAL CIRCULATION 2
FIGURE 2. STRUCTURES OF FOLATE 7
FIGURE 3 VITAMIN B_{12} ABSORPTION AND TRANSPORT. 10
FIGURE 4. VITAMIN-B_{12} 12
FIGURE 5 THREE NATURAL FORMS OF VITAMIN-B_6 AND THE ACTIVE COENZYME PYROXIDINE PHOSPATE 13
FIGURE 6. A SIMPLIFIED SCHEME OF ONE-CARBON METABOLISM. 15
FIGURE 7. A SCHEMATIC DIAGRAM SHOWING HOW DEFECTS IN ONE-CARBON METABOLISM CAN LEAD TO UPI 24
FIGURE 8 PREGNANCY GROUP BASED ON CLINICAL OUTCOME 30
FIGURE 9. CYTOKINESIS BLOCK MICRONUCLEUS CYTOME ASSAY 68 HOUR CULTURE PROTOCOL FOR WHOLE BLOOD. 38
FIGURE 10. THE VARIOUS ENDPOINTS SCORED USING THE CBMN CYTOME ASSAY. 40
FIGURE 11. VARIOUS CELL ENDPOINTS. 44
FIGURE 12. FREQUENCY OF MN-BN IN LOW RISK AND HIGH RISK PREGNANCY GROUPS....................48
FIGURE 13. NDI AT 20 WEEKS GESTATION IN LOW RISK AND HIGH RISK PREGNANCY GROUPS...49
FIGURE 14. FREQUENCY OF MN-BN IN CLINICALLY NORMAL PREGNANCIES AND UPI....................52
FIGURE 15. FREQUENCY OF APOPTOTIC CELLS IN LOW RISK HEALTHY PREGNANCIES (CONTROLS) AND UPI 52
FIGURE 16. FREQUENCY OF MN-BN IN LOW RISK HEALTHY PREGNANCIES (CONTROLS) AND PREECLAMPSIA....................53
FIGURE 17. FREQUENCY OF MN-BN IN LOW RISK HEALTHY PREGNANCIES (CONTROLS) AND IUGR....................53
FIGURE 18. FOLIC ACID, VITAMIN-B_{12} AND B_6 SUPPLEMENT INTAKE....................68
FIGURE 19. HCY CONCENTRATIONS IN WOMEN WHO HAD A NORMAL PREGNANCY OR DEVELOPED UPI WITHIN THE HIGH RISK PREGNANCY GROUP. 77
FIGURE 20. PLASMA HOMOCYSTEINE CONCENTRATION IN WOMEN WITH LOW RISK HEALTHY PREGNANCIES (CONTROLS) AND WOMEN WHO DEVELOPED UPI.....................79
FIGURE 21. PLASMA HOMOCYSTEINE CONCENTRATION IN WOMEN WITH LOW RISK HEALTHY PREGNANCIES (CONTROLS) AND WOMEN WHO DEVELOPED IUGR. 80
FIGURE 22. RED CELL FOLATE CONCENTRATION IN LOW RISK HEALTHY PREGNANCIES (CONTROLS) AND WOMEN WHO DEVELOPED IUGR. 80

FIGURE 23. A SIMPLIFIED SCHEME OF ONE-CARBON METABOLISM. 89
FIGURE 24 EPIGENETIC SILENCING OF TRANSCRIPTION. 106
FIGURE 25 MSP I AND HPA II METHYLATION SENSITIVE RESTRICTION ENZYMES. 109
FIGURE 26 SCHEMATIC MECHANISM FOR DEVELOPMENT OF UPI 124
FIGURE 27. ALLELEIC DISCRIMINATION OUTPUT 167

1 ABSTRACT

Pregnancy complications associated with placental development affect approximately one third of all human pregnancies. Genome health is essential for placental and fetal development, as DNA damage can lead to pregnancy loss and developmental defects. During this developmental phase rapid DNA replication provides an increased opportunity for genome and epigenome damage to occur[1].

Maternal nutrition is one of the principal environmental factors supporting the high rate of cell proliferation and differentiation. Folate functions in one-carbon metabolism and regulates DNA synthesis, DNA repair and gene expression[1]. Deficiencies or defects in gene-nutrient interactions associated with one-carbon metabolism can lead to inhibition of cell division, cell cycle delay and an excessive apoptotic or necrotic cell death rate[2], which may affect placentation.

This study is the first to investigate the association between genomic damage biomarkers in late pregnancy complications associated with uteroplacental insufficiency (UPI) including preeclampsia and intrauterine growth restriction (IUGR). The results indicate that genome damage in the form of micronucleated cells in peripheral blood lymphocytes at 20 weeks gestation is significantly increased in women at risk of developing an adverse pregnancy outcome. The observed OR for the high micronuclei frequency may be the highest observed for any biomarker selected in relation to risk of pregnancy complications to date (15.6 – 33.0). In addition, reduced apoptosis was observed in association with increased micronuclei, suggesting that the cells may have escaped specific cell-cycle checkpoints allowing a cell with DNA damage to proceed through mitosis.

This study demonstrated that an increase in plasma homocysteine concentration at 20 weeks gestation is associated prospectively with the subsequent development of UPI, indicating a causal relationship. The *MTR* 2756 GG genotype was significantly associated with increased plasma homocysteine concentration and UPI. Furthermore, the *MTHFD1* 1958 single nucleotide polymorphism was associated with increased risk for IUGR.

2 DECLARATION

This work contains no material which has been submitted for the award of any other degree or diploma in any University or other tertiary institution, and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University of Adelaide Library, being made available in all forms of media, now and hereafter known.

……………………… ……./……./…….......

Denise Furness

3 ACKNOWLEDGEMENTS

I wish to express my sincerest thanks to Dr Michael Fenech for his invaluable guidance, his comprehensive training in the scientific process and the opportunity to challenge myself. I would like to thank Prof Gus Dekker for his encouragement, enthusiasm and for the opportunity to continue this work into a post-doctoral position. I would also like to thank Prof Yee Khong for his patience and support, especially while writing the thesis. Also, I would like to acknowledge Dr Bill Hague for his kindness and support. I am very grateful to have had such wonderful supervisors, each with different strengths, and I believe we were a great team and have managed to accomplish a great project.

I was blessed with meeting Sasja Beetstra, who took me under her wing when I first arrived in Adelaide to pursue this PhD project. I thank you for your support in both my work and personal life. I would also like to thank Bianca Benassi for helping to keep me sane while writing this thesis. I will never forget the fun times in our office “The Hot Spot”, one can only dream of having such caring and genuine people to share an office with. I would also like to acknowledge staff and students at CSIRO Human Nutrition who have provided assistance or entertained me while on the road to completion of this PhD, including Olgatina Bucco, Jane Bowen, Michelle Zucker, Nathan O’Callaghan, Maryam Hor, Guy Abell and Pamela Tyrone.

I am grateful to have worked with such a wonderful team at the Women’s and Children’s Hospital. My deepest thanks go to Denise Healy for helping to get this project up and running, for her comic relief and ongoing support through this PhD. I must express many thanks to Michelle Cox for her positive nature and great organisational skills. It has been a pleasure spending time with you in and out of our work environment. I would also like to thank Nayana Parange for her wonderful hugs, friendship and advice through all the ups and downs. Furthermore, I must thank all of the pregnant women who donated their time, blood and placentas for this research, especially Megan Veal for allowing me to view the birth of her beautiful baby.

I am very grateful to Derek Hamer who, while dealing with his own PhD horrors, has offered support and continually listened to both my personal and work related concerns. I would also like to thank my new colleagues Rachael Nowak, Amanda Sferruzzi-Perri, Kirsty Pringle and Gary Heinemann for putting up with my stress levels while finalising this thesis. I would

especially like to thank A/Prof Claire Roberts for her encouragement and patience over these last few months, I am truly grateful for your assistance and support.

A special thank you to Rafal Radzikowski, you were the closet person to me during my PhD experience and despite all that has happened I truly value the time we had together. I thank you for coming into CSIRO every weekend and waiting while I completed my experiments. I thank you for picking me up late at night and dropping me home, even though I insisted I didn't need a lift home, it was nice to have someone so concerned for my welfare. I thank you for convincing me that I needed at least one day off and for inviting me to Polish lunch with your family every Sunday. I thank your family, especially your Mum Teresa, for making me feel like I had a family here in Adelaide regardless of the language barrier and cultural differences.

Last, but not least I would like to thank my Father Raymond Leslie Furness. This journey would not have been possible without the love and support from both you and Mum. I'm sorry that I have not been home to help care for Mum. I appreciate your selflessness and encouragement to continue my studies and focus on my career. The past four years have been an internal struggle as I battle with thoughts that I should be with Mum in these precious times. However, you have always insisted that this is my life and I need to do what is best for me. Thank you for everything, you are an amazing father, I love you very much.

4 ABBREVIATIONS

AST: Aspartate amino-transferase

B_{12}: Vitamin-B_{12} (cobalamin)

B_6: Vitamin-B_6

BN: Binucleate cell

BMI: Body mass index

CBMN: Cytokinesis-block micronucleus assay

CH_3: Methyl group

CpG: Cytosine-guanine dinucleotide

DHF: Dihydrofolate

DNA: Deoxyribonucleic acid

Dnmt: DNA methyltransferase genes

FBP: Folate binding protein

$_dH20$: Distilled water

Hcy: Homocysteine

HUMN: The international collaborative project on micronucleus frequency in human populations

IL-8: Interleukin 8

IUGR: Intrauterine growth restriction

(ICF) Immunodeficiency centromeric region instability and facial anomalies syndrome

MCP-1: Monocyte chemoattractant protein 1

MeCP2: Methyl CpG binding protein 2

mCyt: Methylated cytosines

MN: Miconuclei

MN-BN(s): Micronucleated binucleate cell(s)

MTHFR: Methylenetetrahydrofolate reductase

MTHFD1: Methylenetetrahydrofolate dyhydrogenase

MTR: Methionine synthase

MTRR: Methionine synthase reductase

NBUD: Nuclear bud

NBUD-BN(s): binucleated cells with nuclear bud(s)

NDI: Nuclear division index

NPB(s): Nucleoplasmic bridge(s)

NPB-BN(s): Binucleated cells with nucleoplasmic bridge(s)

NTDs: Neural tube defects

NO: Nitric oxide

PBS: Phosphate buffered saline

PE: Preeclampsia

PlGF: Placental growth factor

PLP: Pyridoxal 5-phosphate

RBC: Red blood cell

RCF: Red cell folate

RDI: Recommended daily intake

SAM: S-adenosylmethionine

SF: Serum folate

SGA: Small for gestational age

sFlt-1: Soluble fms-like tyrosine kinase-1

SNPs: Single nucleotide polymorphisms

dTMP: Thymine

THF: Tetrahydrofolate;

dUMP: Uracil

UPI: Uteroplacental insufficiency

VEGF: Vascular endothelial growth factor

5 PUBLICATIONS

Furness DL., Fenech MF., Khong TY., Hague WM., Dekker GA**.** *Evaluation of the use of the CBMN assay to determine inter-individual variation in spontaneous and folate deficiency-induced genome damage in humans.* Proc Nutr Soc Aust 2004, Vol. 28. Asia Pacific Journal of Clinical Nutrition, 2004; 13 (Suppl):S56 - Abstract

Furness D., Dekker G., Khong Y., Hague B., Fenech M. *The Role of Genome Damage and Nutrigenomics in Uteroplacental Insufficiency.* American Journal of Obstetrics and Gynecology, 2006, Dec; 195(6):S14 - Abstract

Furness D., Parange N., Dekker G., Fenech M. (2006) *Role of Genome Damage and Uterine Artery Doppler in Prediction of Uteroplacental Insufficiency*. American Journal of Obstetrics and Gynecology, 2006, Dec; 195(6):S221 - Abstract

Parange N., **Furness D.,** Fenech M., Wilkinson C., Dekker G. *Role of uterine artery doppler and the folate metabolic pathway in prediction of uteroplacental insufficiency.* American Journal of Obstetrics and Gynecology, 2006, Volume 195, Issue 6, Pages S207-S207-Abstract

6 GENERAL INTRODUCTION

6.1 Pregnancy and uteroplacental insufficiency

When considering the fact that couples, who have intercourse regularly without the use of contraception, only have a 25–30% chance of becoming pregnant[3] on a monthly basis, that 70-75% of blastocysts created are able to implant and that only 58% of the blastocysts that implant survive past the second week[4], one comes to understand the miracle of reproduction. Even after these hurdles are overcome, there are still many variables that can affect the likelihood of a successful pregnancy. Preeclampsia (PE)[5-13], intrauterine growth restriction (IUGR)[13-15] and placental abruption[16-18] are common pregnancy complications, characterised by impaired trophoblast invasion and inadequate remodelling of the uterine spiral arteries. These pregnancy complications are of concern in both western and developing countries[19].

Invasive trophoblasts are the key to remodelling the uterine spiral arteries[5, 12, 20-24], which is an essential step in healthy pregnancy. Trophoblasts are unique cells derived from the outer cell layer of the blastocyst which mediate implantation and placentation[25]. Cytotrophoblast stem cells of the placenta give rise to the differentiated forms of trophoblasts depending on their subsequent function *in vivo*, i.e 1) hormonally active villous syncytiotrophoblasts, 2) extravillous anchoring trophoblastic cell columns, or 3) invasive intermediate trophoblasts[5, 12]. The action of the invasive trophoblasts on the maternal spiral arteries leads to the conversion of the normally narrow-calibre spiral arteries to a very low resistance uteroplacental circulation which facilitates the marked increase in blood flow seen in these arteries at term[5, 24, 26] (Figure 1).

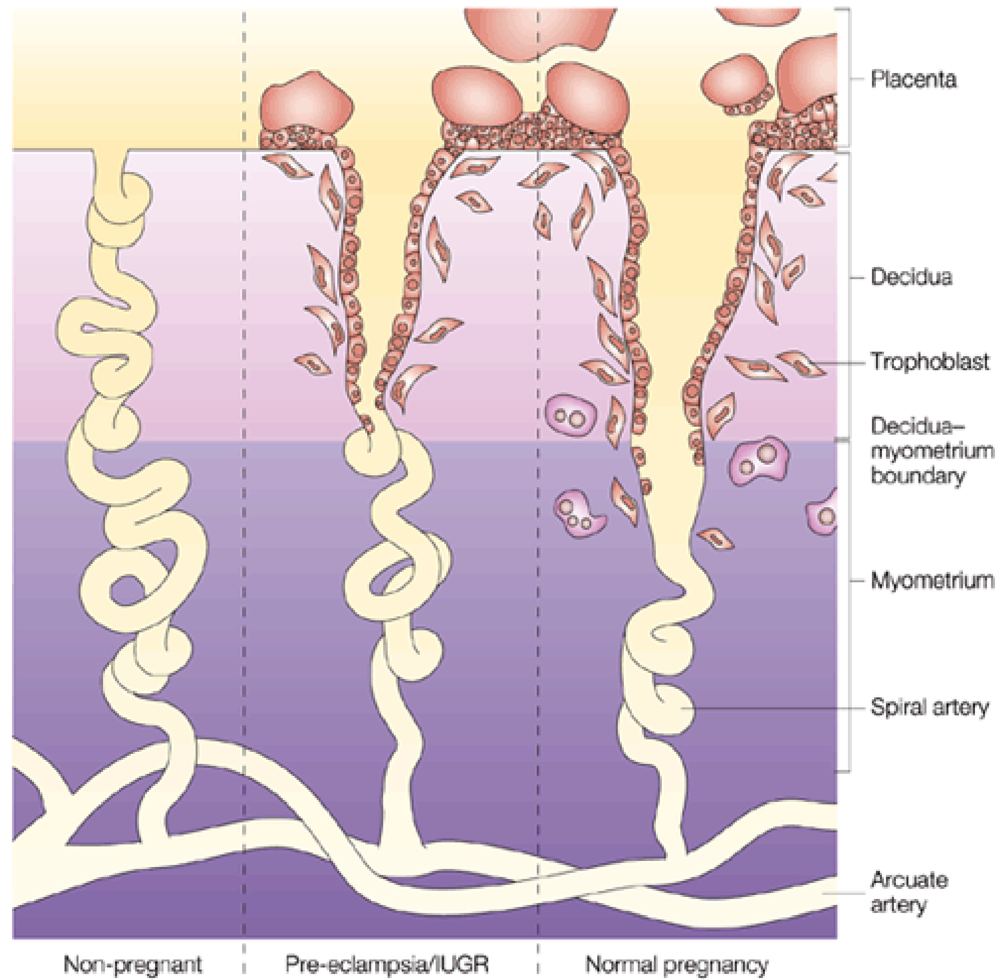

Figure 1. Uteroplacental circulation

A comparison between uninvaded arteries (non-pregnant), and arteries complicated with PE/IUGR, and normal pregnancy arteries. The extent and depth of trophoblast invasion is less in PE/IUGR compared with normal pregnancy, which results in inadequate transformation of the spiral arteries in the former and consequently, reduced blood flow to the feto-placental unit and poor fetal growth.

Adapted from Moffet-King [27].

In the non-pregnant state the uterine arteries carry < 1% of the maternal cardiac output[28] and the uterus weighs approximately 50 g. At term the same arteries support a uterus, placenta and fetus that can collectively weigh up to 5000 g[12, 29]. Comparison of spiral arteries in the non-pregnant state to those at term demonstrates that these arteries can increase up to tenfold in diameter, resulting in an increase of blood flow by a factor of 10,000[12] (Figure 1). Reduced blood flow from the uterus to the placenta, termed uteroplacental insufficiency (UPI)[30], is caused by incomplete transformation of the maternal spiral arteries[8, 15] and can result in decreased nourishment and oxygen to the fetus and, consequently, malnutrition, hypoxia and, ultimately, asphyxia[31]. Decreased or absent trophoblast invasion of the maternal spiral arteries is the most common clinical finding in pathological studies of spiral arteries from pregnancies complicated by PE[5-13, 32, 33]. In addition, several studies have reported the same morphological abnormalities in spiral arteries in pregnancies complicated with IUGR[14, 15].

6.1.1 Preeclampsia

Preeclampsia (PE) is a leading cause of maternal and fetal morbidity and mortality (15–20%) in developed countries[34]. Known risks for PE include diabetes, pre-existing hypertension, kidney disease, or having previously had the condition during an earlier pregnancy. Symptoms associated with PE include hypertension and proteinuria, which is the result of proteins, normally confined to the blood, spilling into the urine due to damaged blood vessels in the kidneys[35]. In some cases, PE may progress to eclampsia, a series of potentially fatal seizures. In modern obstetrics eclampsia has almost disappeared due to medically induced premature deliveries in uncontrolled or threatening cases of PE[36, 37]. Although the hypertension accompanying PE can be treated with blood pressure lowering drugs, the only effective treatment for the condition is delivery of the placenta, hence the central role of the placenta in the pathogenesis of PE is undisputed[7, 38-49]. Pathophysiological processes underlying PE are very complex and poorly understood. To date studies have related oxidative stress, placental ischaemia, maternal-fetal immune maladaption and possible genetic factors (such as polymorphisms in MTHFR) with the development of this disease[46].

Preeclampsia is characterised by endothelial cell damage and vasospasm which decreases blood flow causing a reduction in maternal oxygen supply to vital organs such as the brain,

kidneys, liver[50] and the placenta[51]. In addition, decreased blood supply, can also reduce the fetal nutrient supply which is required for normal development.

Studies[34, 46] have shown that the interaction between endovascular trophoblast and decidual leukocytes, especially natural-killer cells, results in substantial angiogenic growth factor release. Raised concentrations of angiogenic growth factors, such as placental growth factor (PlGF) and vascular endothelial growth factor (VEGF) are important in maintaining normal endothelial function[52], in particular PlGF, which is essential for early placental development and has been associated with both PE and IUGR. Maynard et al.[52] showed that placenta-derived soluble fms-like tyrosine kinase-1 (sFlt-1), an antagonist of VEGF and PlGF, is upregulated in PE, leading to increased systemic amounts of sFlt1 that fall after delivery. Raised circulating sFlt1 in PE is associated with lowered circulating concentrations of free VEGF and PlGF, resulting in endothelial dysfunction. The magnitude of increase in sFlt correlates with disease severity[34, 46, 52, 53], suggesting that the balance of VEGF and soluble Flt is closely implicated in one of the final pathophysiological pathways associated with PE.

Several biomarkers for predicting PE have been tested in a variety of patient populations, including activin A[54] and fibronectin[55], with generally unsatisfactory results[56]. Current studies are exploring the use of increased fetal cell trafficking and cell-free DNA from 20 weeks gestation in the maternal circulation as a biomarker for women who developed PE[57, 58]; however, these biomarkers appear to have a low sensitivity and specificity[59].

6.1.2 Intrauterine growth restriction

Birthweight is a key indicator of a baby's health status and also of their future health as adults. A low birth weight is defined as less than 2,500 g and very low birthweight as less than 1,500 g[60]. Low birth weight babies have a greater risk of poor health and dying, often requiring longer hospitalisation after birth and are more likely to develop disabilities[61, 62]. For most growth restricted infants, brain growth is spared relative to the rest of the body; however, as adults these individuals are at risk for multiple morbidities including cardiovascular disease[19, 63], hypertension[64], stoke[65] and type-2-diabetes[63, 66].

In the late 1960's, two landmark studies reported intrauterine growth patterns for the first time[67, 68]. Two subsequent studies reported that disproportionate growth at a given gestational age was related to neonatal mortality risk[69, 70]. These studies have led to the

use of reference growth curves to characterise an infant's growth relative to gestational age for over 30 years[71].

Intrauterine growth restriction (IUGR) implies that fetal growth is being inhibited and that the fetus does not attain its full growth potential. This functional definition seeks to identify fetuses at risk for modifiable but otherwise poor outcomes. IUGR is the second leading cause of perinatal morbidity and mortality[72]. In Australia approximately 6.5 % of babies are born growth resticted[60]; however, the incidence varies depending on the population under examination (including its geographic location) and the standard growth curves used as a reference[73]. In addition, the term IUGR excludes fetuses that are small for gestational age (SGA). SGA is defined as growth at the 10th or less percentile for weight of all fetuses at that gestational age[74]. Not all fetuses that are SGA are pathologically growth restricted. Similarly, not all fetuses that have not met their genetic growth potential are less than the 10th percentile for estimated fetal weight[74].

Multiple factors have been identified in relation to IUGR; however, studies have shown that aside from uteroplacental insufficiency the maternal environment is a major determinant of newborn weight[75]. Clinical factors associated with IUGR include multiple gestation, chromosomal anomalies, infections and various maternal disorders including anaemia, severe chronic asthma and hypertension[76]. Furthermore, maternal stress factors[77], including narcotic addiction[78, 79], cigarette smoking[80, 81] and alcoholism[82] have been associated with fetal growth restriction.

To date the relationship between reduced uteroplacental circulation, IUGR and the maternal syndrome of PE is not clear. Factors that may differentiate growth restriction from the maternal syndrome of PE include obesity[83], insulin resistance[84], thrombophilia[85] and hyperhomocysteinaemia[86]. In addition to PE and IUGR, reduced uteroplacental circulation has been associated with placental abruption[16-18].

6.1.3 Placental abruption

Placental abruption describes the early separation of a normal placenta from the wall of the uterus. Premature separation of the placenta occurs in 1% of all pregnancies[87] and this is associated with excessively high rates of stillbirth, preterm birth, reduced fetal growth and maternal death[87-91]. Placental abruption accounts for 20–25% of antepartum haemorrhages[87]. The perinatal mortality rate associated with placental abruption varies

between 2% and 67%, depending on gestational age, fetal weight, and the degree of abruption[91]. Over 50% of all perinatal deaths occur before delivery[92]. While the aetiology of placental abruption remains largely speculative, risk factors include advanced maternal age, multiparity, cocaine use during pregnancy, PE, intrauterine infection, oligohydramnios, prolonged rupture of membranes and prior abruption[87, 91, 93-95].

As for PE and IUGR, the cause(s) of placental abruption are unknown, but these pregnancy complications are associated with abnormal placental development leading to inadequate uteroplacental circulation[96] and can be grouped under the collective term uteroplacental insufficiency (UPI).

6.2 Maternal nutrition

Maternal nutrition is one of the principal environmental factors supporting the high rates of cell proliferation and differentiation necessary for placental and fetal development[97]. Nutrition is important for DNA synthesis as cofactors for enzymes involved in DNA synthesis and substrates for base synthesis are derived from the diet. Moreover, B-vitamins are involved in synthesis of DNA precursors (i.e dTTP and 5-methy cytosine); however, many individuals do not meet their recommended daily intake (RDI) in B-vitamins. Moreover, B-vitamin absorption is affected by substances such as alcohol, drug intake and possibly, to a lesser extent, oral contraceptives[98], making supplementation essential in people with inadequate dietary consumption, malabsorption, alcoholism and drug use. In addition, recent studies have suggested that regular use of a multivitamin supplements in the periconceptional period may help to prevent PE[99] and lower rates of severe preterm births and extreme SGA[100].

6.2.1 Folate

Folates are a group of inter-convertible substrates, differing by their oxidation state, number of glutamic acid moieties and one-carbon substitutions[101] (Figure 2). They are involved in amino acid metabolism, purine and pyrimidine synthesis and methylation of a large number of nucleic acids, proteins and lipids[102, 103]. The term folate represents all forms of vitamin-B_9, including the many derivatives found in biological systems. Folic acid (pteroylmonoglutamic acid) is the synthetic but more stable form and, therefore, is typically used in tablets and fortified foods. The function of folate is to provide one-carbon units (methyl, methylene, methenyl, formyl, or formimino)[104] for biosynthetic purposes.

Folate is present in a wide variety of foods, such as green-leafy vegetables, liver, bread, yeast and fruits[105]. After absorption from the gastrointestinal tract, folate is hepatically converted to tetrahydrofolic acid which is a cofactor in the biosynthesis of purines and thymidylates of nucleic acids. The liver contains about 50% of total body folate stores[106].

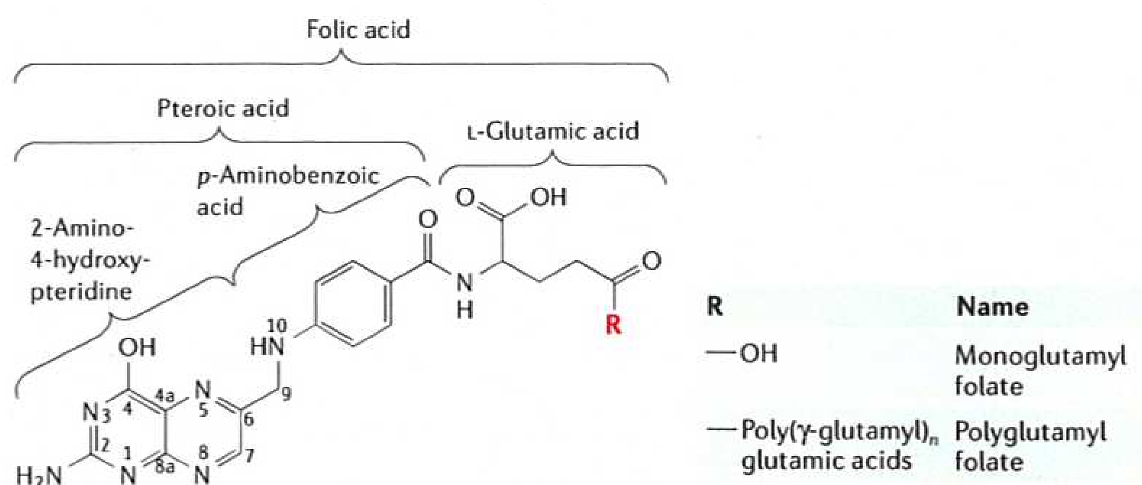

Figure 2. Structures of folate

Folic acid is the monoglutamated form of folate. Polyglutamation prevents transport of folate out of the cell.

The influence of folate nutritional status on various pregnancy outcomes has long been recognised[107]. Studies have shown that women receiving folic acid supplementation had better quality oocytes and a higher degree of mature oocytes compared to women who did not receive folic acid supplementation[108]. Moreover, it has been shown that folic acid present in preimplantation embryos is absolutely essential for early embryo development[101]. Furthermore, placental abruption, recurrent pregnancy loss, IUGR and PE which are all thought to arise due to defects within the placental vascular bed, have been associated with deficiencies in folate intake or metabolism[109].

Prenatal and periconceptional folic acid supplementation and folic acid fortification of staple foods may well be ranked among the most significant public health measures for the prevention of pregnancy-related complications[110-112]. Chanarin et al[113], reviewed many early studies on folate in pregnancy with the overall conclusion that pregnancy was associated

with an increased folate demand and that circulating folate concentrations declined (~10 nmol/L serum folate) in pregnant women who do not supplement their diet with folic acid[113-116]. Furthermore, the significance of folate in preventive medicine was shown in a series of papers by the Medical Research Council Vitamin Study Group in 1991, which documented how periconceptional folate prevents spina bifida[117].

6.2.2 Homocysteine

Homocysteine (Hcy), the demethylated derivative of the essential amino acid methionine, is a thiol (SH)-containing amino acid, involved in two major metabolic pathways in human physiology[118]. Under normal conditions, the intracellular concentration of Hcy is maintained at a low level as a result of remethylation reactions and catabolism via the transsulfuration pathway; however, several genetic and nutritional factors can alter the normal state of Hcy metabolism[119, 120]. These factors include deficiencies of folate, vitamin-B_{12}, vitamin-B_6 and single nucleotide polymorphisms (SNPs) within enzymes involved in one-carbon metabolism that result in decreased enzyme activity[118, 121].

In 1995 a meta-analysis of 27 studies with a total of more than 4000 patients provided evidence that Hcy was an independent, graded risk factor for atherosclerotic disease in the coronary, cerebral and peripheral vessels[122]. Elevated Hcy has been associated with an increased risk for placental diseases as it can manifest within maternal veins (thrombosis), arteries or spiral arteries supplying the placenta[123]. Moreover, excess Hcy has been linked with a number of developmental abnormalities, in particular neural tube defects (NTDs)[124, 125], IUGR[123], PE[126-130], placental abruption[109, 131], preterm birth, repeated miscarriage[132] and intrauterine fetal death[129, 133-135]. In addition, Khong and Hague[133] observed the absence of trophoblast-induced remodelling of spiral arteries, which is a feature of UPI, in the placenta from women with hyperhomocysteinemia.

Accumulation of circulating Hcy may have multiple biological effects. It has been reported to impair methylation activity and is believed to be thrombogenic and atherogenic[136]. The mechanism behind these vascular effects has not been identified but platelet abnormalities, stimulated coagulation or inhibited fibrinolysis, smooth muscle cell proliferation, LDL oxidation and endothelial dysfunction may be associated with these vascular effects, as demonstrated in experimental systems[137]. In addition, vascular inflammation has been shown to be induced by Hcy enhancing the expression of pro-inflammatory cytokines, such as monocyte chemoattractant protein 1 (MCP-1), which regulates migration and activation of

monocytes/macrophages, and interleukin 8 (IL-8), which is an important chemoattractant for neutrophils and T-lymphocytes[138]. A recent clinical trial demonstrated that Hcy can interfere with the function of nitric oxide (NO)[139, 140], one of the major endothelium-dependent vasodilators, through its pro-oxidant effects[141].

There is increasing evidence suggesting that Hcy is a mediator of endothelial cell dysfunction and that it may be the cause of vascular complications associated with UPI[123, 126, 142, 143]. The mechanism of endothelial cell dysfunction relies either on auto-oxidation of the highly reactive thiol group of Hcy[144] or on the formation of intracellular superoxide anions (O_2-) and hydrogen peroxide (H_2O_2) radicals with concomitant inhibition of cellular antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase[145]. Endothelial cell dysfunction may cause arteriolar vasoconstriction in hepatic, cardiac, and cerebral circulations which and is a central feature of the pathophysiological mechanism of PE and IUGR[56, 146].

6.2.3 Vitamin-B_{12}

Cobalamin (vitamin-B_{12}), an organometallic compound in which a cobalt atom is situated within a corrin ring, is the most chemically complex of all the vitamins and is the only known biomolecule with a stable carbon-metal bond[147]. Vitamin-B_{12} is an essential cofactor for two enzymes in human cells. In the methionine synthase (MTR) reaction, Hcy is converted to methionine allowing for the “recycling” of 5-methyl-tetrahydrofolate (THF) to 5,10 methylene-THF which is needed for the *de novo* synthesis of dTMP and ultimately, for DNA synthesis. Since conversion of 5,10-methylene-THF to 5-methyl-THF is irreversible, when Hcy is limited folate is trapped as 5-methyl-THF. Concurrently, Hcy accumulates while methionine decreases, leading to a decrease in S-adenosylmethionine (SAM), which may limit DNA methylation affecting gene expression[148, 149].

An understanding of the vitamin-B_{12} absorption cycle helps illuminate the potential causes of deficiency. During gastric digestion, vitamin-B_{12} is released from the ingested food and forms a stable complex. Upon entering the duodenum, the complex is digested releasing the vitamin-B_{12}, which then binds to intrinsic factor (IF), a glycoprotein produced by the parietal cells of the stomach. The vitamin-B_{12}–IF complex is resistant to proteolytic digestion and reaches the distal ileum, where it is absorbed by specific receptors. In the ileal mucosal cell the IF is destroyed and the vitamin-B_{12} is transferred to transcobalamin (TC) II (Figure 3). The vitamin-B_{12}–TCII complex is then secreted into the circulation, from which it is rapidly

taken up by the liver, bone marrow, and other cells[150]. In addition, an increased bacterial load can bind significant amounts of vitamin-B12 in the gut, preventing its absorption [151, 152].

NOTE: This figure is included on page 10 in the print copy of the thesis held in the University of Adelaide Library.

Figure 3 Vitamin B12 absorption and transport.

The acidic environment of the stomach facilitates the breakdown of vitamin-B12 which is bound to food. Intrinsic factor (IF), which is released by parietal cells in the stomach, binds to vitamin-B12 in the duodenum and subsequently aids in the absorption of vitamin-B12 in the terminal ileum. Adapted from Oh et al. [148]

A number of cross-sectional, case-control and longitudinal studies reported that blood concentration of vitamin-B_{12} drops during normal pregnancy[153-155]. Haemodilution, altered renal function, hormonal changes, changes in the concentration of vitamin-B_{12} binding proteins, and maternal-fetal vitamin-B_{12} transfer are normal physiological consequences of pregnancy that affect plasma vitamin-B_{12} concentrations in the mother. In addition, there is an increased requirement for vitamin-B_{12} in pregnancy[156] due to increasing maternal-fetal erythropoiesis and uptake into tissues[157].

Vitamin-B_{12} can not be made by plants or by animals, as the only type of organisms that have the enzymes required for the synthesis of vitamin-B_{12} are bacteria and archea[158, 159]. Therefore, vitamin-B_{12} dietary intake is exclusively from animal sources, which have consumed foods contaminated with vitamin-B_{12}[160]. Clinically significant vitamin-B_{12} deficiency meriting intervention during pregnancy has only been reported in pregnant women with vegetarian diets[161, 162]. Low dietary intake or malabsorption of vitamin-B_{12} may be the reason for the high risk of NTDs in countries such as India[163]and Mexico[164] where the reported incidence of NTDs in some regions is nearly ten times higher than that observed in the United States[164, 165]. In such a population, increased folate intake offers minimal protection against birth defects[136]. A recent study by Ray et al.,[166] demonstrated almost a 3-fold increase in the risk for NTDs in the presence of low maternal vitamin-B_{12} concentration. Furthermore, the harmful effect of vitamin-B_{12} deficiency is not confined to birth defects but may also cause growth restriction[167], delayed psychomotor development and, in some instances, permanent effects on the developing brain[168].

Vitamin-B_{12} deficiency is less defined as a risk factor for UPI, compared to folate and Hcy; however, a large study[169] of 406 patients demonstrated that vitamin-B_{12} deficiency is associated with elevated serum Hcy concentrations. In addition, studies have shown that inadequate vitamin-B_{12} status has been associated with adverse pregnancy outcomes such as NTDs[170-173], preterm birth[174], IUGR[167, 175] and recurrent miscarriage[176]; however, a review by Ray and Laskin[109] concluded that prospective studies are needed to confirm these findings due to the limited quality of these data.

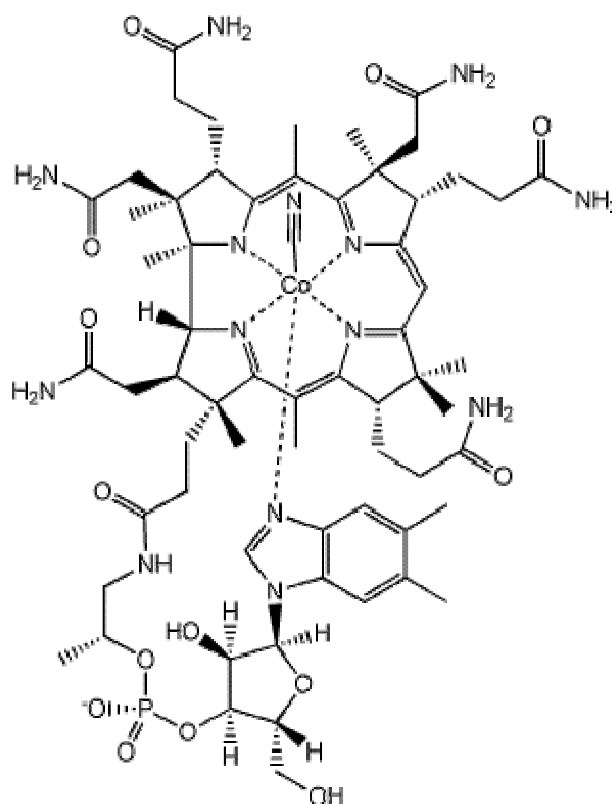

Figure 4. Vitamin-B_{12}

The core of vitamin-B_{12} is a corrin ring with 4 pyrrole subunits, coordinated to the central cobalt atom. The pyrrole subunits are joined on opposite sides by a C-CH_3 methylene link, on one side by a C-H methylene link, and with the two of the pyrroles joined directly. The sixth coordination partner varies, being a cyano group (-CN), a hydroxy group (-OH), a methyl group (-CH_3) or a 5-deoxyadenosyl group to yield four vitamin-B_{12} forms.

6.2.4 Vitamin-B_6

Vitamin-B_6 is the common name for three naturally occurring compounds: pyridoxine, pyridoxal, and pyridoxamine (Figure 5). All three can be phosphorylated to the active vitamin-B_6 coenzyme, pyridoxal 5-phosphate (PLP)[177]. Vitamin-B_6 is thought to be the most versatile coenzyme by participating in more than 100 biochemical reactions[98, 178]. Within humans PLP plays a role in haeme (component of haemoglobin) synthesis and activates several pathways in amino acid metabolism, including the formation of neurotransmitters (such as serotonin, norepinephrine and gama-amino butyric acid)[179]. In addition, PLP aids the normal development of the central nervous system and also influences cognitive function[179, 180]. Moreover, vitamin-B_6 plays a major role in one-carbon metabolism[181] and is involved in lowering the concentration of Hcy which, as stated previously, is considered a risk factor for cardiovascular and placental disease[122, 123, 182, 183].

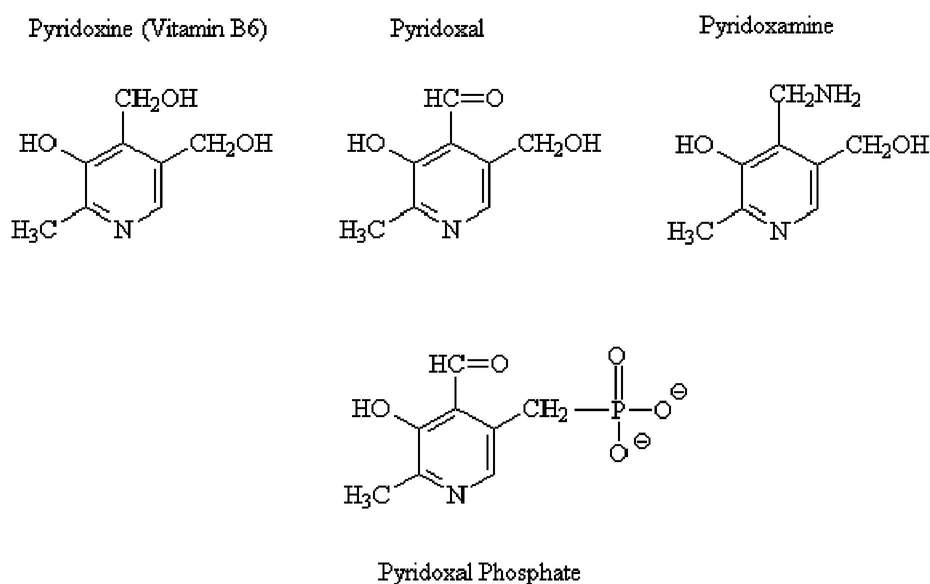

Figure 5 Three natural forms of vitamin-B_6 and the active coenzyme pyroxidine phospate

Pyridoxal-phosphate (PLP) is a coenzyme of many reactions. It is the active form of vitamin- B_6 which comprises three natural organic compounds, pyridoxal, pyridoxamine and pyridoxine. By replacing the -CH_2OH group on position 4 of the pyridoxine molecule with -CH_2NH_2 and -CHO respectively, two related compounds, pyridoxamine and pyridoxal can be formed which are also vitamin active.

The richest sources of vitamin-B_6 include meats, poultry, fish, potatoes, legumes, bananas and liver[184]. Vitamin-B_6 is well absorbed from the gastrointestinal tract[184]. During pregnancy circulating vitamin-B_6 levels decrease during pregnancy, especially during the third trimester; however, there is insufficient evidence to determine whether this is indicative of a deficient state with associated clinical implications, or a normal physiological response to pregnancy[185].

Limited studies have reported on the association of pregnancy risks associated with vitamin-B_6 deficiency. Earlier studies demonstrated that vitamin-B_6 supplementation during pregnancy (2 mg/day or higher) is associated with increased birthweights[186], reduced incidence of PE[187] and preterm birth[174]. Recent studies also suggest a protection against congenital malformations[188, 189], recurrent early pregnancy losses[190] and placental abruption[191]; however, the 2006 Cochrane review on vitamin-B_6 in pregnancy stated that the few trials reviewed did not have clear trial methodology or significant clinical outcomes. Therefore, future trials assessing vitamin-B_6 and pregnancy complications, such as PE and other adverse events, are required[185].

6.3 One-carbon metabolism

One-carbon metabolism, i.e the pathway involved in the exchange of carbon moieties, is controlled by folate and related B-vitamins which act as cofactors for key enzymes in this pathway. One-carbon is a biochemical term for functional groups containing only one-carbon in addition to other atoms[192]. Folate substrates act as acceptors and donors of one-carbon units such as methyl (-CH3), methylene (-CH2-), formyl (-CH=O), formimino (-CH=NH), and methenyl (-CH=)[104] in a variety of reactions critical for nucleic and amino acid metabolism[193]. There are two main branches of the pathway, one consists of reactions involving dTMP (thymine) synthesis, and the other is responsible for Hcy remethylation, to form methionine and *S*-adenosylmethionine (SAM) required for methylation[194]. In addition, Hcy can be converted to cysteine using a vitamin-B_6 dependent enzyme. Thus, indicating that the amount of circulating Hcy in the blood is regulated by one-carbon metabolism[98].

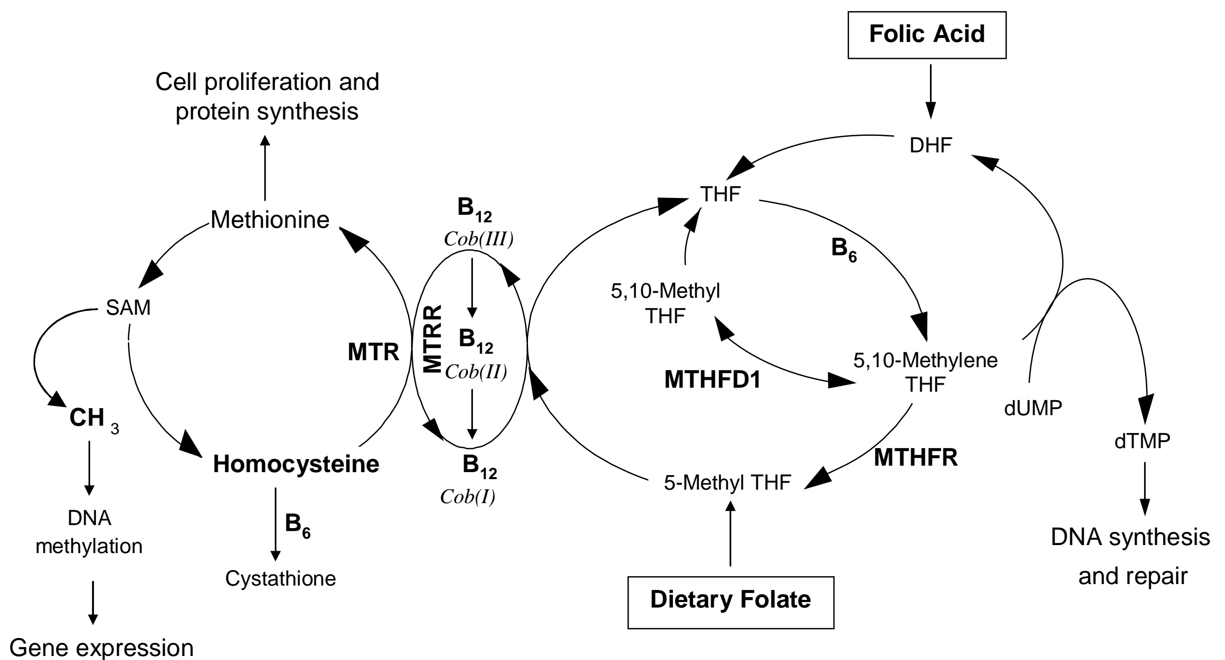

Figure 6. A simplified scheme of one-carbon metabolism.

Unmetabolised folic acid is reduced to DHF and THF. The β-carbon of serine is transferred to THF, forming glycine. At a major branch point between transmethylation reactions and nucleotide biosynthesis, 5,10-methylene-THF can be reduced to 5-methyl-THF by MTHFR or transfer a methyl group to dUMP to form dTMP. In a reaction catalysed by the vitamin-B_{12} containing enzyme, MTR, the methyl group of 5-methyl-THF is transferred to Hcy, generating methionine as well as regenerating THF. The vitamin-B_{12} dependent MTR is methylated by 5-methyl-THF to form methylcobalamin and demethylated by Hcy to form cob(I)alamin. Occasional oxidation of the cob(I)alamin intermediate produces an inactive cob(II)alamin enzyme, which is reactivated by MTRR. Methionine is further transformed into SAM, the universal methyl donor for methylation of nucleic acids, proteins, polysaccharides, phospholipids. After release of the methyl group, SAM is reversibly hydrolysed into Hcy. Hcy is also metabolised into cystathionine by a vitamin-B_6 dependent enzyme which is further cleaved into cystein. Abbreviations, **DHF**, Dihydrofolate; **THF**, Tetrahydrofolate; **MTHFR**, Methylenetetrahydrofolate Reductase; **MTHFD1**, Methylenetetrahydrofolate Dyhydrogenase; **MTR,** Methionine Synthase; **MTRR**, Methionine Synthase Reductase; **SAM**, S-Adenosylmethionine; **B_{12},** Vitamin-B_{12} (cobalamin, in various oxidative forms); **B_6,** Vitamin-B_6; **dUMP**, Uracil; **dTMP,** Thymine; **CH_3,** Methyl group.

Defects in one-carbon metabolism can result in reduced methionine formation. If methionine is limited, MTHFR activity will be stimulated, thereby providing more 5-methylTHF for the enzyme methionine synthase (MTR)[101]. During vitamin-B_{12} deficiency, the MTR enzyme function remains impaired and a situation referred to as the folate trap develops[195] in which 5-methylTHF accumulates at the expense of the other cellular folates, and the uptake of folate from serum is prevented. Thus, the biochemical effects of both folate and vitamin-B_{12} deficiency are similar and include a functional folate deficiency, hyperhomocysteinemia, and a low methionine levels[196].

6.3.1 One-carbon metabolism enzymes

The importance of adequate folate status for reproductive health has prompted investigations to evaluate how genetic variations in key enzymes may influence folate (one-carbon) metabolism. The most widely studied polymorphism is a cytosine to thymine transition in the gene for 5,10-methylenetetrahydrofolate reductase (MTHFR)[197, 198] at bp 677, resulting in an alanine to valine substitution in the enzyme and reduced activity[121]. MTHFR is required to catalyze the reduction of 5,10-methyleneTHF to 5-methylTHF, the primary methyl donor in the remethylation of Hcy to methionine. MTHFR directs the folate pool toward the remethylation of Hcy at the expense of DNA and RNA synthesis[199]; besides its conversion to 5-methylTHF by MTHFR, 5,10-methyleneTHF is further metabolised in several one-carbon transfer reactions during the synthesis of thymine (methylation of deoxyuridine 5'-monophosphate (dUMP) to deoxythymidine 5'-monophosphate (dTMP)), as well as the synthesis of purines (Figure 6).

The presence of the MTHFR 677TT genotype is important in women of reproductive age because of its reported association with an increased risk for neural-tube defects (NTDs)[200] and pregnancy complications[197, 201]. Another polymorphism within MTHFR occurs when adenine is replaced with cytosine at bp 1298, yielding a substitution of alanine for glutamate in the enzyme[202, 203]. Although the variant MTHFR 1298CC genotype has not been reported to affect birth defect risk, compound heterozygosity for the 677 and 1298 MTHFR polymorphisms has been associated with higher risk for NTDs[200], Down syndrome[204] and IUGR[205]. The MTHFR 677TT genotype is associated with elevated plasma Hcy when folate status is low [121, 206, 207]. Although the MTHFR A1298C polymorphism was not reported to affect Hcy status, double heterozygosity for both MTHFR polymorphisms was cited as a risk factor for hyperhomocysteinemia[208].

The enzyme methionine synthase (MTR) is required for the conversion of Hcy to methionine using vitamin-B_{12} as a cofactor and 5-methylTHF as methyl donor. Methionine is required for the production of S-adenosylmethionine (SAM), the principal methyl donor to control cellular proliferation, cellular migration, differentiation, and cell to cell recognition[2]. Thus, by limiting the supply of SAM, as a result of defects in one-carbon metabolism, DNA can become hypomethylated, which can lead to abnormal placental development[135]. A common polymorphism in MTR, A2756G, changes an aspartic acid to glycine at the C-terminal end of the alpha/beta domain of the enzyme and has been associated with Down syndrome[209]; however, no association has been detected in relation to PE[210]. During the MTR reaction, transfer of the methyl group from methylcob(III)alamin results in the formation of the highly reactive cob(I)alamin, which may become oxidized to cob(II)alamin, resulting in MTR inactivation[211], an event that may occur every 100 methyl transfer cycles[212]. Methionine synthase reductase (MTRR) is required for the reductive methylation of cob(II)alamin, which reactivates MTR[213].

Wilson and colleagues [214] identified a common variant of methionine synthase reductase MTRR (A66G) in which methionine replaces isoleucine in the enzyme[213-215]. Heterozygosity and homozygosity for this polymorphism were associated with an increased risk for NTDs[216, 217]. Furthermore, the MTRR 66GG genotype, in association with the MTHFR 677TT genotype, was related to an increased risk for NTDs[200, 214].

Polymorphisms in either MTR or MTRR are expected to produce similar effects on genome methylation as those induced by vitamin-B_{12} deficiency. Recent studies indicated that the MTRR A66G variant allele was not associated with an increased risk for placental abruption[218] or with NTDs[219]; however, the MTRR 66GG genotype was associated with an increased risk for having a pregnancy affected with NTD when maternal vitamin-B_{12} concentrations are low[214]. In addition, MTRR A66G polymorphism has been associated with chromosomal instability and Down syndrome[220]. In addition, polymorphisms at the MTRR A66G loci also were associated with an increase in plasma Hcy, with the GG genotype having a greater effect than the AG genotype[213]; however, data are limited and additional research is warranted to further characterise this association.

A study by Doolin et al.[221], designed to address questions regarding both maternal and embryonic genetic risk factors for NTDs, investigated MTR A2756G and MTRR A66G polymorphisms and provided evidence that the variant alleles influence the risk of spina

bifida via the maternal rather than the embryonic genotype. These findings highlight the importance of considering both the maternal and embryonic genotypes when evaluating pregnancy complications.

The methylenetetrahydrofolate dehydrogenase (*MTHFD1*) gene encodes a trifunctional protein comprising 5,10-methylenetetrahydrofolate dehydrogenase, 5,10-methenyltetrahydrofolate cyclohydrolase, and 10-formyltetrahydrofolate synthetase. These three enzymes are involved in sequential reactions enabling the interconversion of one-carbon derivatives of tetrahydrofolate (THF) which are substrates for methionine, dTMP and *de novo* purine syntheses[222]. The *MTHFD1* A1958G polymorphism results in replacement of the arginine residue by glutamine. To date this polymorphism has not been studied in relation to PE or IUGR; however, the *MTHFD1* 1958A allele was recently linked with placental abruption[223] and NTDs[224-226], suggesting that *MTHFD1* G1958A polymorphism could represent an attractive candidate gene in UPI aetiology.

6.4 Genome damage and nutrigenomics

Placentation is orchestrated between cell proliferation, cell differentiation and cell invasion[227]. During this developmental phase rapid DNA replication provides an increased opportunity for genome and epigenome damage to occur[1]. Damage to the genome can lead to inhibition of cell division, cell cycle delay and an excessive apoptotic or necrotic cell death rate[2], causing defective trophoblast invasion and abnormal placentation. Genome damage arises from a variety of mechanisms including point mutations, base modifications, chromosome breakage, chromosomes rearrangement, chromosome loss or gain, gene silencing due to inappropriate methylation of CpG dinucleotides at promoter sequences, expression of parasitic DNA due to reduced methylation of CpG dinucleotides and telomere shortening[1].

Gene-nutrient interactions (nutrigenomics) in one-carbon metabolism regulate DNA synthesis, DNA repair and gene expression[1]. Micronutrient deficiency (in particular folate and vitamin-B_{12}) can cause genome damage of the same magnitude, if not greater, than environmental genotoxins such as chemical carcinogens[1, 228-231]. In addition, nutrigenomic mechanisms regulate the transformation from dUMP (uracil) to dTMP (thymine) for DNA synthesis using 5,10-methyleneTHF. Low levels of 5,10-methyleneTHF decreases synthesis of dTMP[232], increasing the cellular dUMP/dTMP ratio and consequently DNA polymerase-mediated dUTP misincorporation into DNA[233]. Blount et

al[234] demonstrated that folate deficiency induced uracil incorporation into human DNA, which can ultimately cause DNA strand breaks and chromosome instability[235].

Excess Hcy causes an increase in oxidative stress through the overproduction of hydrogen peroxide[236]. Reactive oxygen species such as hydrogen peroxide can directly or indirectly damage DNA[237, 238] causing whole chromosome loss, chromosome breaks, chromosome rearrangements, DNA base deletions and substitutions[239]. There is mounting evidence that oxidative stress in uteroplacental tissues plays a pivotal role in the development of UPI[240], and is therefore considered to be a key intermediary step in the pathogenesis of PE[241, 242]. Oxidative stress has also been regarded as major pathogenetic driver of apoptosis causing the release of placental debris into the maternal circulation, a key pathogenic event in the progression of PE[243]. Previous studies[244, 245] have shown that Hcy causes apoptosis in vascular smooth muscle cells *in vitro*, while the administration of folic acid diminished apoptotic effects. Steegers-Theunissen et al.[246] observed that folate deficiency resulted in significant apoptosis in human trophoblasts. In addition, several other investigators have observed similar results in various cell types[247-249].

Genome damage has been associated with infertility and developmental defects[250-257]. DNA damage within sperm has been shown to increase infertility, pregnancy loss, and childhood disease[258]. In addition research has shown that sperm from infertile men have abnormal chromatin structure, microdeletions in chromosomes, aneuploidy and DNA strand breaks[259]. This damage is poorly characterised, but is known to involve hypomethylation of key genes, oxidative stress, endonuclease-mediated cleavage and the formation of adducts with xenobiotics and the products of lipid peroxidation[260]. A recent study found a 74% increase in normal sperm count in subfertile men after supplementation of folic acid in combination with zinc sulphate[261]. Little research has focussed on the effect of maternal genome damage on reproductive success and in relation to late pregnancy complications associated with UPI[256, 257].

6.4.1 Epigenetic modifications

Epigenetics refers to modifications in gene expression that are controlled by heritable but potentially reversible changes in DNA methylation and/or chromatin structure[262]. DNA methylation is the process in which a methyl group is added at the carbon 5' position of cytosine within the cytosine-guanine (CpG) dinucleotide[262-264]. It is responsible for establishing and maintaining the diverse patterns of gene expression that distinguish different cell and tissue types[265, 266]. CpG dinucleotides are unequally distributed across the human genome. Vast stretches of DNA are deficient of CpGs, but are interspersed by CpG clusters known as CpG islands, which are usually unmethylated[267]. Unmethylated CpG-rich islands can be found in the promoter region of housekeeping genes in all cell types, whereas tissue-specific genes are methylated in all tissues except the tissue where the gene is expressed[268]. Altered DNA methylation is a mechanism, other than uracil misincorporation, that can result in genome damage[269].

Approximately 80% of the CpG sites in the mammalian genome are methylated[270, 271]. Dense methylation of promoter regions has been associated with a compacted chromatin structure and can lead to transcriptional silencing of the associated gene[263, 267, 272, 273]. Transcriptional silencing has a profound effect in development, including protection against intragenomic parasitic DNA[274], genomic imprinting[275] and chromosome X-inactivation[275]. It is becoming increasingly evident that epigenetic gene regulatory mechanisms play an important role in the early developmental stages of life and in the aetiology of human diseases[276].

Environmental factors, such as diet and nutrition can alter epigenetic modifications[277]. Studies have shown that folate deficiency decreases DNA methylation in humans[278, 279] and animal models[79, 104]; however, a folate replete diet may restore DNA methylation status[279]. Reduced availability of methyl-THF, the main circulating form of folate, as a result of decreased folate intake, limits the biosynthesis of SAM, thus limiting the availability of methyl groups for methylation reactions[280]. Moreover, Duerre and Briske-Anderson[281] demonstrated that accumulation of high Hcy levels in the cell, can completely inhibit all methylation reactions. In addition, the common C677T polymorphism in the *MTHFR* gene has been reported to influence DNA methylation status through interaction with folate status[282].

Waterland and Jirtle [283] showed that maternal nutrition (including folate) in mice affects the phenotype of offspring by influencing the degree of CpG methylation at the agouti locus. This indicates the importance of maternal nutrition in relation to methylation status of the offspring as it may have long lasting effects on phenotype[277]

6.5 Genome damage, folate nutrigenomics and uteroplacental insufficiency

Our genome is the most precious possession we have; however, it is constantly under threat. It is becoming increasingly evident that the risk for developmental and degenerative disease increases with DNA damage which in turn is dependent on nutritional status and genetic polymorphisms[284]. In addition, a higher incidence of genome damage in the infertile population is widely recognised[285]. Genome damage can affect fertility due to a reduced production of germ cells as it causes apoptosis, one of the mechanisms by which grossly mutated cells are normally eliminated[286, 287]. When this mechanism fails reproductive cells with genomic abnormalities may survive leading to serious developmental defects[288, 289]. Although it has long been acknowledged that both male and female partners contribute to human reproductive success, the past 20 years have focussed on the importance of the male factor in successful pregnancy[290]. There is extensive literature relating DNA damage in sperm [291] with infertility, but what is not known is how maternal DNA damage affects the growth and development of the placenta which ensures fetal and maternal health.

There is general agreement that defects or deficiencies in gene-nutrient interactions in one-carbon metabolism cause increased genome damage[230, 234, 292-294] and infertility[246, 295-298]. The deleterious effects of defective one-carbon metabolism include reduced cell division (e.g. of oogonia during oogenesis or of granulosa cells during folliculogenesis), inflammatory cytokine production[299, 300], altered nitric oxide (NO) metabolism[297], oxidative stress[295], apoptosis[246] and defective methylation reactions[296]. Of the factors involved in one-carbon metabolism, folate has been most extensively investigated for its unique function as a mediator for the transfer of one-carbon moieties for nucleotide synthesis/repair and biological methylation. In addition, numerous studies have investigated the relationship between Hcy and important enzyme polymorphisms (e.g *MTHFR* 677)[215, 301, 302] or with genome damage[231, 294, 303] or with pregnancy complications[19, 118, 126, 131, 191, 304-307], but to date these factors have not been investigated.

In the future a more comprehensive approach is needed to investigate genome damage and gene-nutrient interactions associated with one-carbon metabolism that may affect trophoblast cell proliferation, differentiation and invasion into the spiral arteries, events which can reduce uteroplacental circulation and lead to pregnancy diseases such as PE and IUGR. Genome damage analysis of peripheral blood lymphocytes has been accepted as a technique for the biological monitoring of genetic damage in somatic cells since the early 1970s[308]. Lymphocytes have a half-life of 3–6 months and circulate around the body and experience physiological and nutrient imbalances within tissues and, thus, DNA damage in those cells provide an integrated estimate of inherent and/or acquired genomic instability[309]. Today, genome damage in human peripheral lymphocytes measured using the cytokinesis-block micronucleus (CBMN) cytome assay is recognised as a valuable biomarker, probably the only one which has been internationally standardised and validated[310-312]. Furthermore, a high frequency of MN in lymphocytes is predictive of an increased risk of cancer[313-315] and has been associated with neurodegenerative diseases, such as Alzheimer's and Parkinson's disease[316, 317].

The CBMN cytome assay is the most robust and commonly used method for measuring micronuclei (MN), marker of chromosome breakage and loss, in cultured human lymphocytes because scoring is specifically restricted to once-divided cells. These cells are recognized by their binucleated (BN) appearance after the inhibition of cytokinesis by cytochalasin-B[318, 319]. Restricting scoring of micronucleated binucleate cells (MN-BNs) prevents confounding effects caused by suboptimal cell division kinetics, which is a major variable in this *ex vivo* assay. The CBMN assay allows the detection of micronuclei, apoptosis, necrosis, nucleoplasmic bridges, and nuclear budding. The latter two endpoints are indicative of chromosome rearrangements and gene amplification, respectively, and is a sensitive marker of folate deficiency [230].

A study assessed MN frequencies using the CBMN cytome assay in lymphocytes of 130 patients (65 couples) with idiopathic infertility or with two or more spontaneous abortions, and 30 healthy fertile donors (15 couples). The frequency of micronucleated cells in the cohort with reproductive failure and healthy controls averaged 14.95+/-6.04 per 1000 and 10.60 +/-2.57 per 1000 ($P<0.0001$), respectively[256]. These results suggest that genome damage biomarkers may be a useful prognostic marker of likelihood of a successful pregnancy as it provides an index of the probable genomic instability of the parental and/or fetal tissues either because of inherited factors predisposing to genomic instability or because of nutritional inadequacies causing genome and epigenome damage.

Currently, women at risk of UPI are identified on the basis of epidemiological and clinical risk factors as there is no reliable test available early enough in pregnancy to identify women at risk for developing these disorders to permit pre-treatment or preventative interventions. A better understanding of underlying mechanisms in reduced uteroplacental circulation and clarifying the effectiveness of nutritional and biochemical factors, such as those in one-carbon metabolism, by determining which factors enable the placenta to function optimally it may be possible to develop a predictive approach for identifying woman at high risk of PE and IUGR and develop rational preventative strategies

Previous studies identified a major obstacle in research into placental diseases in trying to distinguish between primary cause and secondary effects[37, 41-47], which may be attributable to the fact that the common end result of low uteroplacental blood flow may be caused by many primary defects[39, 320-324]. The interaction of key nutrients and genotype (i.e. nutrigenomics[325]) and investigations of genome damage biomarkers may provide the means to develop markers of early, pivotal changes between healthy pregnancy and the development of UPI. Therefore, we hypothesise that genome damage and defects in one-carbon metabolism alter methylation patterns, increase Hcy and chromosome instability, which reduce trophoblast proliferation, differentiation and invasion leading to defective spiral artery remodelling and reduced uteroplacental circulation and to late pregnancy complications, including PE and IUGR (Figure 7).

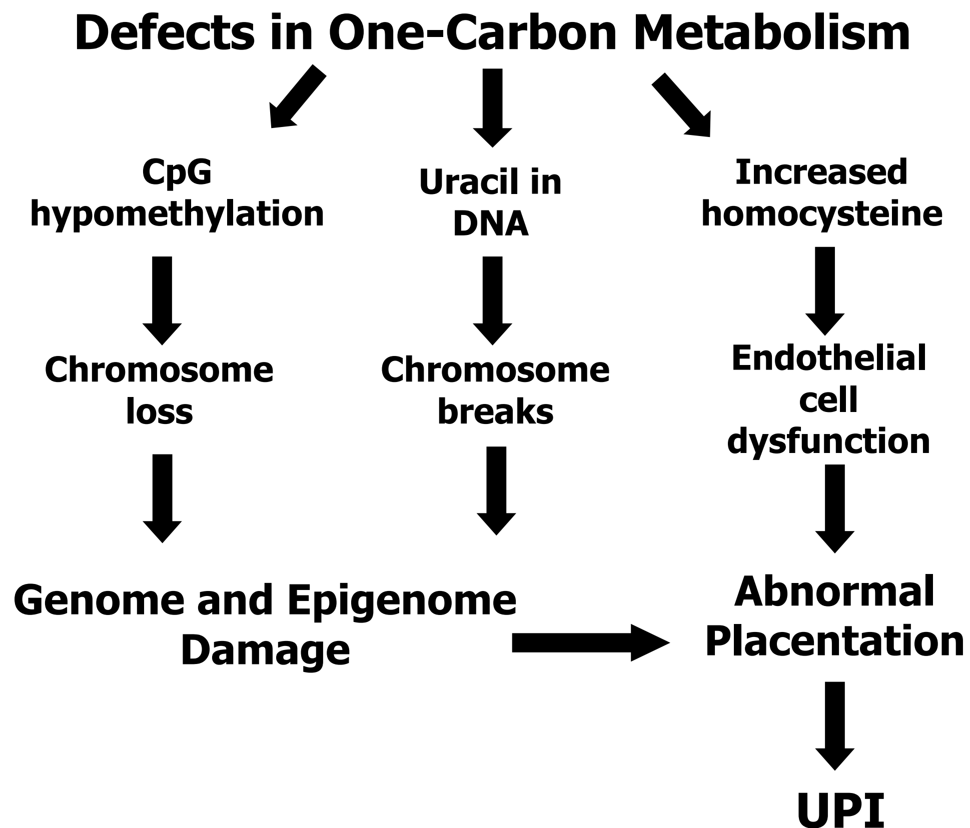

Figure 7. A schematic diagram showing how defects in one-carbon metabolism can lead to UPI

Defects in one-carbon metabolism can reduce the synthesis of SAM causing CpG hypomethylation. Demethylation causes undercondensation of the heterochromatin regions of specific chromosomes and can result in whole chromosomes loss[326]. When uracil is misincoporated into DNA, transient nicks are formed; two opposing nicks can lead to chromosome breaks[234]. Increased circulating Hcy can be cytotoxic, damaging endothelial cells and placental vasculature causing abnormal placentation[123, 327] and possibly UPI.

7 AIMS AND HYPOTHESES

7.1 Aim

The purpose of this PhD thesis was to determine the nutrigenomics basis of increased risk for adverse pregnancy outcome by investigating the association of this risk with DNA damage, B-vitamin status and genetic background in one – carbon metabolism.

7.2 Hypotheses

1. Women at risk of uteroplacental insufficiency (UPI), including preeclampsia (PE) and intrauterine growth restriction (IUGR) exhibit higher rates of genome and epigenome damage

2. Increased rates of genome/epigenome damage in pregnancy are determined by micronutrient deficiency and genetic defects in one-carbon metabolic pathway

3. The risk of PE and IUGR is also determined by B-vitamin deficiency and genetic defects in one-carbon metabolic pathway.

The above hypotheses will be tested by (a) comparing the genome damage markers, cell proliferation, methylation and genetic polymorphisms (MTHFR C677T and A1298C, MTR A2756G, MTRR A66G, MTHFD1 G1958A) in maternal blood and placental tissues and (b) comparing blood and micronutrients (red blood cell (RBC) folate, serum folate, serum vitamin-B_{12}, and red cell vitamin-B_6 and plasma homocysteine (Hcy)) of women with low risk (healthy) pregnancies and women diagnosed with high risk of developing UPI. This will determine if women with increased genome and epigenome damage markers in combination with genetic defects in one-carbon metabolism and micronutrient deficiency have increased risk UPI, including, PE and IUGR.

8 STUDY DESIGN AND GENERAL METHODOLOGY

This chapter outlines the eligibility criteria used to recruit women with high risk pregnancies and low risk (healthy) pregnancies. The general methods section includes protocols performed throughout the study. The experimental methodology that is specific for each chapter is found within the related chapter.

8.1 Study design

This study was a prospective cohort study conducted at CSIRO Human Nutrition and Women's and Children's Hospital (WCH), Adelaide. The study was approved by the Human Experimentation Ethics Committees of both institutions and participants provided informed consent after the aims and protocols of the study were explained. The WCH draws on patients of diverse socioeconomic status. Pregnant volunteers were enrolled in early pregnancy (before 20 weeks gestation), when consent was signed an appointment was made for a fasting blood sample at 20 weeks gestation and given a delivery pack which included the necessary instructions and collection devices for placental and blood collection at the time of delivery. After blood collection at 20 weeks gestation pregnant women were fed breakfast and administered with a questionnaire. Information on diet, socioeconomic factors, and supplement intake, obstetric and medical history was collected. A digital balance was used to record the weights of all mothers to the nearest 100 g. Measurements of height were made using a stadiometer to the nearest 1cm. Maternal body mass index (BMI, kg/m^2) was calculated using height and weight.

8.1.1 Index pregnancies

As part of prior patient care, many of the high risk patients had already been genotyped for *MTHFR* 677 and 1298 polymorphisms; most of these patients were treated with high dose folate, vitamin-B_{12} and vitamin-B_6 supplements. Since these high-dose vitamin supplementations might well mask the phenotypical effects of the MTHFR 677 CT and 1298 AC polymorphism, a separate layer of analysis was introduced based on the so-called index pregnancy, i.e. that prior pregnancy that led the clinicians to genotyping of that particular patients involved (at that stage women were not treated with high-dose vitamins) in an attempt to eliminate the influence of high B-vitamin consumption.

8.1.2 Recruiting criteria

All eligible patients who attended the antenatal clinics of the WCH were invited to participate in this study. Patients were fully informed about the purposes and characteristics of the study and their rights as research subjects. Specifically, the voluntary nature of their participation was explained. Women were provided with a delivery pack containing guidelines for placental and cord blood collection, Vacuette® tubes for blood collection and necessary WCH requisition forms. Consent forms were copied and entered into the patients medical records along with information highlighting the patient's involvement in the study, ensuring hospital staff are aware of procedures to be taken place at the time of delivery.

8.2 Patient classification

8.2.1 High risk classification

The women who were classified as being at high risk of developing an adverse pregnancy outcome were classified based on a variety of risk factors, focusing on obstetric history. A full medical history was recorded by obstetricians at the WCH, Adelaide. The inclusion criteria are listed below.

Risk factors for the development of UPI for this study:

- Previous severe preeclampsia / eclampsia
- Previous severe IUGR
- Previous placental abruption
- Pre-term birth
- Greater than or equal to than 3 miscarriages
- Previous fetal demise
- Diabetes / insulin resistance
- Chronic hypertension / high BMI / PCOS

8.2.2 Low risk classification

Women who were classified as being at low risk of developing an adverse pregnancy outcome were healthy women with no previous pregnancy complications or current risk factors for UPI including hypertension and /or insulin resistance.

8.2.3 Exclusion criteria

Any maternal or fetal condition that requires termination of pregnancy.

Known major fetal anomaly or fetal demise.

Multifetal pregnancy

Any medical disorder requiring systemic steroids

Lack of informed consent

8.3 Clinical diagnosis of pregnancy outcome

8.3.1 Primary outcomes

Primary outcomes were defined according to the criteria of the Australasian Society for the Study of Hypertension in Pregnancy [38]. Gestational hypertension is hypertension arising in pregnancy after 20 weeks gestation without any other feature of the multisystem disorder preeclampsia (PE) and resolves within 3 months postpartum[38]. Gestational hypertension is defined as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg on two occasions ≥ 6 hours apart.

Preeclampsia (PE) is hypertension in pregnancy after 20 week gestation and the onset of one or more of:

- Proteinuria ≥ 300 mg/24 hr or spot urine protein/creatinine ratio ≥ 30mg/mmol
- Renal insufficiency
- Liver disease
- Neurologic symptoms (headache, visual disturbances, persistent hyperreflexia, and HELLP syndrome).
- Haematological disturbances – thrombocytopenia
- Fetal growth restriction
- Eclampsia will be diagnosed if convulsions develop.

Customised centiles were used to identify growth restriction [328]. Use of customised centiles is likely to detect more babies at risk of perinatal morbidity and mortality than would be detected by population centiles [74]. Intrauterine growth restriction (IUGR) was defined as less than the 10th centile according to Australian/NZ criteria [329]. Customised centiles allows calculation of an individual centile for a particular birthweight, after adjusting for maternal height, weight, parity and ethnic group and the sex of the baby. This method is able to improve, for retrospective assessment, the distinction between normal and abnormal

birthweight. Body mass index is also an important indicator for limits between which it is acceptable to adjust for maternal size. Normal BMI limits were derived from the 90th and the 10th centile values for BMI in the population and applied in customised centile calculation software. The purpose of the limits is to avoid over adjustment to extremes which represent pathology, that is, maternal obesity or malnutrition at the upper and lower limits, respectively. The Australian module of the customised growth chart program is available for free download from http://www.gestation.net. The verification and severity of IUGR was based on the reduction of fetal growth velocity on fetal growth trajectory using observed serial ultrasound scans.

8.3.2 Secondary outcomes

Maternal

- gestational age at delivery
- Other measurements (caesarean section rate, other medical complications, death).

Fetal and neonatal outcomes

- Gestational age at delivery
- Histopathological lesions in the placenta.
- Mortality (stillbirth, neonatal mortality and infant mortality)

8.4 Pregnancy groups

One hundred and forty-nine women were recruited via the NIH trial and antenatal clinic at WCH by WCH midwives, obstetricians or via the chief investigator (DF). Of the 149 women recruited 6 were excluded for the following reasons: 1 moved overseas, 3 dropped out, 2 miscarried (just prior to 20 weeks gestation). The study population involved women between the ages of 18 and 54 years. The number of women classified into each group is shown in (Figure 8).

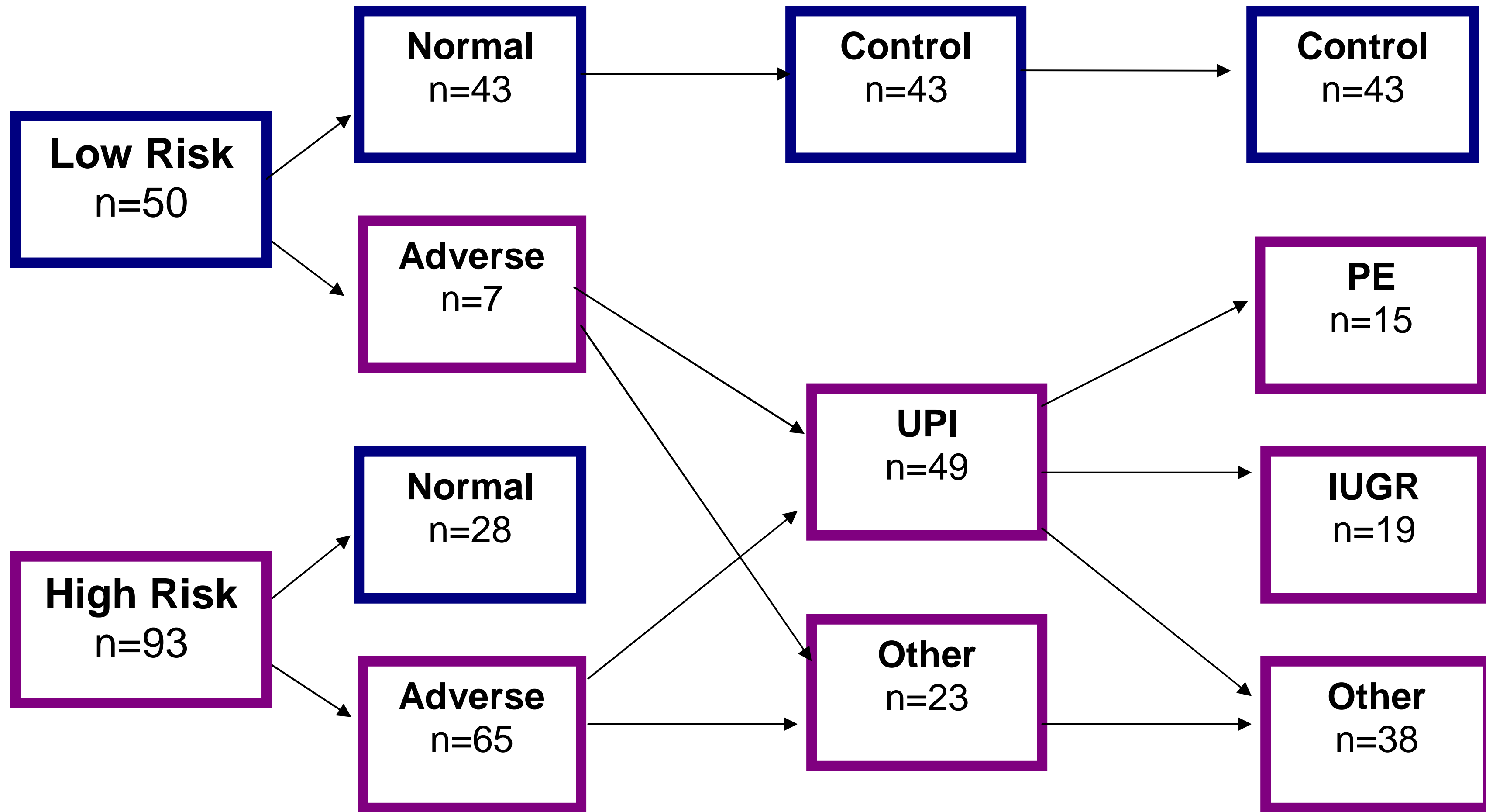

Figure 8 Pregnancy group based on clinical outcome

Low Risk = healthy women with low risk of developing pregnancy complications, High Risk = women with high risk of developing pregnancy complications, Normal = pregnancies with clinically normal outcomes, Adverse = pregnancies with adverse outcome, Control = low risk women with clinically normal outcome, UPI = uteroplacental insufficiency including preeclampsia, IUGR, gestational hypertension and placental abruption, Other = adverse outcome other than UPI e.g. gestational diabetes, PE = preeclampsia, IUGR = intrauterine growth restriction, Other = adverse outcome other than PE or IUGR e.g. placental abruption and gestational hypertension

8.4.1 Demographic and clinical questionnaire

A questionnaire was administered at 20 weeks gestation which included information on demographic, smoking status, vitamin supplementation (including brands), medication and health.

8.4.2 Maternal blood collection

Study participants donated a fasted blood sample at ~20 weeks gestation for genome damage measurements, micronutrient analysis and genotyping. The type of tube and amount of blood for each assay is listed below:

Approximately 20 ml venous blood was collected in 9 ml Vacuette®-Li Heparin tube, 4 ml Vacuette®-K-EDTA and 4 ml Vacuette®-Serum. The Vacuette®-Li Heparin was left at room temperature 1 ml of whole blood was processed within 4 hours of collection for the CBMN cytome assay. 500ul whole blood from the Vacuette®-K-EDTA was transferred to an eppendorf for red cell folate (RCF) analysis. The remaining blood in the Vacuette®-K-EDTA and the 4 ml Vacuette®-Serum were spun at 1200rpm. The plasma removed from EDTA and transferred to cryovial and delivered within 2 hours of collection to the Core Laboratory, Division of laboratory Medicine, Women's and Children's Hospital Adelaide for the quantification of plasma homocysteine. After the serum was separated it was transferred to cryovial for serum folate and vitamin-B_{12} quantification then delivered along with whole blood aliquot for RCF and the remaining packed cells from the Vacuette®-K-EDTA tube for vitamin-B_6 quantification to the Institute of Medical and Veterinary Science (IMVS), Frome Rd, Adelaide. The remaining blood was frozen for future DNA isolation. Vacuettes® were purchased from Greiner Bio-One, Kremsmuenster, Austria.

8.4.3 Placental cord collection

Staff or partners called the chief investigator (DF) or the study mid-wife (Denise Healy) when patient was in labour or soon after delivery in order to collect the placenta. Of the placentas collected 23% were collected in 1 hour, 48% within 3 hours, 18% 5 hours and 11% were collected the > 5 hours after delivery. A small section of cord tissue was cut using sterile scissors and placed in cryovial and placed at -20°C, before DNA extraction for genotyping and CpG methylation analysis.

8.4.4 Placental cord DNA extraction

DNA Isolation from 50 mg-100 mg fresh or frozen tissue

Maternal contamination removal

Cord tissue was thawed and then placed in a sterile Petri dish. The tissue was covered with HBSS, then cut and teased with sterile scissors and forceps to remove maternal blood and tissue**.** The tissue was transferred to a fresh Petri dish with HBSS, cut and teased to remove any residual blood, which may be causing maternal contamination. This step was repeated until placental tissue is transparent and free from maternal contamination.

Cell lysis

Placental cord tissue and Hanks Balanced Salt Solution (Hanks BSS, Trace Scientific, Melbourne, Australia) was placed using a sterile glass pipette into glass homogeniser and grind on ice with 4ml $T_{10}E_5$ (2.5 mL 1 M Tris HCl, 2.5 mL 0.5 M EDTA, 245 mL dH_2O; pH 8.0). The homogenised tissue was transfer into a sterile plastic 10 ml tube (TV10, Technoplas, Adelaide, Australia) before adding 500 µL 10% sodium dodecyl sulphate (SDS; pH 7.2) and inverted several times. 200 µL proteinase K (10 mg/mL; Roche Diagnostics, Mannheim, Germany) was added before incubation at 56ºC overnight.

Protein precipitation

The samples was removed from the incubator and immediately placed on ice for 5 minutes. 600 µL saturated NaCl (6 M) was added and then the tube was vortexed vigorously for 20 sec, then centrifuged at 2800g x 15 mins. This was repeated if necessary to gain protein pellet.

DNA precipitation

The supernatant with DNA was transferred (leaving behind protein pellet) into a clean TV10 tube with 2ml 100% isopropanol (2-propanol). The sample was mixed the by inverting approximately 50 times. The precipitated DNA (looks like cotton wool) was placed into a 1ml eppendorf tube with 400ul 70% ethanol, then centrifuged at 13000rpm x 3mins. The supernatant was discarded and the DNA pellet allowed to air dry on absorbent paper for ~10mins. The DNA pellet was dissolved 300 µL T10E1 $T_{10}E_1$ (2.5 mL 1 M Tris HCl, 0.5 mL 0.5 M EDTA, 247 mL dH_2O; pH 8.0) at 37ºC overnight.

9 MATERNAL GENOME DAMAGE AND UTEROPLACENTAL INSUFFICIENCY

9.1 Aim

The main purpose of this study was to investigate the use of the cytokinesis-block micronucleus (CBMN) cytome assay as a predictive tool for the pregnancy diseases uteroplacental insufficiency (UPI), preeclampsia (PE) and intrauterine growth restriction (IUGR).

The secondary aim was to establish normal range values for the biomarkers of genome damage: micronucleated binucleates (MN-BN), binucleated cells with nucleoplasmic bridges (NPB-BN), binucleated cells with nuclear buds (NBUD-BN), cell death (apoptosis and necrosis) and cellular division using the nuclear division index (NDI) in pregnant women at 20 weeks gestation and identify physiological, genetic, dietary and lifestyle variables affecting these biomarkers in specific pregnancy groups.

9.2 Hypotheses

1. Women predicted to be at higher risk of pregnancy complications exhibit abnormally high rates of genome damage including chromosome loss, chromosome breakage, gene amplification and cell death at 20 weeks gestation compared to women with low risk pregnancies.
2. Women who develop UPI, including preeclampsia and intrauterine growth restriction, exhibit abnormally high rates of genome damage representing chromosome loss, chromosome breakage, gene amplification and cell death at 20 weeks gestation compared to low risk healthy pregnancies (controls).

9.3 Introduction

Pregnancy complications associated with placental development are almost unique to the human species, and affect approximately one third of all pregnancies [240]. Pathological development of the uteroplacental unit is based on the assumption that the development of the placental villous trees, the invasion of extravillous trophoblast or the differentiation of the villous trophoblast is somehow dysregulated [330]. Accumulating evidence shows that trophoblastic invasiveness is regulated by a balance between the local expression of molecules which promote invasion and those that inhibit invasion [331]. For these signalling

molecules to be expressed and accurate angiogenesis to occur, genomic stability is crucial as gene dosage may alter gene expression resulting in altered cellular phenotypes and possibly reduced proliferative potential of cells. Angiogenesis, defined as the formation of new vessels sprouting from pre-existing endothelium, is essential to allow uteroplacental circulation which is necessary for the growing needs of the fetus [332]. This may be altered if endothelial cells experience abnormal DNA damage rates, given that increased DNA damage often results in cell cycle delay, reduced nuclear division rate and cell death [333].

Uteroplacental insufficiency (UPI) refers to insufficient blood flow to the placenta during pregnancy and is characterised by an impaired cytotrophoblast invasion and an inadequate response of the uterine spiral arteries to undergo physiological transformation to allow for greater blood flow for fetal development [330]. UPI can lead to pregnancy diseases including preeclampsia, IUGR and placental abruption [334].

Preeclampsia (PE) is a multisystem disorder of unknown cause and is unique to human pregnancy [34]. PE is a major cause of maternal mortality (15 – 20% in developed countries) and morbidities [34]. Studies have linked PE with pathological placentation driven by immune maladaptation, with reduced concentrations of angiogenic growth factors and increased placental debris in the maternal circulation resulting in a (mainly hypertensive) maternal inflammatory response [335]. Severe PE is more common in patients with anti-phospholipid antibodies [336] as well as in those with resistance to activated protein C [128]. Free radical induced oxidative stress with consequent lipid peroxidation has also been implicated in the pathogenesis of preeclampsia [337]. Studies have suggested that enhanced oxidative stress, which leads to genome damage, may be a mechanism of this disease [338]. The diagnostic criteria for PE remain a matter for debate but, in this work, the Australasian definitions have been used [38].

Intrauterine growth restriction (IUGR) occurs when a fetus does not reach full growth potential, displaying signs of chronic malnutrition and hypoxia leading to brain-sparing effect [339]. Fetal growth restriction is the second leading cause of perinatal morbidity and mortality [72]. The incidence of IUGR is estimated to be approximately 5 percent in the general obstetric population [340]. Defects in placentation may restrict the transportation of required nutrients and hormones affecting fetal growth and also on the function of organs throughout life [63]. IUGR infants exhibit higher rates of coronary heart disease, hypertension, body fat

distribution, stroke [65] and adult type 2 diabetes [63, 66]. The causes of IUGR are multiple, involving many different factors. However, studies in humans and animals have shown that the maternal environment is the most important determinant of newborn weight [340]. Maternal stress factors associated with IUGR include narcotic addiction, cigarette smoking and chronic alcoholism [341]. In addition poor nutritional status of the mother at conception and inadequate energy intakes during pregnancy have also been implicated in IUGR [342]. Currently there are no reliable biomarkers that can predict the subsequent development of either PE or IUGR.

During placental and fetal development a large proportion of cells are in DNA synthesis phase, providing more opportunity for DNA mutations at the base sequence and chromosome level [1]. Genome damage can occur via a variety of mechanisms including point mutations, base modifications, telomere shortening, chromosome breakage and rearrangement, chromosome loss or gain and inappropriate methylation of CpG dinucleotides (cytosine and guanine separated by a phosphodiester bond) which may lead to gene silencing [1].

DNA damage within the sperm has been shown to increase infertility, pregnancy loss, and childhood disease in offspring [258]. To date there are only two published reports in relation to the levels of maternal DNA damage and health outcomes during and after pregnancy. Trokva et al. [256] demonstrated that micronuclei (MN) frequency (a biomarker of chromosome breakage or loss) was higher in infertile couples and those who experienced spontaneous abortion relative to fertile couples. In addition a more recent study by Levario et al. [257] evaluated the formation of MN in umbilical cord blood cells as a potential indicator of in utero exposure to genotoxic agents. This study also showed that the frequency of MN was significantly correlated between mothers and their babies.

The CBMN assay is the most extensively used method for measuring genome damage in human lymphocytes, and is now be considered a “cytome” assay, including measurements of cell proliferation, cell death and chromosomal instability [343]. In this assay a cell with genome damage may either undergo cell death, via apoptosis or necrosis, or may survive and undergo further nuclear division. In the latter case, dividing cells are recognised as binucleated cells (BN) by blocking cytokinesis with cytochalasin-B and then scored for genome damage events: *1*) micronuclei (MN), which originate from lagging whole chromosomes or acentric chromosome fragments and are therefore a marker of chromosome

loss and/or chromosome breakage events, the former being due to defects in centromere/kinetochore complex on the mitotic apparatus (e.g spindle); *2*) nucleoplasmic bridges (NPB), which originate from dicentric chromosomes caused by misrepair of chromosome breaks and/or telomere end fusion and are therefore a marker of chromosome rearrangement; and *3*) nuclear buds (NBUD), which are the mechanism by which the nucleus eliminates amplified DNA resulting from breakage-fusion-bridge cycles generated by NPB. The NBUD level therefore provides a measure of gene amplification. An increase in MN, NPB and NBUD levels is indicative of an increase in genome instability. The CBMN cytome assay also provides an index of mitogen response and proliferative potential of cells by measuring the proportion of mono-, bi- and multinucleated cells from which the nuclear division index (NDI) of viable cells can be calculated.

To test the hypotheses, as described in section 1.2, genome damage and cell death biomarkers were scored in peripheral blood lymphocytes of pregnant women using the CBMN cytome assay.

9.4 Methodology

Subjects were recruited following the criteria as described in section 8.1.2. A total of 143 pregnant women were recruited into the study, including 50 healthy women with no history of abnormal pregnancies and classified as “low risk” and 93 women who were classified as “high risk” in respect of their previous medical history and the likelihood for developing pregnancy complications. From the low risk pregnancy group 43 had normal pregnancy outcomes (no clinical complications) and were allocated into the “control” group. Seven of the “low risk” women and 65 of the “high risk” women had an adverse outcome: 49 of these women developed uteroplacental insufficiency (UPI), including 19 with IUGR, 15 with PE, 14 with gestational hypertension, and 1 with placental abruption. The breakdown of the pregnancy outcomes is summarised in section 8.4, Figure 8.

9.4.1 Study design and power calculations

The study was designed so that the smallest difference that could be detected with 80% power and $P < 0.05$ would be 5 MN-BN per 1000 BNs, assuming a group size of $N = 35$. The power calculation was based on the expected standard deviation for the MN-BN frequency, estimated from previous population studies of MN frequency in females aged between 20 and

60 years (similar to the age range of 18-54 years in this study) which is 7.1 micronuclei per 1000 cells [344]. An increment of 5 micronuclei was chosen as the threshold for concern because this level of genetic damage, in biological dosimetry terms, has a "rad-equivalent" value of 5 rad (0.05Gy) of X-rays [345] and a dose equivalent of 0.05 Sieverts, the annual dose-limit allowed for radiation workers [345].

9.4.2 Rationale for DNA damage assessment in lymphocytes at 20 weeks gestation

DNA damage excess in lymphocytes, a type of leukocyte, provide an estimate of an individual's genome health (stability) and have been shown to correlate with DNA damage in cells in other tissues of the body [346]. Leukocyte infiltration is one of the major cellular events in placentation [34]. Because lymphocytes circulate around the body they experience physiological and nutrient imbalances within tissues and thus DNA damage in those cells may provide an integrated estimate of inherent and/or acquired genomic instability. During early placentation, natural killer cells (a lymphocyte cell type) in the uterus accumulate as a dense infiltrate around the invading cytotrophoblast cells and affect both trophoblast invasion and vascular changes in the maternal placental bed [34]. DNA damage in natural killer cells and cytotrophoblasts may adversely affect placentation if cellular phenotype and viability is altered. Blood collection was timed for 20 weeks as the gestation at which the maternal spiral arteries are being transformed to larger vessels to allow for increased blood volume.

9.4.3 Whole blood 68 hour cytokinesis block micronucleus cytome assay

The whole blood CBMN assay was performed as described by Fenech [255] with the exception that the cells were harvested at 68 hours instead of 72 hours post mitogen stimulation. Venous blood was collected in lithium heparin tubes from 143 pregnant women at 20 weeks gestation as described in section 8.4.2. Whole blood was cultured in RPMI 1640 (Sigma, Sydney, Australia) containing 10% FBS (Trace Scientific, Melbourne, Australia) 2mM L-Glutamine (Sigma, Sydney, Australia), 1mM Sodium pyruvate (Trace Scientific, Melbourne, Australia). Duplicate cultures were set up for each patient. A schematic diagram of the time frame for the CBMN assay protocol is shown in Figure 9.

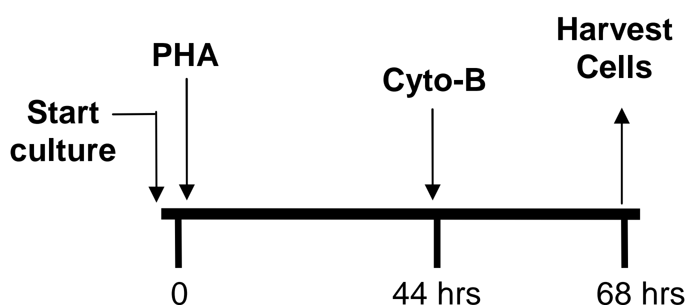

Figure 9. Cytokinesis block micronucleus cytome assay 68 hour culture protocol for whole blood. At time 0 PHA (a mitogen) is added to the culture to stimulate mitosis, 44 hours later Cyto-B is added to prevent cytokinesis. Exactly 24 hours later the cells are harvested and processed for DNA damage analysis. PHA; phyto-haemagglutinin, Cyto-B; Cytochalasin-B.

Five hundred microlitres of heparinised blood was added to 4.5 ml of culture medium in a sterile 30 ml universal container. Mitogenesis was stimulated with phyto-haemagglutinin (PHA, 200 μg/ml, Murex Biotech, Kent, England). Cultures were incubated with caps loose in humidified atmosphere 5% CO_2 at 37°C for. Forty-four hours after PHA stimulation the cultures were removed from the incubator. Cytochalasin-B (6.0μg/ml; Sigma, Sydney, Australia) was added to the culture to block cytokinesis and then re-incubated for a further 24 hours. Twenty-four hours after the addition of cytochalasin-B the cultures were removed from the incubator and resuspended by gentle pipetting to disassociate cell clumps. The total volume of the culture was overlaid onto 1.8 ml of Ficoll-Paque (Amershan BioSciences, Uppsala, Sweden) in a 50 ml falcon tube (Technoplas, Adelaide, Australia) then centrifuged at 400 g for 30 mins at 18 - 20°C. The lymphocyte layer was removed and transferred to a 10 ml sterile conical tube containing 3x removed volume of Hanks Balanced Salt Solution (Hanks BSS, Trace Scientific, Melbourne, Australia) before centrifugation at 180g for 10 mins. The supernatant was discarded and the cell pellet gently resuspended in 300 μl of culture medium. Dimethyl sulfoxide (DMSO; 7.5% v/v of cell suspension) (Sigma, Sydney, Australia) was added to the cell suspension to minimise clumping and to optimise cytoplasmic boundary recognition. Cells were resuspended and incubated for 10 minutes at room temperature (RT). One hundred microlitres of cell suspension was transferred into a cyto-centrifuge sampling cup (attached to a microscope slide) prior to centrifugation at 600 rpm for 5 minutes using a cyto-centrifuge (Model Cytospin 3; Shandon Southern Products, Cheshire, England). The microscope slides were removed and air-dried for 10 minutes at RT. The cells were fixed in Diff Quick fixative (Lab Aids, Narrabeen, Australia) for 10 minutes before staining with the Diff Quick red dye, followed by the blue

dye (8 - 10 dips per stain) and rinsed with tap water. The slides were air dried slides for 10 minutes at RT and cover slipped using DePeX mounting medium (BDH Laboratory Supplies, Poole, England). One slide contained two sample spots from each duplicate culture.

9.4.4 Slide scoring criteria

The scoring criteria for the various cell endpoints (displayed in

Figure 10 and Figure 13) were as described by Fenech [255] and Fenech *et al* [311]. CBMN cytome slides were scored using 1000x magnification (10x eyepiece magnification, 100x oil immersion objective magnification) using a conventional light microscope (Model Leica DM LB2; Leica Microsystems, Wetzlar, Germany).

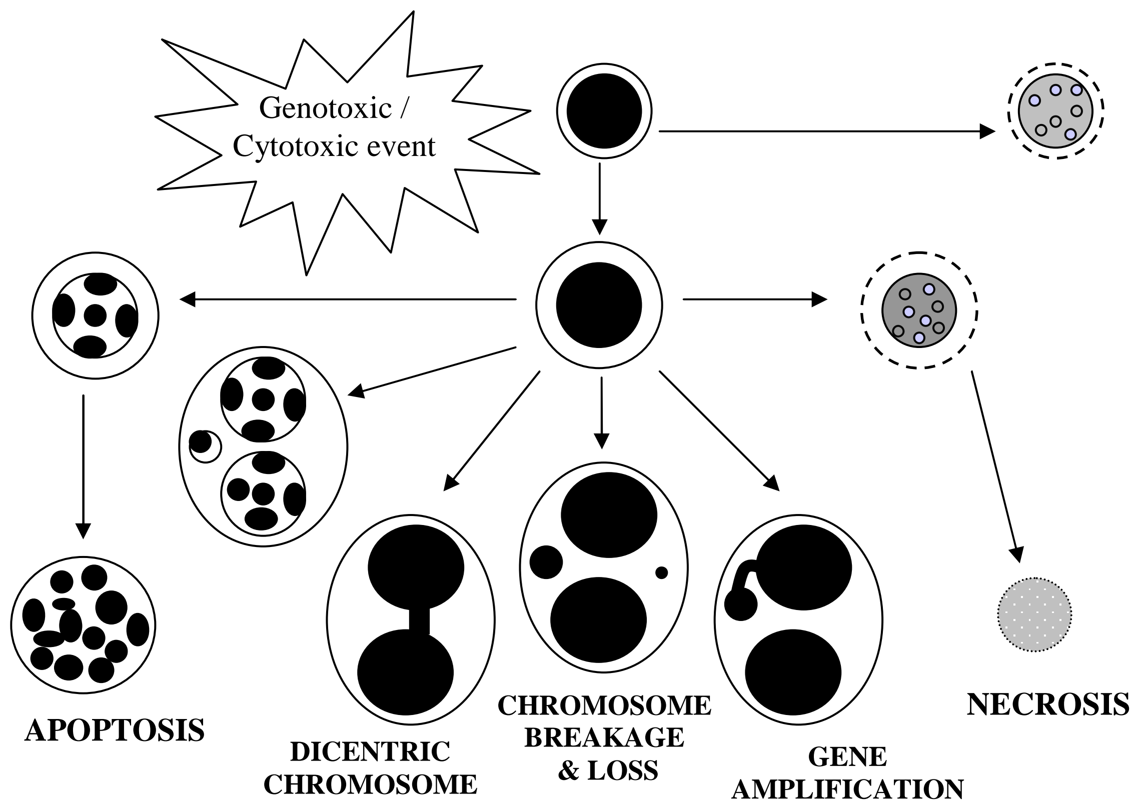

Figure 10. The various endpoints scored using the CBMN cytome assay.

From left to right; Using the CBMN assay it is possible to detect cell death in the form of apoptosis, chromosome rearrangements, such as dicentric chromosomes (nucleoplasmic bridges), chromosome breakage and loss (micronuclei), gene amplification (nuclear buds) and necrosis, another biomarker of cell death. Adapted from Fenech, 2003 [311].

Mononucleated, binucleated (BN) and multinucleated cells along with apoptotic and necrotic cells were scored until 200 cells per spot were classified; 2 spots from each duplicate culture, 200 cells per spot, 400 cells per culture, total 800 cells per patient were scored. The frequency of mono-, bi- and multinucleated viable cells was used to calculate the nuclear division index (NDI). This is a useful parameter to evaluate the viable cells capacity to respond to a mitogen and divide. NDI was calculated using the equation of Eastmond and Tucker [347]

$$NDI = \frac{(M1 + 2xM2 + 3xM3)}{N}$$

Where M1-M3 represent the number of viable cells with one, two and three or more nuclei, respectively, and *N* is the total number of viable cells recorded.

At least 500 BNs per spot (1000 BNs per culture, total 2000 BNs per patient) were scored for the presence of micronuclei (MN), nucleoplasmic bridges (NPB) and nuclear buds (NBUD).

Criteria for identifying binucleates (BN) for measuring micronuclei (MN-BN), nucleoplasmic bridges (NPB-BN) and nuclear buds (NBUD-BN)

The cytokinesis-blocked cells that were scored for MN-BN, NPB-BN and NBUD-BN frequency should have the following characteristics:

1. The cells should be binucleated.
2. The two nuclei in a BN cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.
3. The two nuclei in a BN cell should be approximately equal in size, staining pattern and staining intensity.
4. The two nuclei within a BN cell may be attached by a fine nucleoplasmic bridge which is no wider than one-fourth of the largest nuclear diameter.
5. The two main nuclei in a BN cell may touch but ideally should not overlap each other. A cell with two overlapping nuclei can be scored only if the nuclear boundaries of each nucleus are distinguishable.
6. The cytoplasmic boundary or membrane of a BN cell should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.

Photographic examples of mono, bi and multinucleated cells are shown in Figure 11A-C.

Criteria for scoring micronuclei (MN-BN)

MN are morphologically identical to but smaller than the main nuclei. They also have the following characteristics:

1. The diameter of MN in human lymphocytes usually varies between 1/16 and 1/3 of the mean diameter of the main nuclei which corresponds to 1/256 and 1/9 of the area of one of the main nuclei in a BN cell, respectively.
2. MN are round or oval in shape.
3. MN are non-refractile and they can therefore be readily distinguished from artefacts such as staining particles.
4. MN are not linked or connected to the main nuclei.
5. MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary.
6. MN usually have the same staining intensity as the main nuclei but occasionally staining may be more intense.

An example of MN-BN is illustrated in Figure 11F.

Criteria for scoring nucleoplasmic bridges (NPB-BN)

Nuceoplasmic bridges (NPB) are sometimes observed in binucleated cells following exposure to clastogens or as a result of telomere end fusion. They are thought to originate from rearranged chromosomes with more than one centromere, e.g. dicentric chromosomes. They have the following characteristics:

1. NPBs are a continuous nucleoplasmic link between the nuclei in a binucleated cell.
2. The width of a nucleoplasmic bridge may vary considerably but usually does not exceed one-fourth of the diameter of the nuclei within the cell.
3. NPBs should have the same staining characteristics of the main nuclei.
4. On rare occasions more than one nucleoplasmic bridge may be observed within one binucleated cell.
5. A binucleated cell with a nucleoplasmic bridge (NPB-BN) may or may not contain one or more micronuclei.

An example of NPB-BN is illustrated in Figure 11G.

Criteria for scoring nuclear buds (NBUB-BN)

Nuclear buds (NBUD) are a biomarker of gene amplification. NBUDs are morphologically similar to MN, but are attached to the nucleus via a nucleoplasmic connection. Shimizu *et al* [348, 349] showed that amplified DNA is concentrated to a peripheral point in the nucleus during S-phase, after which it is budded out from the nucleus to form a MN. The MN is subsequently pushed out of the cell, forming a mini cell. NBUD-BN were scored using the following criteria:

1. NBUDs should have a diameter between 1/16 and 1/3 of the main nuclei.
2. the staining intensity should be similar to the intensity of the main nuclei.
3. NBUDs are joined to the nucleus by a nucleoplasmic connection that is thinner than the NBUDs

An example of a NBUD-BN is illustrated in Figure 11H

Criteria for scoring apoptotic and necrotic cells

Apoptotic cells

1. Early apoptotic cells can be identified by the presence of chromatin condensation within the nucleus and intact cytoplasmic and nuclear boundaries.

2. Late apoptotic cells exhibit nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane.

3. Staining intensity in the nucleus, nuclear fragments and cytoplasm is usually greater than in viable cells.

Necrotic cells

1. Early necrotic cells can be identified by the presence of a pale cytoplasm with numerous vacuoles (mainly in the cytoplasm and some in the nucleus) and damaged cytoplasmic membrane with a fairly intact nucleus.

2. Late necrotic cells exhibit loss of cytoplasm and damaged/irregular nuclear membrane with only a partially intact nuclear structure and often with nuclear material leaking from the nuclear boundary.

3. Staining intensity of the nucleus and cytoplasm is usually less than that observed in viable cells. Examples of an apoptotic and necrotic cell are shown in Figure 11D and E.

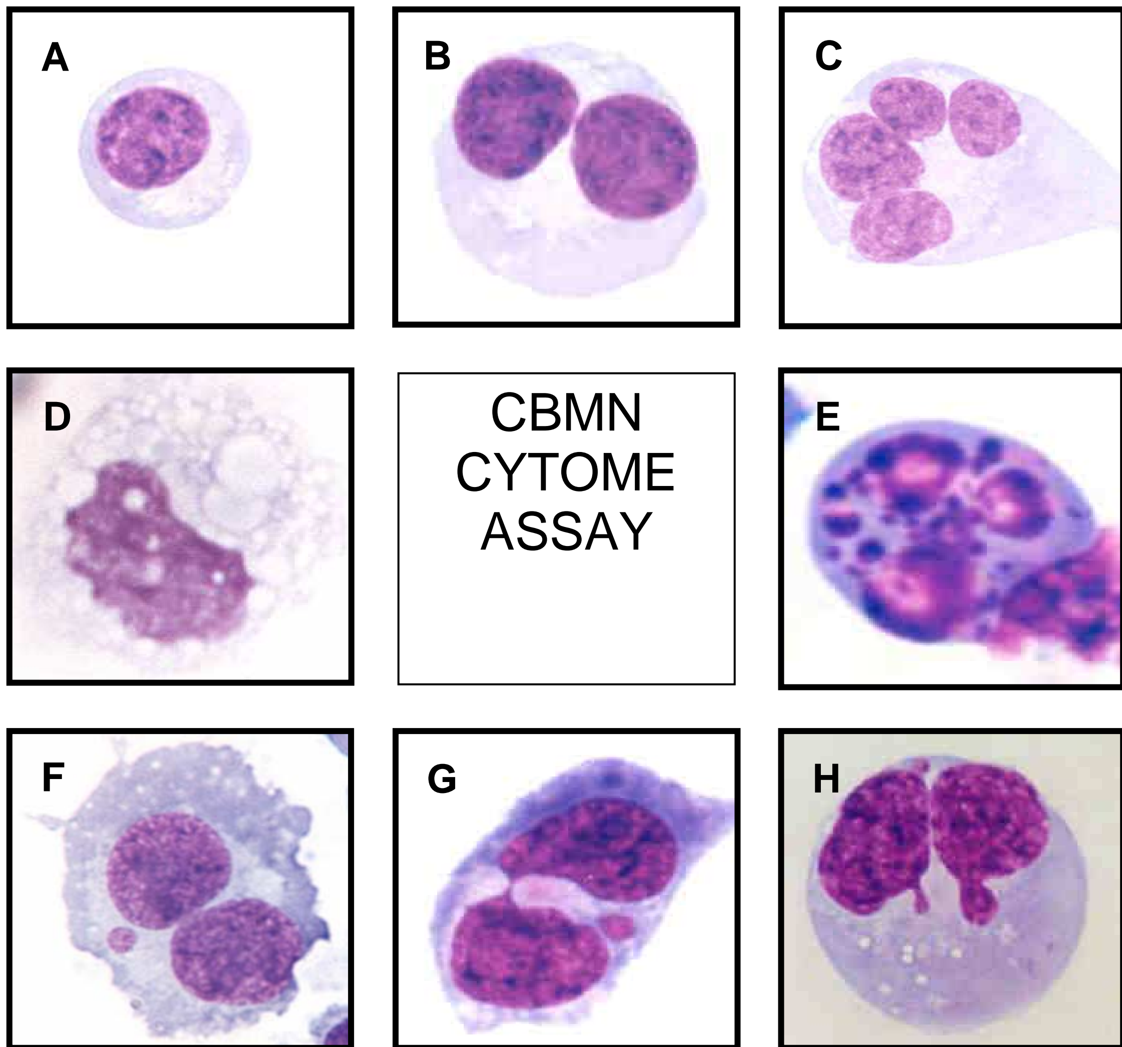

Figure 11. Various cell endpoints. A: mononucleated cell, B: binucleated cell, C: multinucleated cell D: necrotic cell, E: apoptotic cell, F: micronucleated binucleated cell (MN-BN), G: binucleated cell with nucleoplasmic bridge (NPB-MN), H: binucleated cell with nuclear buds (NBUD-BN).

9.4.5 Statistics

The association between pregnancy outcomes and genome damage biomarkers with age, BMI and smoking status was determined by Pearson's correlation analysis and/or 2 - tailed independent t-tests. Differences between groups were compared using independent sample t-tests and logistic regression: $P < 0.05$ was considered statistically significant. Logistic regression was used to correct for the effects of other variables that might be different between groups and might affect genome damage biomarkers. All data analyses were performed by means of the computer based statistical package of Statistical Product and Service Solution (SPSS) version 14.0 (SPSS, Chicago, IL, USA). Results were reported as mean ± S.E. Sensitivity, specificity, positive and negative predictive values and odds ratios for specific threshold values of MN-BN cell frequencies were determined using GraphPad PRISM 4.0 (GraphPad, San Diego, CA, USA).

9.5 Results

9.5.1 Age, BMI and smoking status in study groups

There was no difference in age between the control groups and the groups with high risk and adverse pregnancy outcomes. Independent t-test did not show an association with respect to age and adverse pregnancy outcome. BMI and the proportion of smokers were, significantly lower in the low group risk and control groups (Table 1).

Table 1. Comparison of Age, BMI and smoking status

	Age (y)	BMI (kg/M^2)	% Smokers*
Low Risk (n=50)	31.5 ± 0.8	26.6 ± 0.6	5.9%
High Risk (n=93)	33.8 ± 0.8	29.5 ± 0.8	19.6%
t-test (2-tailed) P	0.097	**0.009**	**0.001**
Control (n=43)	31.9 ± 0.8	26.8 ± 0.6	4.6%
UPI (n=49)	32.5 ± 1.0	29.9 ± 1.1	30.6%
t-test (2-tailed) P	0.696	**0.011**	**0.001**
Control (n=43)	31.9 ± 0.8	26.8 ± 0.6	4.6%
PE (n=15)	35.1 ± 1.9	31.6 ± 2.3	33.3%
t-test (2-tailed) P	0.077	**0.009**	**0.002**
Control (n=43)	31.9 ± 0.8	26.8 ± 0.6	4.6%
IUGR (n=19)	30.9 ± 1.4	31.5 ± 2.1	36.8%
t-test (2-tailed) P	0.507	**0.007**	**0.001**

All data represented as mean ± SEM
*% Smokers was calculated by dividing the number of smokers by the total number of women in each specific group

9.5.2 Genome damage biomarker correlations with age, BMI and smoking

Age and smoking were correlated with MN-BN frequency (r = 0.253, P = 0.003 and r = 0.241, P = 0.005 respectively). In addition all measured genome damage biomarkers were positively correlated with each other (P < 0.005) and negatively correlated with NDI; Pearson's correlation matrix is displayed in appendix 1. As smoking is categorical independent t-tests were performed to investigate the mean difference in MN-BN frequency in smokers and non-smoker both Pearsons correlation matrix and t-tests showed a P value of 0.005 (Table 2).

Table 2. The difference in genome damage markers between smokers and non-smokers

	‰ MN-BN	‰ NPB-BN	‰ NBUD-BN	NDI	‰ Apoptosis	‰ Necrosis
Smoker						
No (n=115)	19.5 ± 0.8	6.8 ± 0.5	27.3 ± 2.1	2.0 ± .02	136.9 ± 5.0	86.8 ± 2.6
Yes (n=21)	26.3 ± 3.4	7.0 ± 0.8	25.9 ± 3.8	2.1 ± .03	139.9 ± 15.1	88.5 ± 7.7
P (2-tailed)	**0.005**	0.843	0.783	0.372	0.821	0.806

9.5.3 Genome damage and cytotoxicity markers in low risk and an at risk population for pregnancy complications at 20 weeks gestation

The descriptive statistics (Table 3) show the spread of the CBMN genome damage biomarkers in both the low risk and high risk pregnancy groups.

Table 3. Description of MN-BN, NPB-BN, NBUD-BN, NDI, apoptosis and necrosis in low risk and high risk pregnancy groups at 20 weeks gestation

	‰ MN-BN	‰ NPB-BN	‰ NBUD-BN	NDI	‰ Apoptosis	‰ Necrosis
Low risk (n=50)						
Minimum	4.93	1.50	6.43	1.38	57.00	25.00
Mean	**15.98**	**7.54**	**26.53**	**2.00**	**149.32**	**87.28**
Maximum	31.97	34.70	95.73	2.38	324.00	138.50
Std. Deviation	5.45	6.97	19.12	0.30	56.96	26.53
High risk (n=93)						
Minimum	5.90	1.00	5.45	1.54	21.30	16.50
Mean	**22.83**	**6.41**	**27.39**	**2.00**	**131.27**	**87.00**
Maximum	73.28	33.00	153.00	2.37	290.30	164.80
Std. Deviation	11.46	4.18	23.17	0.20	54.48	29.69

The mean values of the genome damage biomarkers MN-BN, NPB-BN and NBUD-BN in women with a low risk of pregnancy complications in comparison with that found in women with high risk pregnancies at 20 weeks gestation are shown in Table 4. The results show a highly significant increased MN-BN frequency in high risk pregnancies compared to women with low risk pregnancies ($P < 0.0001$). This remained significant after controlling for age, smoking and BMI ($P = 0.004$; Figure 12). A reduction in mitogen response is shown by the lower nuclear division index (NDI) in the high risk pregnancy group ($P = 0.017$; Figure 13). Apoptosis was marginally reduced in women with predicted high risk pregnancies.

Table 4. Frequency of MN-BN, MN-NPB, MN-NBUD, NDI, apoptosis and necrosis in high risk and low risk pregnancy groups

	‰ MN-BN	‰ NPB-BN	‰ NBUD-BN	NDI	‰ Apoptosis	‰ Necrosis
All cases 20 weeks						
Low Risk (n=50)	16.0 ± 0.8	7.5 ± 1.0	25.5 ± 2.8	2.1 ± .04	149.3 ± 8.4	87.3 ± 3.9
High Risk (n=93)	22.8 ± 1.2	6.4 ± 0.4	27.4 ± 2.4	2.0 ± .02	131.3 ± 5.7	87.0 ± 3.1
t-test (2-tailed) P	**<0.001**	0.225	0.829	**0.025**	0.074	0.958
Adjusted P value*	**0.004**			**0.017**	0.076	

All data represented as mean ± SEM

‰ represents frequency per 1000 BN cells

*Controlled for age, smoking and BMI using logistic regression analysis

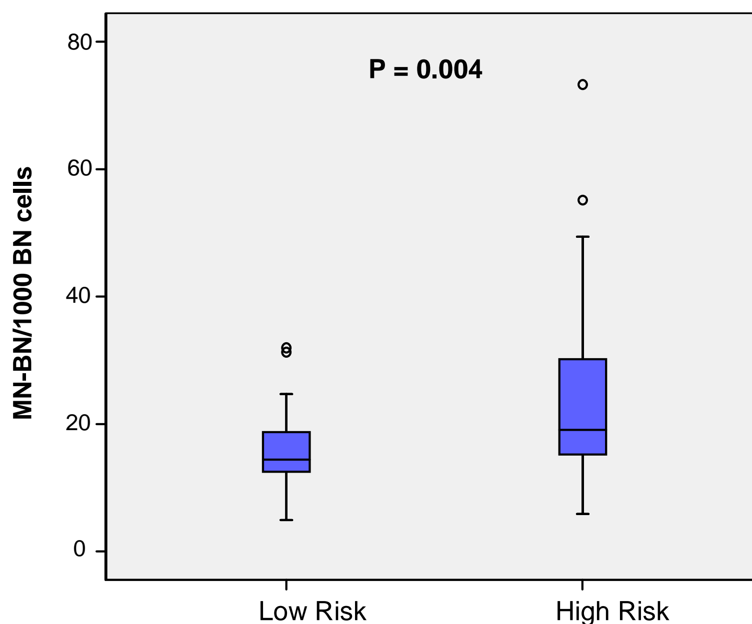

Figure 12. Frequency of MN-BN in low risk and high risk pregnancy groups

The box plot shows the median (the central horizontal black line) and measures of dispersion - inter-quartile range (blue box), range (vertical lines) and outliers (circles).

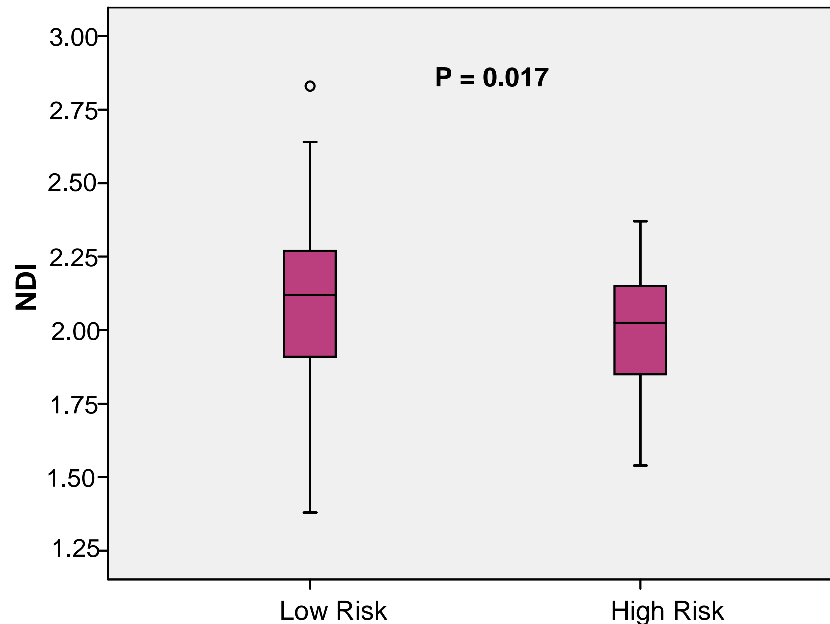

Figure 13. NDI at 20 weeks gestation in low risk and high risk pregnancy groups

The box plot shows the median (the central horizontal black line) and measures of dispersion - inter-quartile range (blue box), range (vertical lines) and outliers (circles).

9.5.4 Genome damage and cytotoxicity markers in a low risk pregnancy population that developed normal and adverse outcomes

There were no significant differences when comparing genome damage and cytotoxicity markers amongst the low risk pregnancy group with normal or adverse outcome; however, this comparison was not significantly powered due to the small numbers in the adverse pregnancy outcome group n = 7 (Table 5).

Table 5. Frequency of MN-BN, NPB-BN, NBUD-BN, NDI, apoptosis and necrosis in a low risk pregnancy group

	‰ MN-BN	‰ NPB-BN	‰ NBUD-BN	NDI	‰ Apoptosis	‰ Necrosis
Low risk only						
Normal (n=43)	16.1 ± 0.9	7.4 ± 0.9	27.9 ± 3.2	2.1 ± .05	153.6 ± 9.4	88.1 ± 4.4
Adverse (n=7)	16.2 ± 1.8	8.3 ± 4.4	28.1 ± 3.2	2.2 ± .07	125.6 ± 16.0	82.3 ± 8.0
t-test (2-tailed) P	0.969	0.770	0.810	0.550	0.236	0.622

All data represented as mean ± SEM
‰ represents frequency per 1000 BN cells

9.5.5 Genome damage and cytotoxicity markers in a high risk pregnancy population that developed normal and adverse outcomes including UPI, PE and IUGR

Within the high risk population a significant difference was observed between the MN-BN frequencies in women who developed UPI, PE and IUGR (Table 6); however, there was no difference with regards to other genome damage and cytotoxic markers. Trends for increased DNA damage were evident in the high risk sub-group with adverse pregnancy outcomes relative to the women who had normal outcomes, but this was not significant.

Table 6. Frequency of MN-BN, NPB-BN, NBUD-BN, NDI, apoptosis and necrosis in high risk pregnancies that developed adverse outcomes

	‰ MN-BN	‰ NPB-BN	‰ NBUD-BN	NDI	‰ Apoptosis	‰ Necrosis
High risk only						
Normal (n=28)	20.5 ± 1.3	5.2 ± 0.5	22.8 ± 3.5	2.0 ± .04	140.3 ± 9.0	93.3 ± 5.9
Adverse (n=65)	23.5 ± 1.5	6.9 ± 0.6	29.1 ± 3.1	2.0 ± .02	127.8 ± 7.1	84.6 ± 3.7
t-test (2-tailed) P	0.387	0.077	0.252	0.508	0.331	0.211
Normal (n=28)	20.5 ± 1.3	5.2 ± 0.5	22.8 ± 3.5	2.0 ± .04	140.3 ± 9.0	93.3 ± 5.9
UPI (n=46)	25.0 ± 1.9	6.6 ± 0.5	28.0 ± 2.8	2.0 ± .03	132.0 ± 8.2	86.3 ± 4.4
t-test (2-tailed) P	**0.035**	0.774	0.997	0.323	0.751	0.848
Adjusted P value*	**0.046**					
Normal (n=28)	20.5 ± 1.3	5.2 ± 0.5	22.8 ± 3.5	2.0 ± .04	140.3 ± 9.0	93.3 ± 5.9
PE (n=15)	29.9 ± 3.2	7.1 ± 1.1	30.5 ± 3.7	2.0 ± .03	153.9 ± 14.3	95.4 ± 8.6
t-test (2-tailed) P	**0.009**	0.522	0.593	0.827	0.070	0.194
Adjusted P value*	**0.019**					
Normal (n=28)	20.5± 1.3	5.2 ± 0.5	22.8 ± 3.5	2.0 ± .04	140.3 ± 9.0	93.3 ± 5.9
IUGR (n=19)	24.7 ± 3.0	6.1 ± 1.0	29.7 ± 3.7	2.0 ± .05	145.1 ± 13.8	86.9 ± 6.3
t-test (2-tailed) P	**0.010**	0.434	0.327	0.591	0.513	0.895
Adjusted P value*	**0.190**					

All data represented as mean ± SEM
‰ represents frequency per 1000 BN cells
*Controlled for age, smoking and BMI using logistic regression analysis

9.5.6 Genome damage and cytotoxicity markers in a in low risk healthy pregnancy (controls) and those who developed UPI including PE and IUGR

Maternal peripheral lymphocyte MN-BN cell frequency was increased in UPI including PE and IUGR (Table 7). When controlled for age, smoking and BMI, the association remained significant (P = 0.002; Figure 14; P = 0.005; Figure 16; P = 0.023, Figure 17). Apoptosis was significantly reduced in women who developed UPI (P = 0.025; Figure 16); however, this was not demonstrated in individual PE and IUGR groups.

Table 7. Frequency of MNed BNs, NPBs, NBUDs, NDI, apoptosis and necrosis in controls and women who developed UPI

	‰ MN-BN	‰ NPB-BN	‰ NBUD-BN	NDI	‰ Apoptosis	‰ Necrosis
Control (n=43)	15.8 ± 0.8	7.3 ± 0.9	27.9 ± 3.2	2.1 ± .04	153.8 ± 9.4	87.8 ± 4.5
UPI (n=49)	24.1 ± 1.7	6.9 ± 0.7	26.7 ± 2.5	2.0 ± .03	130.5 ± 7.5	85.5 ± 4.0
t-test (2-tailed) P	**<0.0001**	0.693	0.768	0.286	**0.049**	0.631
Adjusted P value*	**0.002**				**0.025**	
Control (n=43)	15.8 ± 0.8	7.3 ± 0.9	27.9 ± 3.2	2.1 ± .04	153.8 ± 9.4	87.8 ± 4.5
PE (n=15)	29.9 ± 3.2	7.1 ± 1.1	30.5 ± 3.7	2.0 ± .03	153.9 ± 14.3	95.4 ± 8.6
t-test (2-tailed) P	**<0.0001**	0.797	0.467	0.173	0.995	0.397
Adjusted P value*	**0.005**					
Control (n=43)	15.8 ± 0.8	7.3 ± 0.9	27.9 ± 3.2	2.1 ± .04	152.6 ± 9.6	87.1 ± 4.4
IUGR (n=19)	24.7 ± 3.0	6.1 ± 1.0	29.7 ± 3.7	2.0 ± .05	145.13 ± 13.8	86.9 ± 6.3
t-test (2-tailed) P	**0.001**	0.346	0.858	0.344	0.769	0.983
Adjusted P value*	**0.023**					

All data represented as mean ± SEM
‰ represents frequency per 1000 BN cells
*Controlled for age, smoking and BMI using logistic regression analysis

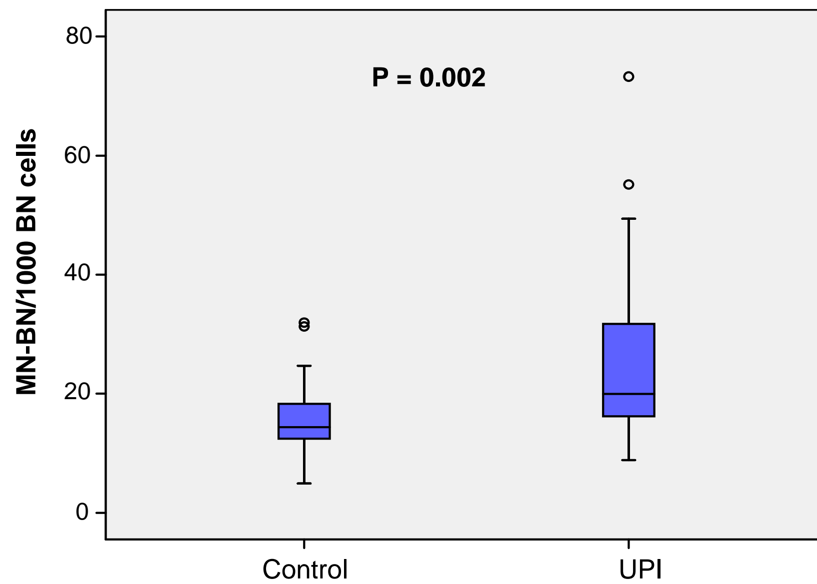

Figure 14. Frequency of MN-BN in clinically normal pregnancies and UPI

The box plot shows the median (the central horizontal black line) and measures of dispersion - inter-quartile range (blue box), range (vertical lines) and outliers (circles).

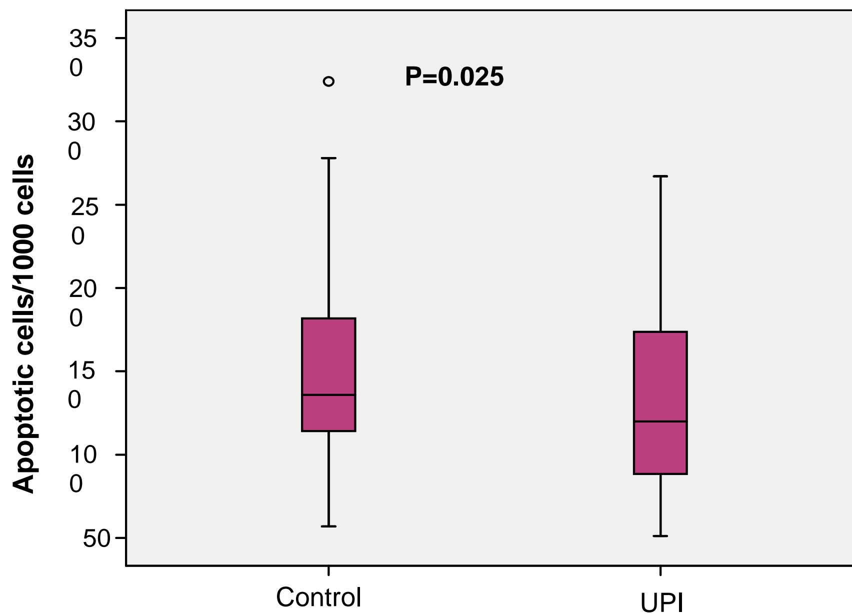

Figure 15. Frequency of Apoptotic cells in low risk healthy pregnancies (controls) and UPI

The box plot shows the median (the central horizontal black line) and measures of dispersion - inter-quartile range (blue box), range (vertical lines) and outliers (circles).

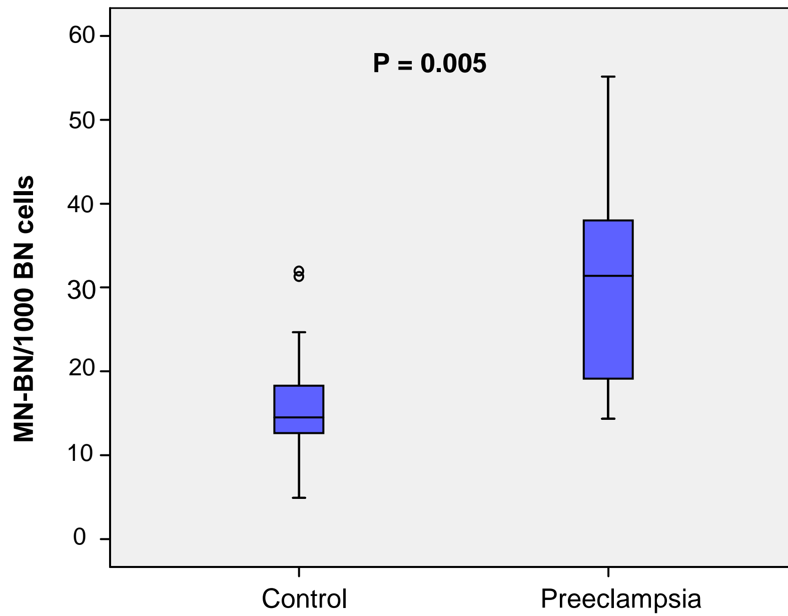

Figure 16. Frequency of MN-BN in low risk healthy pregnancies (controls) and preeclampsia

The box plot shows the median (the central horizontal black line) and measures of dispersion - inter-quartile range (blue box), range (vertical lines) and outliers (circles).

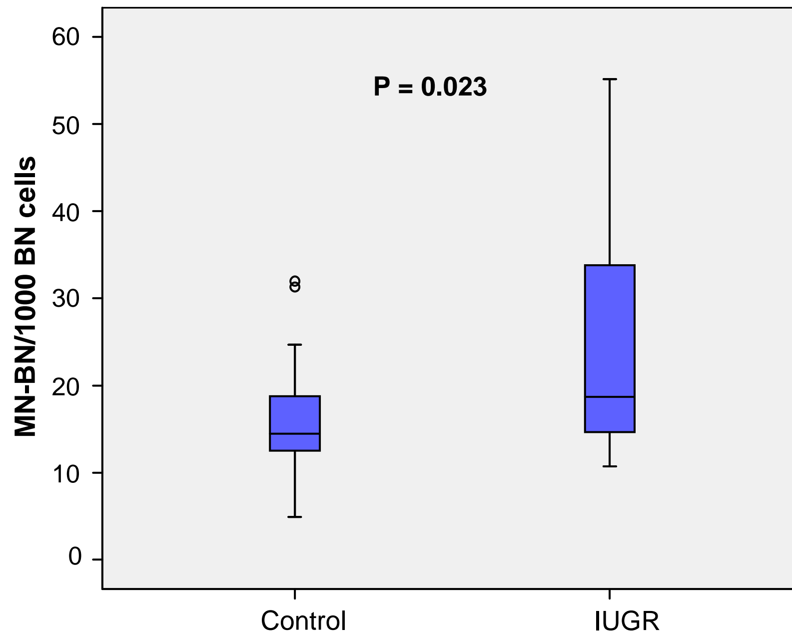

Figure 17. Frequency of MN-BN in low risk healthy pregnancies (controls) and IUGR

The box plot shows the median (the central horizontal black line) and measures of dispersion - inter-quartile range (blue box), range (vertical lines) and outliers (circles).

9.5.7 Predictive values

The potential of MN-BN frequency to be a predictive marker for risk of abnormal pregnancy (i.e. UPI, PE and IUGR) was determined by calculating the positive predictive values (PPV), negative predictive values (NPV), sensitivity, specificity and odds ratio (OR). PPV, NPV, sensitivity, specificity and OR at increasing threshold values starting at > 20 MN-BN per 1000 BNs are shown in Table 8. The results indicate that a pregnant woman at 20 weeks gestation with a MN-BN frequency > 35 per 1000 BNs has 100% chance of experiencing a pregnancy complication.

Table 8. Positive predictive value (PPV), negative predictive value (NPV), sensitivity, specificity, odds ratio (OR) and P values at defined cut offs of MN-BN frequency for high risk women and women who developed UPI, PE and IUGR

	PPV	NPV	Sensitivity	Specificity	OR (95% CI)	P Value
High Risk						
MN > 20	81.1%	43.4%	47.8%	78.3%	3.3 (1.5-7.4)	0.0032
MN > 25	81.6%	39.8%	34.4%	84.8%	2.9 (1.2-7.3)	0.0181
MN > 30	92.3%	40.0%	26.7%	95.7%	8.0 (1.8-35.6)	0.0017
MN > 35	100.0%	37.7%	15.6%	100.0%	17.6 (1.0-303)	0.0047
UPI						
MN > 20	76.5%	55.2%	50.0%	80.0%	4 (1.6-10.3)	0.0031
MN > 25	89.5%	52.1%	32.7%	95.0%	9.2 (2.0-42.9)	0.0011
MN > 30	87.5%	50.0%	26.9%	95.0%	7.0 (1.5-32.9)	0.0060
MN > 35	100.0%	48.8%	19.2%	100.0%	20.0 (1.1-353)	0.0033
PE						
MN > 20	55.6%	86.1%	66.7%	79.5%	7.8 (2.1-29.2)	0.0013
MN > 25	84.1%	80.0%	53.3%	95.0%	21.1 (3.7-121.4)	<0.0001
MN > 30	84.1%	80.0%	53.3%	95.0%	21.1 (3.7-121.4)	<0.0001
MN > 35	100.0%	79.6%	33.3%	100.0%	41.4 (2.1-810)	0.0002
IUGR						
MN > 20	52.9%	75.0%	47.4%	79.0%	3.4 (1.0-11.1)	0.0406
MN > 25	77.8%	75.0%	36.8%	94.7%	10.5 (1.9-57.6)	0.0021
MN > 30	75.0%	73.5%	31.2%	94.7%	8.3 (1.4-46.5)	0.0070
MN > 35	100.0%	71.7%	21.1%	100.0%	22.4 (1.1-441)	0.0034

9.6 Discussion

This study is the first to investigate the association between genomic instability and cytotoxicity biomarkers in the development of uteroplacental insufficiency (UPI) and associated diseases. Our results indicate that genome damage in the form of micronucleated cells (MN-BN) in peripheral blood lymphocytes is significantly increased in women at risk of developing an adverse pregnancy outcome. Almost 80% of women exhibiting more than 20 micronucleated binucleate cells (MN-BN per 1000 binucleates) are at high risk of having some type of pregnancy complication. In addition the results show that, when genome damage frequency increased to 25 MN-BN per 1000 BNs, there is a 95% chance of developing UPI, preeclampsia (PE) or intrauterine growth restriction (IUGR). It is evident that 100% PPV and 100% specificity are achievable at MN-BN frequency greater than 35 but the NPV and sensitivity are substantially less at this threshold value. The odds ratio (OR) values indicate the increased level of risk of higher threshold values, but the confidence intervals increase proportionally. The observed OR for the high MN-BN frequency (> 20 MN-BN‰) may be the highest observed for any biomarker selected in relation to risk of pregnancy complications to date; however, the sensitivity is relatively low (15.6 - 33%).

Studies have shown that smoking during pregnancy is associated with a decreased risk of developing hypertensive disorders in pregnancy of up to 56% [350]. Our data differ from previous studies and show smoking to be associated with the development of PE. This may be due to the selected high risk cases that have developed previous PE and other risk factors making them more likely to develop the disease, regardless of smoking status. In addition IUGR was also linked with smoking during pregnancy, which has been previously demonstrated [351].

In this study increased body mass did have an effect on both PE and IUGR. The literature states that the risk for PE increases with a greater BMI [352, 353] while a low pre-pregnancy BMI is one of strongest predictors of IUGR [354]. The higher BMI in relation to IUGR may have been due to poor dietary choices and/or lifestyle affecting placental and fetal development.

Base-line genome damage biomarker frequencies provide an index of accumulated genetic damage which has occurred during the life span of the lymphocytes (half life approximately 6 months [355]) as well as being a marker of inherited or acquired defects in genome

maintenance. The baseline MN-BN rate for women in the low risk pregnancy group was 15.98 per 1000 BN at 20 weeks gestation (Table 3). The difference between the minimum and maximum frequencies in the control group was 4.93 - 31.97 MN-BN per 1000 BN. This inter-individual variation may be explained by lifestyle factors including environmental exposures such as genotoxic or cytotoxic agents or genetic susceptibility factors [356, 357]. The minimum and maximum frequency of MN-BN in women in the high risk pregnancy group was much higher 5.90 – 73.28 per 1000 BNs (2). Other studies have shown mean MN-BN from lymphocytes of healthy non-pregnant women in a similar age bracket (18-54) to be 10 – 30 MN-BN per 1000 BN cells [358, 359].

The only other known study [360] to measure micronuclei (MN) in maternal lymphocytes during pregnancy was performed at 7 - 8 weeks gestation to test the effect of gestogens in pregnancy. The maternal MN frequency was lower than the observed values in our study, 6.79 ± 0.69‰ in the control group, 11.83 ± 1.33‰ and 15.67 ± 3‰ for the two high risk groups [360]. Trkova et al. [256] related MN frequency with infertility demonstrating a significant difference between couples with reproductive failure (14.95 ± 6.04 per 1000 BN) and healthy fertile (control) couples (10.60 ± 2.57 per 1000 BN). Fenech et al. [359] found that the MN frequency in females is, on average, 1.4 times greater than that in males. This has also been confirmed by the HUMN project study in which data from 25 laboratories were analysed [312]. It is thought that the loss of the X chromosome contributes to the MN frequency in females [361] and Hando et al [362] proved this showing that the X chromosome was present in 72.2% of the MN scored in women, with other chromosomes being involved to a lesser extent [363].

MN are formed from whole chromosomes and chromosomal fragments that lag behind in anaphase and are left outside daughter nuclei in telophase [364]. Fragments may occur due to direct double-strand DNA breakage, conversion of single stranded breaks into double stranded breaks after cell replication, or inhibition of DNA synthesis [365]. Misrepair of two chromosome breaks may lead to asymmetrical chromosome rearrangement, producing a dicentric chromosome and an acentric fragment [343]. The centromeres of the dicentric chromosomes can be pulled to opposite poles of the cells at anaphase resulting in the formation of NPBs between the daughter nuclei and an acentric fragment that lags behind to form a MN [366, 367]. MN can also arise by gene amplification via breakage-fusion-bridge cycles when amplified DNA is selectively localized to specific sites at the periphery of the nucleus and eliminated via NBUDs during the S-phase of the cell cycle [366]. In this study

the frequency of NPB-BN and NBUD-BN did not differ significantly between healthy or pathologic pregnancies leading to the assumption that mis-repair of chromosome breaks and gene amplification may not be linked with these diseases, leaving whole chromosome loss as a plausible explanation.

Whole chromosome loss can be caused by defects in chromosome segregation machinery, such as deficiencies in the cell cycle controlling genes (e.g. hCDC4 and BUBR1 [368]), mitotic spindle failure, defects in kinetochore proteins [310] or hypomethylation of centromeric DNA, which leads to fragility and despiralisation of chromosomes 1, 9 and 16 [366].

The majority of publications related to MN provide evidence that higher frequencies are found in the populations exposed to genotoxic agents [310]. Therefore, we can speculate that women with an abnormally high frequency of MN-BN within the high risk and pathologic groups may have been chronically exposed to a mutagen or aneugen, are deficient in micronutrients that affect chromosome segregation, or have mutations in genes that code for important enzymes involved in genome maintenance, such as MTHFR. For example, inheritance of the MTHFR C677T variant allele is associated with aneuploidy [369], Down syndrome [370] and folate deficiency, the last of which has been shown to increase chromosome malsegregation [371]. Moreover, hypomethylation of DNA which may lead to centromeric DNA dysfunction can also cause aneupoidy [333]. Identification of MN content by telomere and centromere detection using FISH technology may add useful information allowing clarification of the mechanisms involved in the formation of MN in pregnancy complications.

The elevated MN-BN cell frequency in high risk pregnancies and those who devloped UPI occurred together with a reduced apoptosis and lower NDI. The lower NDI indicates that the maternal lymphocytes may be less responsive to mitogen response and taking longer to divide. The lower apoptotic rate could mean cells with malsegregation of chromosomes and other genome damage events leading to MN formation are being permitted to survive, suggesting a possible problem in the cell cycle checkpoint and apoptosis processes. If these defects are due to inherited or acquired mutations then it may be that events observed in lymphocytes could be indicative of the proliferative potential and genome instability in cytotrophoblasts and other cells involved in the placentation process. Given the established

association of DNA damage and abnormal apoptosis and altered cellular proliferation in developmental and degenerative diseases it seems plausible that increased DNA damage may be an underlying cause of UPI.

9.7 Conclusion

The data from this study are in keeping with the hypotheses and provide preliminary evidence that increased MN-BN frequency is associated respectively with increased risk for UPI including PE and IUGR.

10 B-VITAMINS AND HOMOCYSTEINE IN UTEROPLACENTAL INSUFFICIENCY

10.1 Aim

1. To determine whether folate (vitamin-B_9), cobalamin (vitamin-B_{12}), pyridoxine (vitamin-B_6) and homocysteine (Hcy) concentrations in pregnant women at 20 weeks gestation are associated with the development of uteroplacental insufficiency (UPI), including preeclampsia (PE) and intrauterine growth restriction (IUGR).

2. To establish the range of B-vitamin and Hcy concentrations at 20 weeks gestation in pregnant women who may or may not be supplementing with B-vitamins.

3. To compare the maternal circulating folate, vitamin-B_{12}, vitamin-B_6 and Hcy concentrations with genome damage markers in peripheral lymphocytes at 20 weeks gestation.

10.2 Hypotheses

1. Women with increased Hcy at 20 weeks gestation have increased risk of UPI including PE and IUGR.

2. Women with reduced blood concentrations of folate (vitamin-B_9), cobalamin (vitamin-B_{12}) and pyridoxine (vitamin-B_6) have increased risk of UPI, including PE and IUGR.

3. Decreased circulating folate, vitamin-B_{12}, vitamin-B_6 and/or increased Hcy concentration are associated with increased chromosome breaks, chromosome loss, gene amplification and cell death in peripheral blood lymphocytes in the mother at 20 weeks gestation.

10.3 Introduction

The maternal diet, and therefore the nutrient supply to the developing oocyte, embryo or fetus, is one of the major environmental factors influencing the developmental process and supports the high rates of cellular proliferation and DNA replication that take place during the fetal stages of life [97]. Optimal DNA stability is essential for normal cell differentiation and

growth. Diet is a key factor in determining genomic stability as it affects all relevant developmental pathways, i.e. activation/detoxification of chemicals preventing DNA oxidation, DNA repair, apoptosis and DNA synthesis [372].

Folic acid and the related vitamin-B_{12} and vitamin-B_6 have many fundamental and diverse biological properties [298]. Folate, vitamin-B_{12} and vitamin-B_6 participate in one carbon metabolism [373]. In this metabolism, a carbon unit from serine or glycine is transferred to tetrahydrofolate (THF) to form methylene-THF. Subsequently methylene-THF is used for the synthesis of thymidine or is oxidized to formyl-THF, which in turn is utilised for the synthesis of purines that are precursors for DNA synthesis. In addition, formyl-THF can be reduced to methyl-THF, which donates a methyl group to enable homocysteine (Hcy) to convert to methionine[1] (section 6.3, Figure 6). Methionine provides the methyl groups required for maintenance of DNA methylation of CpG sites which determines gene expression via S-adenosylmethionine (SAM) [98] and structural stability of chromosomes. Folate, vitamin-B_{12} and vitamin-B_6 deficiency causes a decrease in dependent reactions (e.g methionine synthesis) and an inadequate supply of required substrates and/or the accumulation of Hcy which can inhibit cell growth [104]. These events can contribute to placental vascular dysfunction which may be associated with uteroplacental insufficiency (UPI) [118].

Elevated blood Hcy concentration, termed hyperhomocysteinaemia, is a thrombophilic condition associated with reduced CpG methylation [374], increased DNA damage [231], cardio vascular disease [375], neural tube defects [376], congenital heart defects [135], miscarriages [132] and UPI including PE and IUGR [109, 118, 128, 130, 183, 377, 378]. The association of hyperhomocysteinemia and PE was initially suggested by Dekker et al. [128]. Studies by Dekker et al. [128] and Hague et al. [130] in Amsterdam and Adelaide, respectively, have shown that hyperhomocysteinemia is present in up to 30% of women with early onset PE and other pregnancy complications associated with placental development [183].

Hcy is thought to be associated with disease through endothelial dysfunction as well as arteriolar vasospasm in hepatic, cardiac, and cerebral circulations [379]. Endothelial dysfunction appears to be a central feature of the pathophysiological mechanism for PE and IUGR, leading to altered vascular reactivity, activation of the coagulation cascade, and a loss of vascular integrity [56]. Although the exact mechanism of endothelial damage is not known, there is growing evidence that Hcy exerts its pathological effects through oxidative damage [142]. In addition to Hcy, folate has been shown to modify endothelial oxidative metabolism

by increasing the availability of tetrahydrobiopterin, a cofactor for nitric oxide synthase, and by reducing superoxide production [380]. Excessive trophoblast apoptosis may also represent a pathogenic mechanism by which increased Hcy can cause placental diseases [378, 381]. In addition studies by Di Simone et al. [377] suggest that folic acid plays a protective role in the prevention of trophoblast apoptosis [246, 377], which may be caused by reduction in Hcy as research has shown that folic acid supplementation significantly reduces plasma Hcy concentrations [382].

Genetic polymorphism in genes involved in one-carbon metabolism (folate - Hcy metabolic pathway) can also lead to excess Hcy. Common variants in *methylenetetrahydrofolate reductase* (*MTHFR*), C677T and A1298C, are associated with decreased enzyme activity leading to mild hyperhomocysteinaemia in humans [121, 208]. This provides evidence that the level of MTHFR activity and the availability of folate and vitamins-B_6 and -B_{12} are all interrelated and affect Hcy concentrations, suggesting that these factors should be investigated together.

Pregnant women have a high folate requirement because of increased folate utilisation by the fetus [383]; it is therefore expected that a reduced red cell or serum folate status and subsequent higher plasma Hcy concentration would be present during pregnancy; however, studies [107, 384] have reported that Hcy concentrations are lower during pregnancy. This may occur because of the higher rate of Hcy consumption to produce methionine for methylation reactions via SAM and for polyamine and protein synthesis [385].

We hypothesised that deficiencies in folate, vitamin-B_{12}, vitamin-B_6 and elevated Hcy at 20 weeks gestation may alter one-carbon metabolism that can increase genome instability the risk for developing UPI including PE and IUGR.

10.4 Methodology

Demographic, clinical and vitamin supplementation information was obtained from the cohort before blood collection. Vitamin supplement brand, type, dose and frequency of intake were recorded.

10.4.1 Blood, plasma and serum collection

At 20 weeks gestation a fasted blood sample was collected from study participants in vacuatainers by venepuncture. Approximately 18 ml venous blood was collected from each patient in one 9 ml Vacuette®-Lithium Heparin tube, one 4 ml Vacuette®-K-EDTA and one 4 ml Vacuette®-Serum. The blood in the Vacuette®-Lithium Heparin was left at room temperature and processed within 4 hours of collection for the cytokinesis block micronucleus cytome (CBMN) assay and the remaining blood was frozen for future DNA isolation.

Bloods taken for micronutrient analysis were processed within 30 minutes of collection. To test for red cell folate (RCF) 500 µl of whole blood from the Vacuette®-K-EDTA was transferred to an eppendorf tube. Both the Vacuette®-K-EDTA with remaining blood and the Vacuette®-Serum tubes were spun at 1200 rpm for 10 minutes. The plasma was removed from the Vacuette®-K-EDTA and transferred to a cryovial and delivered within 2 hours of collection to the Core Laboratory, Division of laboratory Medicine, Women's and Children's Hospital Adelaide for the quantification of Hcy. Serum was removed from the Vacuette®-Serum and transferred to cryovial for folate and vitamin-B_{12} quantification then delivered along with the whole blood aliquot for RCF analysis and remaining packed cells in the Vacuette®-K-EDTA tube for vitamin B_6 quantification to the Institute of Medical and Veterinary Science (IMVS), Frome Road, Adelaide. All Vacuettes® were purchased from Greiner Bio-One, Kremsmuenster, Austria.

10.4.2 Quantification of serum and red blood cell folate

Serum and red blood cell (RCF) folate were quantified using the ARCHITECT® folate assay (Abbott Laboratories, Abbott Park, IL, USA), a chemiluminescent microparticular folate binding protein assay, on the ARCHITECT® *i* System. The ARCHITECT® folate assay has a total imprecision of < 10% within the calibration range (0.0 – 20.0 ng/mL). The analytical sensitivity of the assay is ≤ 0.8 ng/mL. Prior to quantification of the serum and RBC samples, a calibration curve was generated for the ARCHITECT® folate assay. Subsequently, every 24 hours, a single sample of all control levels was tested to ensure the control values were within

the concentration range as specified by the manufacturer. The assay was recalibrated when control(s) were out of range or when a new reagent kit with a new lot number was used.

To convert RBC-bound folate to measurable folate a manual pre-treatment is performed before the following process. Two pre-treatment steps mediate the release of folate from endogenous folate binding protein. First, serum/RBC and a pre-treatment reagent (dithiothreitol in acetic acid buffer with EDTA) were aspirated and dispensed into a reaction vessel. Second, an aliquot of the pre-treatment solution and potassium hydroxide were aspirated and dispensed into a second reaction vessel. Subsequently, an aliquot of the second pre-treatment solution was mixed with a TRIS buffer with protein stabilisers (human albumin) containing monoclonal mouse anti-folate binding protein coupled to microparticles affinity bound with bovine folate binding protein (FBP). Folate present in the serum/RBC binds to the FBP coated microparticles. After washing with phosphate buffered saline (PBS) solution, pteroic acid–acridinium labelled conjugate (in MES buffer with porcine protein stabiliser) was added, which binds to unoccupied sites on the FBP coated microparticles. Pre-trigger (1.32% w/v hydrogen peroxide) and trigger (0.35 N sodium hydroxide) solutions were then added, resulting in a chemiluminescent reaction that can be measured in relative light units (RLUs). An inverse relationship exists between the amount of folate in the serum/RBC and the RLUs detected by the ARCHITECT® *i* optical system. The concentration of folate in serum and RBCs was expressed as nmol/L. Quantification of folate was performed by Division of Clinical Biochemistry, Institute of Medical and Veterinary Science, Adelaide.

10.4.3 Quantification of vitamin-B_{12} in serum

Vitamin-B_{12} in serum was quantified using the ARCHITECT® B12 assay (Abbott Laboratories, Abbott Park, IL, USA), a chemiluminescent microparticular intrinsic factor assay, on the ARCHITECT® *i* System. The ARCHITECT® B12 assay exhibits total imprecision of < 10% within the calibration range (0.0 – 1476 pmol/L). The analytical sensitivity of the assay is ≤ 44.27 pmol/L. Prior to quantification of the serum samples of the participants, the ARCHITECT® vitamin-B_{12} assay was calibrated using the test calibrators supplied by the manufacturer and a calibration curve was generated. Subsequently, every 24 hours, a single sample of all control levels was tested to ensure the control values were within the concentration range as specified by the manufacturer. The assay was recalibrated when control(s) were out of range or when a new reagent kit with a new lot number was used.

In a reaction vessel serum was combined with three pre-treatment reagents, containing either a) 1.0 N sodium hydroxide with 0.005% potassium cyanide, b) alpha monothioglycerol and EDTA or c) cobinamide dicyanide in borate buffer with protein stabilisers (avian).

Subsequently, an aliquot of the pre-treatment solution and potassium hydroxide (KOH) were aspirated and dispensed into a second reaction vessel. Subsequently, an aliquot of the pre-treatment solution was mixed in a new reaction vessel with assay diluent (borate buffer with EDTA) and intrinsic factor (porcine) coated microparticles (in borate buffer with bovine protein stabilisers). Vitamin-B_{12} present in the serum binds to the intrinsic factor coated microparticles. After washing with a PBS solution, B_{12}–acridinium labelled conjugate (in MES buffer) was added, which binds to unoccupied sites on the intrinsic factor coated microparticles. Pre-trigger (1.32% w/v hydrogen peroxide) and trigger (0.35 N sodium hydroxide) solutions were then added to the reaction mixture, resulting in a chemiluminescent reaction that can be measured in relative light units (RLUs). An inverse relationship exists between the amount of vitamin-B_{12} in the serum and the RLUs detected by the ARCHITECT® *i* optical system. Vitamin-B_{12} concentrations of the samples were determined from the standard (calibration) curve by matching the absorbance readings with the corresponding B_{12} concentrations. The concentration of vitamin B_{12} was expressed as pmol/L. Quantification of vitamin-B_{12} in serum was performed by the Division of Clinical Biochemistry, Institute of Medical and Veterinary Science, Adelaide.

10.4.4 Quantification of vitamin-B_6 status in red blood cells via red cell aspartate amino-transferase (AST)

Vitamin-B_6 was tested using Red Cell Aspartate Amino-Transferase (AST) activation by pyridoxal phosphate [386]. AST requires pyridoxal phosphate to be active. Addition of pyridoxal phosphate to red cell AST will cause an increase in pyridoxal phosphate activity; the extent of the increase is a reflection of the deficiency of pyridoxal phosphate (vitamin-B_6) in the patient. Pyridoxal phosphate activation (PPA) activity is expressed as a percentage and greater than 63% represents vitamin-B_6 deficiency [386]. Quantification of Vitamin-B_6 in RBC was performed by the Division of Clinical Biochemistry, Institute of Medical and Veterinary Science, Adelaide.

10.4.5 Quantification of total L-Homocysteine in plasma

L-homocysteine in plasma was quantified using the AxSYM® homocysteine (Hcy) assay (Abbott, Wiedbaden, Germany), a fluorescence polarisation immunoassay (FPIA), on the AxSYM ® system. The AxSYM® Hcy assay exhibits total imprecision of < 6% within the calibration range (0.0 – 50 µmol/L). Prior to quantification of the Hcy samples of the participants, the AxSYM® Hcy assay was calibrated using the test calibrators supplied by the manufacturer and a calibration curve was generated. Subsequently, every 24 hours, a single

sample of all control levels was tested to ensure the control values were within the concentration range as specified by the manufacturer. The assay was recalibrated when control(s) were out of range or when a new reagent kit with a new lot number was used.
In the assay homocystine, mixed disulfide, and protein-bound forms of Hcy are reduced to form free Hcy by dithiothreitol (DTT). Subsequently, free Hcy is converted to S-adenosylHcy (SAH) by the use of SAH hydrolase and excess adenosine. Under physiological conditions, SAH hydrolase converts SAH to Hcy. Excess adenosine in the pre-treatment solution drives the conversion of Hcy to SAH by SAH hydrolase. Briefly, plasma was pre-treated in a reaction vessel with 0.1 M phosphate buffer, bovine SAH hydrolase (in phosphate buffer with bovine protein stabilizers) and a pre-treatment solution containing DTT and adenosine in citric acid. An aliquot of this solution was mixed with monoclonal mouse anti-S-adenosyl-L-Hcy (in phosphate buffer with bovine protein stabilisers) and 0.1 M phosphate buffer, and transferred to the cuvette of the reaction vessel. In succession, a second aliquot of the pre-treated plasma solution was mixed with S-adenosyl-L-cysteine fluorescence tracer (in phosphate buffer with bovine protein stabiliser) and 0.1 M phosphate buffer, and transferred to the cuvette. SAH and the labelled fluorescent tracer compete for the sites on the antibody molecule. The intensity of the resulting polarised fluorescent light was measured by the FPIA optical assembly. Total L-Hcy concentrations of the samples were determined from the standard (calibration) curve by matching the absorbance readings with the corresponding total L-Hcy concentrations. The concentration of total L-Hcy was expressed as μmol/L. Quantification of total L-Hcy in plasma was performed by the Core Laboratory, Division of laboratory Medicine, Women’s and Children’s Hospital.

10.4.6 Statistics

Low risk healthy pregnancies were chosen for the control group. High risk pregnancies with normal outcomes were not included into the control group due to abnormal medical and obstetric histories (section 8.2.1). Assuming a standard deviation of 171 nmol/L and 2.9 μmol/L of RCF and Hcy respectively [231] this study had 80% power to detect a difference of 96.9 nmol/L and 1.4 μmol/L of RCF and Hcy between groups with $N = 50$ at $P < 0.05$. The association between pregnancy outcome folate, vitamin-B_{12} and vitamin-B_6, Hcy concentration, age, BMI and smoking status were determined by Pearson’s correlation analysis and/or 2-tailed independent t-tests. Differences between groups were compared using independent sample t-tests and logistic regression. Logistic regression was used to correct for the effects of confounding variables such as age, smoking and BMI that may be different between groups and may affect micronutrient concentrations. Data analyses were performed

by means of the computer based statistical package of Statistical Product and Service Solution (SPSS) version 14.0 (SPSS, Chicago, IL, USA). Sensitivity, specificity, positive and negative predictive values and odds ratios were calculated for Hcy in relation to pregnancy outcome and RCF with IUGR based on the significant t-test results. Predictive values using specific cut offs for Hcy and RCF concentrations were determined using GraphPad PRISM 4.0 (GraphPad, San Diego, CA, USA). Results were reported as mean ± S.E. Effects of $P < 0.05$ were considered statistically significant.

10.5 Results

10.5.1 B-vitamin supplement results

10.5.1.1 B-vitamin supplementation in women with low risk and high risk pregnancies

From the cohort of 143 patients 27 (18.9%) reported no vitamin supplement intake. The recommended daily intake (RDI) for pregnant women is 600 µg/day of folic acid, 2.6 µg/day of vitamin-B_{12} and 1.9 mg/day of vitamin-B_6 [387]. The two most common supplements used were Elevit (Bayer Australia Ltd, NSW, Australia) and Blackmores Pregnancy and Breastfeeding (Blackmores, NSW, Australia). Elevit's daily dosage is one tablet which contains 800 µg folic acid, 4 µg vitamin-B_{12} and 2600 µg vitamin-B_6, which are higher than the RDI's for pregnant women in Australia [387]. Blackmores Pregnancy and Breastfeeding recommends a daily dosage of two tablets that contained 250 µg folic acid, 1.5 µg vitamin-B_{12} and 750 µg Vitamin-B_6 each, holding less than the RDI of vitamin-B_6 [387] and folic acid. A large percentage of the women classified as having a high risk pregnancy supplemented with Megafol 5mg folic acid tablets.

The women in the low risk pregnancy group recorded supplement intakes of folic acid and vitamin-B_6 slightly higher than the RDI; however, their vitamin-B_{12} was on average 3 times the RDI. The women in the high risk pregnancy group recorded a daily supplement intake over 5 times the RDI for folic acid, 9 times the RDI for vitamin-B_{12} and almost twice the RDI for vitamin-B_6. Figure 18 shows the daily supplement intake levels in RDI equivalents. Independent t-tests demonstrated a significant difference between the low and high risk pregnancy groups with regard to intake of folic acid ($P < 0.001$) and vitamin-B_{12} ($P < 0.001$).

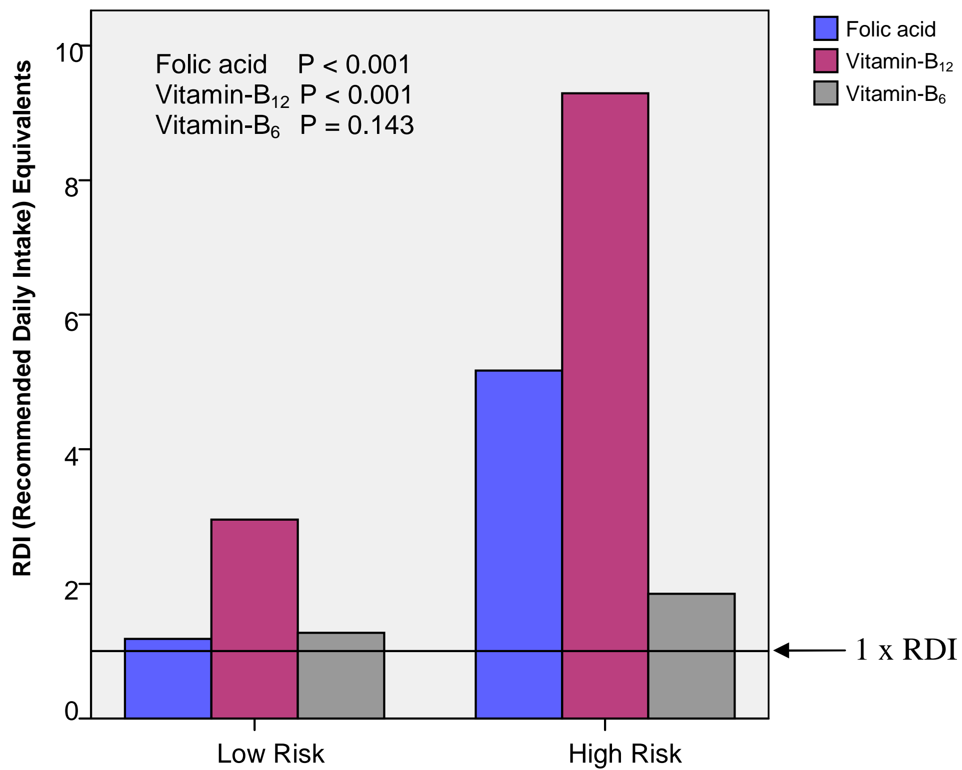

Figure 18. Folic acid, vitamin-B_{12} and B_6 supplement intake

The supplement intake for women classified as having low risk and high risk pregnancies has been expressed in RDI equivalents. The horizontal line represents the current RDI for pregnant women in Australia. Blue, Folic acid; Pink, vitamin-B_{12}; Grey, vitamin-B_6.

10.5.1.2 Pearsons correlation with age, BMI and smoking in pregnant women

Age was shown to have a significant positive association with supplement intakes for all three B-vitamin. BMI and smoking status did not demonstrate a significant correlation with vitamin supplement intake (Table 9).

Table 9. Pearsons correlation of B-vitamin supplement with age, BMI and smoking

	Folic Acid	Vitamin-B_{12}	Vitamin-B_6
Age (y)			
r value	0.428	0.419	0.279
P value	**<0.001**	**<0.001**	**0.001**
BMI			
r value	0.067	-0.015	-0.077
P value	0.435	0.870	0.386
Smoking			
r value	0.115	-0.164	-0.143
P value	0.182	0.064	0.105

10.5.1.3 The association between vitamin supplements and circulating concentrations of folate, vitamin-B_{12}, vitamin-B_6 and homocysteine

Independent t-tests showed a significant increase in all circulating B-vitamins in women who supplemented with folic acid (RCF, P = <0.001; SF, P = <0.001; Vitamin-B_{12}, P = 0.003; Vitamin-$B_{6,}$ P = 0.008). Mothers who reported supplementing with vitamin-B_{12} and vitamin-B_6 had significantly higher serum folate, serum vitamin-B_{12} and red cell vitamin-B_6 (Table 10).

Table 10. B-vitamin supplements and circulating concentrations of B-vitamins and homocysteine

	Hcy µmol/L	RCF nmol/L	SF nmol/L	B_{12} pmol/L	B_6 status*
Folic Acid					
Yes (n = 110)	4.5 ± 0.1	589.5 ± 20.2	29.1 ± 0.8	255.9 ± 14.9	39.9 ± 1.6
No (n = 27)	5.0 ± 0.4	339.9 ± 34.2	18.5 ± 2.2	174.0 ± 21.5	48.5 ± 2.6
P value	0.097	**<0.001**	**<0.001**	**0.003**	**0.008**
Vitamin-B_{12}					
Yes (n = 65)	4.3 ± 0.3	671.0 ± 42.8	34.3 ± 1.5	390.5 ± 35.6	20.5 ± 2.6
No (n = 64)	4.7 ± 0.1	523.4 ± 21.7	25.1 ± 0.9	200.7 ± 10.4	47.4 ± 1.2
P value	0.211	0.067	**<0.001**	**<0.001**	**<0.001**
Vitamin-B_6					
Yes (n = 65)	4.3 ± 0.3	639.9 ± 47.7	34.6 ± 1.4	383.7 ± 36.3	19.9 ± 2.4
No (n = 64)	4.7 ± 0.1	524.3 ± 22.0	25.1 ± 0.9	2.2.5 ± 10.6	47.5 ± 1.2
P value	0.270	0.125	**<0.001**	**<0.001**	**<0.001**

* Based on PPA activity which is inversely related to vitamin-B_6 concentration in RBCs
All data are represented as mean ± SEM
Hcy, L-Homocysteine in plasma; RCF, Red Cell Folate; SF, Serum Folate; B_{12}, Serum vitamin-B_{12}; $B_{6,}$ Red Cell vitamin-B_6

10.5.2 Circulating B-vitamins and homocysteine results

10.5.2.1 Pearson correlation with age and BMI

A significant positive association was observed between circulating concentrations of RCF, serum folate, vitamin-B_{12} and vitamin-B_6 with maternal age ($P < 0.001$). Circulating plasma Hcy concentrations demonstrated a negative association with age ($P = 0.030$). A higher BMI was significantly associated with increased levels of Hcy (Table 11).

Table 11. Pearsons correlation of circulating micronutrients with age and BMI

	Hcy µmol/L (n = 136)	RCF nmol/L (n = 136)	SF nmol/L (n = 137)	B_{12} pmol/L (n = 136)	B_6 status* (n = 132)
Age (y)					
r value	-0.187	0.326	0.351	0.307	-0.423
P value	**0.030**	**<0.001**	**<0.001**	**<0.001**	**<0.001**
BMI					
r value	0.209	0.003	-0.017	-0.105	-0.147
P value	**0.015**	0.976	0.845	0.225	0.093

* Based on PPA activity which is inversely related to vitamin-B_6 concentration in RBCs
All data are represented as mean ± SEM
Hcy, L-Homocysteine in plasma; RCF, Red cell Folate; SF, Serum Folate; B_{12}, Serum vitamin- B_{12}; B_6, Red Cell vitamin-B_6.

10.5.2.2 Circulating micronutrients and smoking

Women who smoked during pregnancy had significantly lower RCF and SF concentrations ($P < 0.001$; $P = 0.043$, respectively) and higher Hcy ($P < 0.001$) concentrations in comparison to non-smoking women (Table 12).

Table 12. Mean difference in circulating B-vitamins and homocysteine in smokers and non-smokers

Smoker	Hcy µmol/L	RCF nmol/L	SF nmol/L	B_{12} pmol/L	B_6 status*
No (n = 115)	4.4 ± 0.1	686.7 ± 20.2	27.3 ± 0.9	244.1 ± 13.5	41.5 ± 1.6
Yes (n = 21)	6.0 ± 0.5	462.9 ± 52.1	22.6 ± 2.2	208.9 ± 31.0	44.5 ± 3.1
P (2 - tailed)	**<0.001**	**<0.001**	**0.043**	0.317	0.448

* Based on PPA activity which is inversely related to vitamin-B_6 concentration in RBCs
All data are represented as mean ± SEM
Hcy, L-Homocysteine in plasma; RCF, Red Cell Folate; SF, Serum Folate; B_{12}, Serum vitamin- B_{12}; B_6, Red Cell vitamin-B_6.

10.5.2.3 Pearsons correlation of circulating folate, vitamin-B_{12}, vitamin-B_6 and homocysteine

Lower plasma Hcy concentrations were significantly correlated with increased levels of RCF, SF and serum vitamin-B_{12}. There was no significant correlation between vitamin-B_6 and Hcy. Vitamin-B_6 was negatively associated with the other tested B-vitamins. Both RCF and SF were positively correlated with serum vitamin-B_{12} levels (Table 13).

Table 13. Pearsons correlation with circulating micronutrients and homocysteine concentrations

		RCF nmol/L	SF nmol/L	B_{12} pmol/L	B_6 status*
Hcy µmol/L	r value	- 0.375	- 0.285	- 0.228	0.023
	P value	**> 0.001**	**0.001**	**0.008**	0.798
	N	135	136	135	131
RCF nmol/L	r value		0.628	0.324	- 0.384
	P value		**> 0.001**	**> 0.001**	**> 0.001**
	N		136	135	131
SF nmol/L	r value			0.282	- 0.412
	P value			**0.001**	**> 0.001**
	N			136	132
B_{12} pmol/L	r value				- 0.370
	P value				**> 0.001**
	N				132

* Based on PPA activity which is inversely related to vitamin-B_6 concentration in RBCs
Hcy, L-Homocysteine in plasma; RCF, Red cell Folate; SF, Serum Folate; B_{12}, Serum vitamin- B_{12}; B_6, Red Cell vitamin-B_6.

10.5.2.4 Pearsons correlation of fetal weight with circulating B-vitamins and homocysteine concentration

The maternal circulating concentrations of RCF and plasma Hcy at 20 weeks gestation was significantly associated with fetal weight at the time of delivery (RCF, P = 0.016; Hcy, P = 0.044; Table 14).

Table 14. Pearsons correlation of maternal circulating B-vitamins and homocysteine with fetal weight

		RCF nmol/L	SF nmol/L	B_{12} pmol/L	B_6 status*	Hcy μmol/L
Fetal weight	r value	0.206	0.121	0.112	-0.102	-0.174
	P value	**0.016**	0.160	0.196	0.248	**0.044**
	N	136	136	135	131	135

*Based on PPA activity which is inversely related to vitamin-B_6 concentration in RBCs
Hcy, L-Homocysteine in plasma; RCF, Red cell Folate; SF, Serum Folate; B_{12}, Serum vitamin- B_{12}; B_6, Red Cell vitamin-B_6.

10.5.2.5 Pearson correlation of circulating micronutrients with genome damage markers

Table 15 shows the correlation between circulating maternal B-vitamins and plasma Hcy concentrations with measured genome damage markers. Increased plasma Hcy was significantly associated with higher frequencies of micronuclei (MN) and nucleoplasmic bridges (NPB) in maternal lymphocytes.

Table 15. Pearsons correlation of maternal circulating B-vitamins and homocysteine with genome damage markers

		Hcy µmol/L (N = 135)	RCF nmol/L (N = 135)	SF nmol/L (N = 136)	B_{12} pmol/L (N = 135)	B_6 status* (N = 131)
MN-BN	r value	0.179	-0.036	0.109	0.056	-0.143
	P value	**0.038**	0.681	0.205	0.517	0.104
NPB-BN	r value	0.171	0.003	-0.028	0.020	-0.074
	P value	**0.047**	0.975	0.746	0.821	0.402
NBUD-BN	r value	0.002	-0.090	0.001	0.043	0.047
	P value	0.981	0.301	0.986	0.623	0.596
Apoptosis	r value	-0.108	-0.132	-0.142	0.085	0.036
	P value	0.214	0.127	0.100	0.325	0.687
Necrosis	r value	-0.090	-0.125	-0.025	0.075	0.048
	P value	0.299	0.150	0.776	0.390	0.589
NDI	r value	0.021	-0.041	-0.033	-0.162	0.052
	P value	0.808	0.634	0.701	0.060	0.555

* Based on PPA activity which is inversely related to vitamin-B_6 concentration in RBCs

Hcy, L-Homocysteine in plasma; RCF, Red Cell Folate; SF, Serum Folate; B_{12}, Serum vitamin- B_{12}; B_6, Red Cell vitamin-B_6; MN, # Micronuclei per 1000 binucleated cells; NPB, # Nuclear plasmic bridges per 1000 binucleated cells; NBud, # Nuclear Buds per 1000 binucleated cells; NDI, Nuclear Division Index.

10.5.2.6 Circulating micronutrient concentrations at 20 weeks gestation in women with low and high risk pregnancies

Thirty-nine women were vitamin-B_{12} deficient (i.e. serum vitamin-B_{12} < 150 pmol/L) of which only four reported taking vitamin-B_{12} supplements. Eight mothers had vitamin-B_{12} concentrations below 100 pmol/L and of these none reported vitamin supplement intake. Vitamin-B_6 deficiency was observed in five women (PPA activity > 63%). Fourteen women were folate deficient (SF < 14 nmol/L); of these folate deficient women eleven did not take folic acid supplements. When comparing the micronutrient concentrations in women with low risk and high risk pregnancies a non significant elevation in plasma Hcy was observed relative to the low risk group (Table 16). The induced increment in PPA activity was significantly lower in the high risk pregnancy group reflecting higher vitamin-B_6 concentrations ($P = 0.035$, Table 16).

Table 16. Circulating B-vitamin and homocysteine concentratiuons at 20 weeks gestation in low risk and high risk pregnancies

	Hcy µmol/L	RCF nmol/L	SF nmol/L	B_{12} pmol/L	B_6 status*
Low Risk (N = 51)					
Mean ± SEM	4.3 ± 0.1	655.3 ± 32.0	25.4 ± 1.2	227.6 ± 18.0	47.6 ± 1.5
Min - Max	3.0 - 6.6	203.0 - 900.0	6.2 - 45.0	65.0 - 809.0	15.2 - 66.5
High Risk (N = 92)					
Mean ± SEM	4.8 ± 0.2	650.5 ± 25.6	27.1 ± 1.1	244.9 ± 16.4	39.0 ± 1.9
Min - Max	2.7 - 11.4	171.0 - 900.0	2.6 – 45.0	57.0 - 811.0	0.4 - 77.9
t-test (2-tailed) P	0.084	0.910	0.338	0.507	**0.003**
Adjusted P value**					**0.035**

* Based on PPA activity which is inversely related to vitamin-B_6 concentration in RBCs
**Controlled for age, smoking and BMI using logistic regression analysis
All data are represented as mean ± SEM
Hcy, L-Homocysteine in plasma; RCF, Red Cell Folate; SF, Serum Folate; B_{12}, Serum vitamin- B_{12}; B_6, Red Cell vitamin-B_6.

10.5.3 Circulating B-vitamins in low risk pregnancies and those at high risk of developing UPI

Within the low risk pregnancy group no significant difference was detected in B-vitamin or plasma Hcy concentrations in relation to normal and adverse pregnancy outcomes including UPI, PE or IUGR. Women who were at high risk of developing an adverse outcome, but delivered clinically normal babies had significantly lower Hcy concentrations than those who developed UPI (P = 0.012, after controlling for age, smoking and BMI). After controlling for confounding factors the significant association between vitamin-B_6 and UPI was lost. In addition after controlling for confounding factors the association detected between increased plasma Hcy concentrations and PE was lost. The women at high risk of developing adverse pregnancy outcomes that developed IUGR showed trend for lower concentrations for all circulating B-vitamins at 20 weeks gestation (Table 17).

Table 17. Circulating micronutrient concentrations at 20 weeks gestation in high risk pregnancies that developed normal outcomes or UPI, PE and IUGR

	Hcy µmol/L	RCF nmol/L	SF nmol/L	B12 pmol/L	B6 status*
Normal (N = 28)	3.9 ± 0.2	707.0 ± 42.0	27.9 ± 1.6	263.8 ± 30.3	33.2 ± 3.7
UPI (N = 45)	5.4 ± 0.3	607.3 ± 38.3	25.9 ± 1.7	236.5 ± 24.8	41.3 ± 2.7
t-test (2-tailed) P	**0.001**	0.085	0.471	0.543	**0.042**
Adjusted P value**	**0.012**				0.452
Normal (N = 28)	3.9 ± 0.2	707.0 ± 42.0	27.9 ± 1.6	263.8 ± 30.3	33.2 ± 3.7
PE (N = 15)	4.8 ± 0.4	664.3 ± 66.7	29.6 ± 2.6	319.0 ± 54.9	41.5 ± 5.5
t-test (2-tailed) P	**0.043**	0.577	0.549	0.349	0.164
Adjusted P value**	0.258				
Normal (N = 28)	3.9 ± 0.2	707.0 ± 42.0	27.9 ± 1.6	263.8 ± 30.3	33.2 ± 3.7
IUGR (N = 19)	5.9 ± 0.6	560.7 ± 71.2	22.9 ± 3.1	216.4 ± 37.8	42.0 ± 4.6
t-test (2-tailed) P	**<0.001**	0.084	0.148	0.364	0.085
Adjusted P value**	0.056				

* Based on PPA activity which is inversely related to vitamin-B_6 concentration in RBCs
**Controlled for age, smoking and BMI using logistic regression analysis
All data are represented as mean ± SEM
Hcy, L-Homocysteine in plasma; RCF, Red Cell Folate; SF, Serum Folate; B_{12}, Serum vitamin- B_{12}; B_6, Red Cell vitamin-B_6.

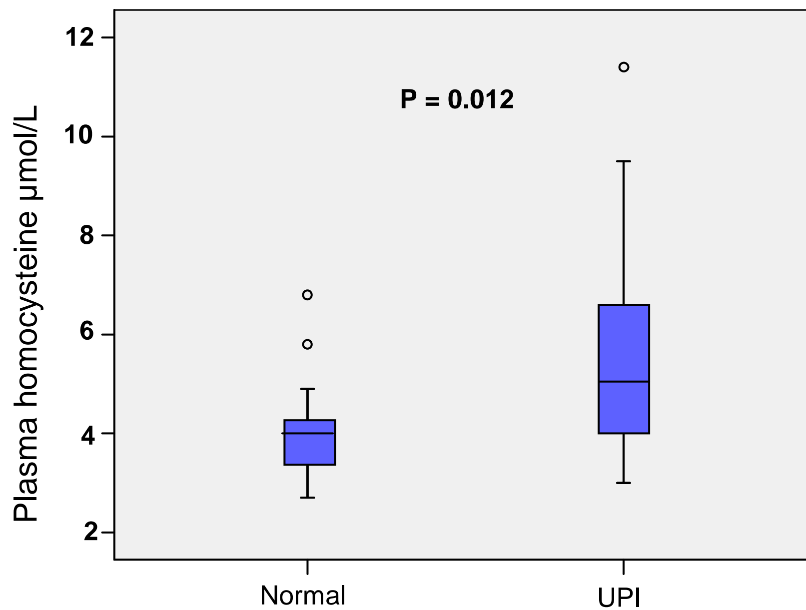

Figure 19. Hcy concentrations in women who had a normal pregnancy or developed UPI within the high risk pregnancy group.

The box plot shows the median (the central horizontal black line) and measures of dispersion - inter-quartile range (blue box), range (vertical lines) and outliers (circles).

10.5.4 Comparison of circulating micronutrient concentrations in low risk healthy pregnancies (controls) and women who developed uteroplacental insufficiency

At 20 weeks gestation there was a significant increase in plasma Hcy and vitamin-B_6 (lower PPA activity) concentrations in women who developed UPI (P = 0.002, P = 0.039 respectively) compared to low risk healthy (control) pregnancies. After controlling for age, smoking and BMI plasma Hcy remained significantly elevated (Figure 20); however, the significant association with vitamin-B_6 (represented as PPA activity) was lost (P = 0.094; Table 18). No significant difference was detected when comparing micronutrient levels and Hcy concentration in low risk healthy pregnancy controls and PE cases. Women who developed IUGR demonstrated a significant association with low red cell folate (RCF) concentration and increased plasma Hcy concentration at 20 weeks gestation as shown in Table 18, Figure 21 and Figure 22.

Table 18. Blood micronutrient concentrations at 20 weeks gestation in low risk healthy pregnancy controls and women who developed uteroplacental insufficiency

	Hcy µmol/L	RCF nmol/L	SF nmol/L	B_{12} pmol/L	B_6 status*
Control (N = 43)	4.3 ± 0.1	669.2 ± 32.6	25.7 ± 1.2	238.2 ± 20.6	47.3 ± 1.7
UPI (N = 49)	5.2 ± 0.3	603.7 ± 36.4	26.1 ± 1.6	242.2 ± 22.7	40.9 ± 2.5
t-test (2-tailed) P	**0.002**	0.149	0.798	0.758	**0.039**
Adjusted P value**	**0.027**				0.094
Control (N = 43)	4.3 ± 0.1	669.2 ± 32.6	25.7 ± 1.2	238.2 ± 20.6	47.3 ± 1.7
PE (N = 15)	4.8 ± 0.4	664.3 ± 66.8	29.6 ± 2.7	319.0 ± 54.9	41.5 ± 5.5
t-test (2-tailed) P	0.132	0.941	0.123	0.08	0.195
Control (N = 43)	4.3 ± 0.1	669.2 ± 32.6	25.7 ± 1.2	238.2 ± 20.6	47.3 ± 1.7
IUGR (N= 19)	5.7 ± 0.5	499.6 ± 63.4	21.9 ± 2.6	199.6 ± 30.4	44.0 ± 3.7
t-test (2-tailed) P	**0.001**	**0.005**	0.126	0.297	0.371
Adjusted P value**	**0.016**	**0.018**			

* Based on PPA activity which is inversely related to vitamin-B_6 concentration in RBCs.
**Controlled for age, smoking and BMI using logistic regression analysis.
All data are represented as mean ± SEM.
Controls were defined as low risk healthy pregnancies with normal outcomes.
Hcy, L-Homocysteine in plasma; RCF, Red Cell Folate; SF, Serum Folate; B_{12}, Serum vitamin- B_{12}; B_6, Red Cell vitamin-B_6.

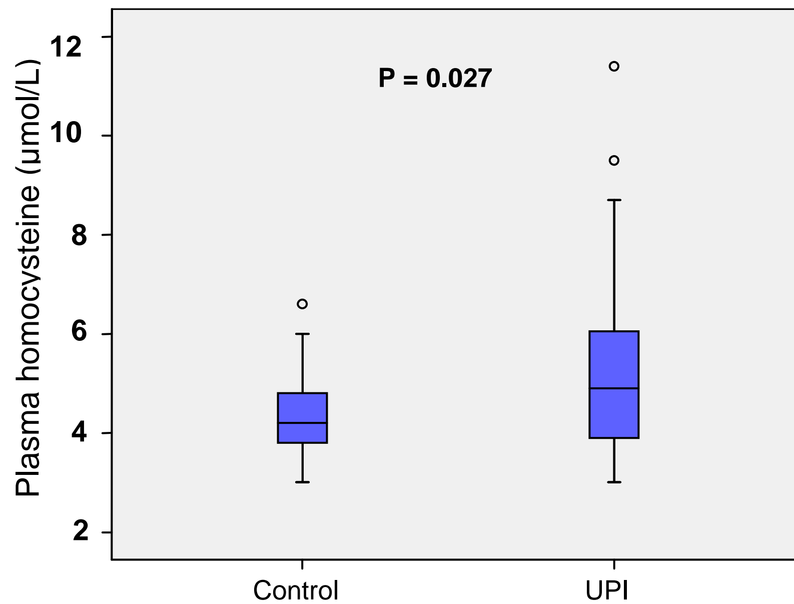

Figure 20. Plasma homocysteine concentration in women with low risk healthy pregnancies (controls) and women who developed UPI. The box plot shows the median (the central horizontal black line) and measures of dispersion - inter-quartile range (blue box), range (vertical lines) and outliers (circles).

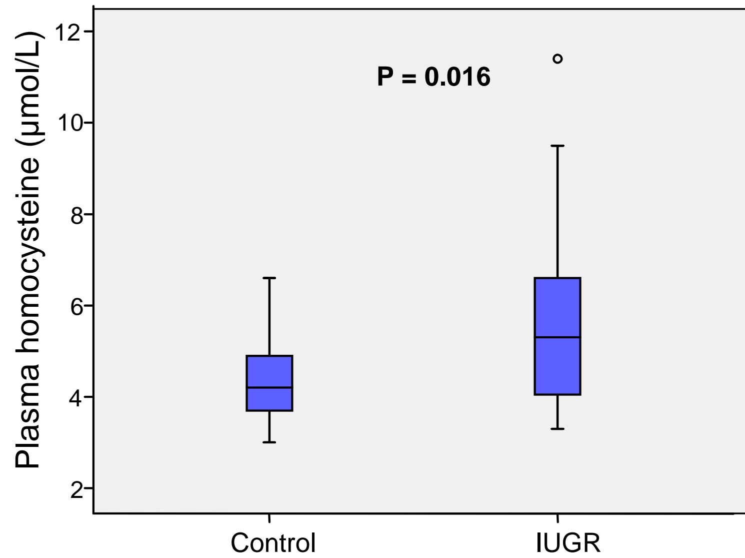

Figure 21. Plasma homocysteine concentration in women with low risk healthy pregnancies (controls) and women who developed IUGR. The box plot shows the median (the central horizontal black line) and measures of dispersion - inter-quartile range (blue box), range (vertical lines) and outliers (circles).One outlier with a plasma Hcy concentration of 11.4 µmol/L was identified; this patient reported no supplement intake.

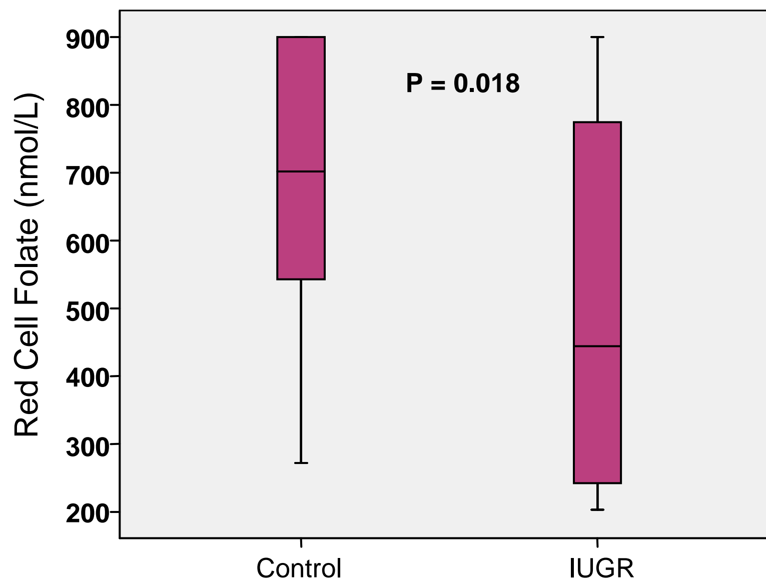

Figure 22. Red cell folate concentration in low risk healthy pregnancies (controls) and women who developed IUGR. The box plot shows the median (the central horizontal black line), two measures of dispersion the inter-quartile range (blue box), and range (vertical lines) and outliers (circles).

10.5.5 Predictive values

The potential of plasma Hcy concentration as a predictive marker for increased risk of developing an adverse pregnancy outcome, UPI, PE and IUGR was measured by calculating the positive predictive values (PPV), negative predictive values (NPV), sensitivity, specificity, likelihood ratio (LR) and odds ratio (OR). PPV, NPV, sensitivity, specificity, LR and OR at various thresholds values starting at plasma Hcy > 5.0 µmol/L are shown in Table 19. ORs greater than 2.8 and LRs greater than 2.4 were obtained for plasma Hcy > 5.5 µmol/L at $P < 0.05$ for all comparisons except in relation to PE as the analysis was underpowered.

Predictive values were calculated in relation to circulating maternal RCF concentration at 20 weeks gestation and risk of developing IUGR (Table 20). A maternal RCF concentration of < 550 nmol/L demonstrated ORs of 4.8 or greater with high significance ($P < 0.01$). The results indicate that at 20 weeks gestation decreased threshold values of maternal circulating RCF concentrations < 250 nmol/L were associated with 100% risk for developing IUGR, however the confidence interval increases substantially (1.5 - 562.6) as shown in Table 20.

Table 19. Positive predictive values (PPV), negative predictive values (NPV), sensitivity, specificity, likelihood ratio (LR), odds ratio (OR) and P values for homocysteine (Hcy) with respect to high and low risk pregnancies, high risk pregnancies with normal or adverse outcomes

	PPV	NPV	Sensitivity	Specificity	LR	OR (95% CI)	P Value
At Risk							
Hcy > 5.0	72.1%	36.6%	34.4%	73.9%	1.3	1.5 (0.7-3.3)	0.3214
Hcy > 5.5	82.1%	38.0%	25.6%	89.1%	2.4	2.8 (1.0-8.0)	**0.0451**
Hcy > 6.0	90.0%	37.9%	20.0%	95.7%	4.6	5.5 (1.2-24.9)	**0.0148**
Hcy > 6.5	93.8%	37.5%	16.7%	97.8%	7.7	9.0 (1.2-70.5)	**0.0131**
UPI							
Hcy > 5.0	71.4%	54.4%	49.0%	75.6%	2.0	3.0 (1.2-7.3)	**0.0156**
Hcy > 5.5	81.8%	52.9%	35.3%	90.2%	3.6	5.0 (1.5-16.4)	**0.0043**
Hcy > 6.0	86.7%	50.7%	25.5%	95.1%	5.2	6.7 (1.4-31.6)	**0.0078**
Hcy > 6.5	91.7%	50.0%	21.6%	97.6%	8.8	11 (1.4-89.3)	**0.0068**
PE							
Hcy > 5.0	37.5%	76.9%	40.0%	75.0%	1.6	2.0 (0.6-7.0)	0.2753
Hcy > 5.5	50.0%	76.6%	26.7%	90.0%	2.7	3.3 (0.7-15.3)	0.1184
Hcy > 6.0	60.0%	76.0%	20.0%	95.0%	4.0	4.8 (0.7-31.8)	0.0848
Hcy > 6.5	66.7%	75.0%	13.3%	97.5%	5.3	6.0 (0.5-71.8)	0.1151
IUGR							
Hcy > 5.0	54.6%	80.6%	63.2%	74.4%	2.5	5.0 (1.5-16.1)	**0.0057**
Hcy > 5.5	69.2%	77.8%	47.4%	89.7%	4.6	7.9 (2.0-31.1)	**0.0015**
Hcy > 6.0	77.8%	75.5%	36.8%	94.9%	7.2	10.8 (2.1-59.2)	**0.0017**
Hcy > 6.5	83.3%	73.1%	26.3%	97.4%	10.3	13.6 (1.5-126.6)	**0.0053**

Table 20. Positive predictive values (PPV), negative predictive values (NPV), sensitivity, specificity, likelihood ratio (LR), odds ratio (OR) and P values for red cell folate with respect to IUGR in comparison to controls

	PPV	NPV	Sensitivity	Specificity	LR	OR (95% CI)	P Value
IUGR							
RCF < 550	54.6%	80.0%	63.2%	73.7%	2.4	4.8 (1.5-15.6)	**0.0071**
RCF < 400	72.7%	76.1%	42.1%	92.1%	5.3	8.5 (1.9-37.7)	**0.0020**
RCF < 250	100%	73.1%	26.3%	100%	-	29.2 (1.5-562.6)	**0.0009**

10.6 Discussion

This study provides evidence that increased plasma homocysteine (Hcy) in early pregnancy may be associated with the development of uteroplacental insufficiency (UPI), in particular intrauterine growth restriction (IUGR). Plasma Hcy concentrations > 6.5 nmol/L provided the highest odds ratios (6 - 13.6) and specificity (97.4% – 97.8%); however, the sensitivity decreased proportionally.

Unlike previous studies [305, 388] plasma Hcy was not significantly increased in women who developed preeclampsia (PE). There have been six published reports on the possible value of using circulating Hcy concentrations in the prediction of PE, all of which were performed in low-risk women [182, 305, 389-392]. Plasma Hcy concentrations were significantly related to the subsequent development of the disease in three of these analyses [182, 305, 389] and not in the other three [390-392]. In our study age was positively associated with the development of PE. The results also show that older women had a higher B-vitamin supplement intake, which may explain the non-significant elevation in circulating B-vitamins in women that developed this disease. In addition B-vitamin supplementation may have reduced maternal plasma Hcy which may explain the non-significant association with PE in this study; however, further controlled studies are needed to clarify this.

Our results show a mean plasma Hcy concentration of 4.3 ± 0.1 μmol/L at 20 weeks gestation, which is lower than previously reported [392, 393]. Increased plasma Hcy was significantly associated with high BMI and increased cigarette smoking. Previous studies [394, 395] have associated smoking with increased plasma Hcy concentrations within the pregnant and general population; however, conflicting data have been published on the relationship between Hcy and BMI [396-399]. In addition the adverse effect of smoking on RCF and SF in the pregnant population [395, 400] and normal adult populations is well documented [401, 402]. RCF status may be influenced by smoking through a number of possible mechanisms as intermediates in one-carbon metabolism are sensitive to the redox balance within the cell. Cigarette smoking is a significant source of oxidative stress [403] and may alter the ability of the cell to metabolise and store folate [404].

Maternal circulating RCF < 400 nmol/L measured in the mother at 20 weeks gestation was significantly associated with the development of IUGR. The present finding of an association with maternal RCF levels and the offsprings birth weight at the time of delivery is in agreement with some [405, 406], but not all [407], previous publications. De Weerd et al. [407] reported no association between maternal RCF concentration and birth weight in a cohort of 240 women; however, their cohort included 15 women with a previous pregnancies with neural tube defect (NTDs) and 119 epileptic women. Both of these conditions are known to be associated with disturbed folate metabolism [408]. Our cohort did not contain any epileptic women or pregnancies complicated with NTDs.

Our results are of particular interest because, despite high supplementation with folic acid, serum vitamin-B_{12} and RBC vitamin-B_6 in women with high risk pregnancies, their plasma

Hcy levels were not significantly lower compared to those with low risk pregnancies with reduced supplement intakes. Increased plasma Hcy concentrations may have been caused by genetic factors such as polymorphisms in MTHFR [409], smoking [394] or other metabolic problems, such as reduced riboflavin [101]. In this case it is possible that high risk patients need supplements to bring them to concentrations that the normal population readily achieve. Three early studies [410-412] associated abnormalities in folate metabolism (one-carbon metabolism) in women who had previous cases of neural tube defects (NTDs); however, Davis et al. [413] claimed that the data was inconclusive due to limitations in the methodology used. Over the past decade little research has been published explaining the absorption and saturation levels of folate. Lucock et al., [414] and Kelly et al., [415] claim that the absorption and biotransformation process of folate is readily saturated at doses of about 400 µg per day; however, more research is needed to confirm these findings especially when an individual's genetic, metabolic and lifestyle factors are considered alone.

There have been contradicting explanations in regards to the placental perfusion studies and the uptake of B-vitamins by the fetus. Although nutrient-transfer via the placenta from the maternal plasma pool must be effective to satisfy the demand for fetal growth, information on placental folate transfer is scarce [416-419]. Our study suggests that RCF < 400 nmol/L may be associated with growth restriction but the extent of maternal folate deficiency that causes deficiency in the fetus remains unknown. Further studies are therefore needed to clarify the maternal-placental transfer of folate and other B-vitamins and at what levels the fetus becomes deficient and whether such thresholds are dependent on one-carbon metabolism genotypes.

Although folic acid is safe and almost free of toxicity [420], the hypothesis has been put forward that increased amounts of folic acid during the periconceptional period could lead to genetic selection by improving survival of embryos carrying the MTHFR C677T polymorphism which may raise Hcy concentrations if folate intake is subsequently restricted in the child [421, 422]; however, during gestation, marginal folate deficiency can impair cellular growth in the placenta and fetus [406]. Therefore it is important to ensure adequate concentrations are available during pregnancy to prevent both maternal and fetal morbidity, including NTD's [423]. Folate is now viewed not only as a nutrient needed to prevent megaloblastic anaemia in pregnancy but also as a vitamin essential for reproductive health [107].

Vitamin-B_6 in its role as a cofactor for cystathion synthase may facilitate the conversion of Hcy to cystathion and make Hcy less available for methionine synthesis; however, Hcy remained high despite high vitamin-B_6 activity in the high risk population.

Fenech proposed the concept that recommended dietary intakes should be based on preventing genomic instability as DNA is the most critical molecule in the cell, particularly in the early developmental process [372]. Studies in non pregnant populations has shown that it is necessary to supplement at higher doses, providing serum vitamin-B_{12} > 300 pmol/L and RCF > 700 nmol/L, plasma Hcy < 7.5 which are associated with minimisation of micronuclei (MN) [102]. In our study a positive association was detected between plasma Hcy and the genome damage markers, MN and nucleoplasmic bridges (NPB), which is consistant with in vitro data showing that MN and NPB are increased with folate-deficiency induced Hcy levels [424].

In the future a more comprehensive approach also targeting DNA methylation or polyamine synthesis via methionine may be required to assess if other abnormalities in one-carbon metabolism in mothers and their fetuses may be the underlying mechanism in the development of UPI.

11 POLYMORPHISMS IN ONE - CARBON METABOLISM GENES AND UTEROPLACENTAL INSUFFICIENCY

11.1 Aim

1. To study the prevalence of single nucleotide polymorphisms (SNPs) within enzymes involved in one-carbon metabolism in maternal and fetal tissue in women with low risk pregnancies that develop normal or adverse pregnancy outcomes.
2. To study the prevalence of single nucleotide polymorphisms (SNPs) within enzymes involved in one-carbon metabolism in maternal and fetal tissue in women with high risk pregnancies that develop normal or adverse pregnancy outcomes.
3. To investigate the possible association between these polymorphism frequencies and the development of uteroplacental insufficiency (UPI), including preeclampsia (PE) and intrauterine growth restriction (IUGR).
4. To study the association between these genotypes with circulating concentrations of red cell folate (RCF), serum folate (SF), serum vitamin-B_{12}, red cell vitamin-B_6 and plasma homocysteine (Hcy) in pregnant women.
5. To assess the association of single nucleotide polymorphisms (SNPs) within enzymes involved in one-carbon metabolism on genome damage in maternal peripheral lymphocytes.

11.2 Hypotheses

1. Women with high risk pregnancies and those who develop UPI have altered frequencies of alleles in *MTHFR* C677T and A1298C, *MTR* A2756G, *MTRR* A66G and *MTHFD1* G1958A genes.
2. UPI including PE and IUGR are associated with higher frequencies of alleles in the fetal genes *MTHFR* C677T and A1298C, *MTR* A2756G, *MTRR* A66G and *MTHFD1* G1958A.
3. Red cell folate (RCF), serum folate (SF), serum vitamin-B_{12}, red cell vitamin-B_6 and plasma homocysteine (Hcy) are regulated by alterations in SNP frequencies in maternal and/or fetal enzymes involved in one carbon metabolism.
4. Biomarkers of DNA damage, cell death and cytostasis in peripheral blood lymphocytes are affected by maternal SNPs within enzymes involved in one carbon metabolism.

11.3 Introduction

In addition to dietary uptake of micronutrients such as folic acid, vitamin-B_{12} and -B_6, genotype can affect folate metabolism (one-carbon metabolism), altering placental and fetal development. The folate - homocysteine (Hcy) metabolic pathway has a number of intermediates whose activity may be altered by polymorphisms in genes coding for enzymes in the pathway. These include SNPs in methylenetetrahydrofolate reductase (*MTHFR),* methionine synthase (*MTR*), methionine synthase reductase (*MTRR*) and methylenetetrahydrofolate dehydrogenase (*MTHFD1*) that all play a critical role in determining availability of folate and methionine for maintenance of methylation of DNA and synthesis of nucleotides such as dTTP [425].

MTHFR activity affects the balance of the pool of the various forms of folate in one-carbon metabolism for DNA synthesis and methylation. The *MTHFR* C677T (A222V) polymorphism has been extensively studied [198, 204, 205, 207, 208, 230, 301, 327, 425-430]. The presence of a T allele at position 677 encodes a thermolabile form of the MTHFR enzyme [427] that has lower affinity for its flavin cofactor [426], thus reducing its activity. Individuals who are TT homozygotes for this allele have reduced levels of serum folate (SF) and red cell folate (RCF) and consequently may have elevated Hcy concentrations [207, 428]. The *MTHFR* 1298C (E429A) variant of the enzyme, like the 677T also has reduced activity [208]. Reduced activity in this enzyme is postulated to provide more folate to be available for synthesis of dTTP from dUMP, preventing uracil incorporation into DNA, but reducing folate availability for methylation of Hcy to form methionine [230]. *MTHFR* C677T and A1298C have been studied and associated with hyperhomocysteinemia and related to vascular disease [198, 301] and pregnancy complications such as PE and IUGR [204, 327, 429, 430]. In addition fetal *MTHFR* 677T genotype has also been linked with cerebral palsy [431] and vascular lesions in the placenta [205].

Elevated plasma Hcy concentrations associated with the *MTHFR* C677T SNP can cause Hcy-induced cellular dysfunction which may lead to UPI via molecular mechanisms such as increased inflammatory cytokine expression, altered nitric oxide bioavailability, induction of oxidative stress and activation of apoptosis [101]. An alternative mechanism is that the conversion of Hcy to methionine via MTR and MTRR which is required for the production of S-adenosylmethionine (SAM) is altered. SAM is a principal methyl donor inside cells and the methyl groups are key for DNA control of cellular proliferation, cellular migration,

differentiation, and cell to cell recognition [385]. Thus by indirectly limiting the supply of SAM, the MTR and MTRR SNPs may be associated with the development of UPI through the mechanism of hypomethylated DNA, failing then to properly regulate cellular growth and development [135].

The association between polymorphisms in MTR and MTRR has also been examined in relation to pregnancy complications [210, 219]. MTR catalyses the methylation of Hcy to methionine using vitamin-B_{12} as a cofactor and 5-methylTHF as methyl donor (Fig. 1) [432]. The *MTR* A2756G polymorphism replaces an aspartic acid with glycine (D919G) at the C-terminal end of the alpha/beta domain of the enzyme. It has been shown that the A2756 mutation in MTR in combination with MTRR A66G has a moderate but significant influence on Hcy (refs) and in coronary artery disease patients. Formation of methionine through this pathway represents an important component of the one-carbon metabolism for synthesis of phospholipids, proteins, myelin, catecholamines, DNA, RNA [167] and SAM [433] which are all important in fetal development.

Methionine synthase reductase (MTRR) plays a critical role in maintaining vitamin-B_{12} (cobalamin) in an active form and consequently may be an important determinant of Hcy concentrations [213]. Vitamin-B_{12} acts as an intermediate methyl carrier between methyl-THF and Hcy during the MTR-catalysed remethylation of Hcy to methionine. Vitamin-B_{12} cycles between cob(I)alamin and methylcob(III)alamin. However, cob(I)alamin is a strong reductant and can be oxidised to produce an inactive cob(II)alamin form of the enzyme. MTRR catalyses the reductive methylation of cob(II)alamin to methylcob(III)alamin using SAM as the methyl donor [434] (Figure 23). The *MTRR* A66G polymorphism is located within the flavin-mononucleotide (FMN) binding domain [293]. Deficiency in this enzyme, caused by the G variant is expected to produce similar effects on genome methylation as those induced by vitamin-B_{12} deficiency [214]. The A66G polymorphism in MTRR appears to represent a risk factor for premature cardiovascular disease [435], neural tube defects [214], and Down syndrome [219]. The location of the A66G polymorphism in the predicted FMN domain suggests that it may influence the interaction between MTRR and its redox partner, MTR [436].

5,10-methylenetetrahydrofolate dehydrogenase (MTHFD1) also plays a key role in folate metabolism. The NADP-dependent enzyme catalyses three sequential reactions in the

conversion of 1-carbon derivatives of tetrahydrofolate, which are substrates for methionine, thymidylate and *de novo* purine synthesis. A polymorphism, (G1958A) R653Q within the MTHFD1 enzyme results in the substitution of a conserved arginine amino acid with a glutamine. Few articles have been published in relation to MTHFD1. To date this SNP has been associated with maternal genetic risk of having a child with neural tube defects [225] and placental abruption [223]; however it has not been studied in relation to PE or IUGR.

Figure 23. A simplified scheme of one carbon metabolism. Key intermediates and enzymes are given. **DHF**, Dihydrofolate; **THF**, Tetrahydrofolate; **MTHFR**, Methylenetetrahydrofolate Reductase; **MTHFD1**, Methylenetetrahydrofolate Dyhydrogenase; **MTR,** Methionine Synthase; **MTRR**, Methionine Synthase Reductase; **SAM**, S-Adenosylmethionine; $\mathbf{B_{12}}$**,** Vitamin-B_{12} (cobalamin, in various oxidative forms); $\mathbf{B_6}$**,** Vitamin-B_6; **dUMP**, Uracil; **dTMP,** Thymine; $\mathbf{CH_3}$, Methyl group.

The purpose of this study was to test five genetic polymorphisms involved in one - carbon metabolism for a potential association with increased risk of developing UPI. Also, results in this PhD project have suggested that micronuclei (MN) and Hcy may be associated with the

development of UPI (chapter 3 and 4). Therefore we have investigated the effect of the genetic polymorphism in relation to maternal genome damage, B-vitamins and Hcy.

11.4 Methodology

One hundred and forty-three pregnant women were included in the cohort. Pregnancies from oocyte donations were excluded from the analyses to avoid unexplained genetic effects. DNA was obtained from 139 mothers and 124 placentas. The missing maternal samples were from one low risk healthy pregnancy with a normal outcome (control) and the remaining from high risk pregnancies, one with a normal outcome and three with adverse outcomes including one case of IUGR. Seventeen placental samples could not be collected and two samples did not yield sufficient DNA. Two of the missing samples were from low risk pregnancies with normal outcomes. The remaining were from high risk pregnancies including seven normal outcomes and ten adverse outcomes. Two were classified as UPI which included one case of placental abruption and one case of IUGR.

In this chapter/study additional analysis was performed using the so-called index pregnancy (previous pregnancy) classification data. Because of their prior obstetrical history, many of the identified high risk patients had previously been genotyped for MTHFR 677 CT and 1298 AC polymorphisms as part of regular patient care and if positive, treated with high dose folate, vitamin-B_{12} and vitamin-B_6. Therefore, a separate layer of analysis was introduced based on the index pregnancies, i.e. the actual pregnancy that had initially led to the so-called thrombophilia analysis (including the MTHFR genotyping) in an attempt to study the relation between maternal genotypes and pregnancy outcome in the untreated patient and in the absence of effect of modification by B-vitamin supplementation.

11.4.1 DNA isolation from maternal granulocytes

Genomic DNA was extracted from thawed granulocytes which had been stored at -20ºC after lymphocyte isolation from whole blood. The experimental procedure for the isolation of DNA from packed nucleated cells is outlined below.

1. 3 mL of packed cell suspension was transferred to a sterile 10 mL conical tube;
2. 7 mL of cold $T_{10}E_{10}$ (2.5 mL 1 M Tris HCl, 5 mL 0.5 M EDTA, 242.5 mL dH_2O; pH 8.0, stored at 4°C) was added to the tubes;
3. Tubes were mixed by inversion and placed at -20°C for 15 minutes to ensure lysis of the erythrocytes;

4. Tubes were then spun at 3000 rpm for 10 minutes;
5. Approximately 9 mL of supernatant was discarded;
6. 2 mL $T_{10}E_{10}$ was added to the tubes and tubes were vortexed to break up and resuspend the pellet;
7. The volume of the solution was then adjusted to 10 mL with $T_{10}E_{10}$;
8. Tubes were spun at 3000 rpm for 10 minutes;
9. The supernatant was removed leaving the pellet pale and with very few erythrocytes. If the pellet still had a red discolouration, an additional spin was performed;
10. 2.5 mL $T_{10}E_5$ (2.5 mL 1 M Tris HCl, 2.5 mL 0.5 M EDTA, 245 mL dH_2O; pH 8.0) was added to the pellet and tubes were vortexed to break up and resuspend the pellet;
11. 250 µL of 10% sodium dodecyl sulphate (SDS; pH 7.2) was the added to the tubes;
12. Solutions were mixed well by gentle inversion until it had become very clear and viscous;
13. 150 µL proteinase K (10 mg/mL; Roche Diagnostics, Mannheim, Germany) was added to the lysate (final concentration: 0.6 mg/mL) and tubes were incubated overnight at 56°C.
14. To extract the DNA from the lysate, approximately 300 µL saturated NaCl (6 M) was slowly added until precipitation occurred;
15. Tubes were gently inverted for approximately 1 minute to mix the solutions;
16. Solutions were then spun at 2800 rpm for 15 minutes;
17. Using a sterile transfer pipette, the supernatant containing the DNA was transferred to a clean and sterile 10 mL conical tube. The tube containing the protein pellet was discarded.
18. Steps 16 and 17 were repeated and the final volume of extracted DNA was estimated;
19. DNA was precipitated by adding one volume of 95% ethanol;
20. Tubes were gently inverted several times to mix the phases, until cotton wool precipitate started to form;
21. Another volume of 95% ethanol was then added and tubes were gently inverted until the cotton wool had condensed into a tight precipitate; to aid in precipitation, tubes were placed at -20°C for 15 minutes;
22. The precipitate was removed from the tubes and transferred into 1.5 mL Eppendorf tubes using a 1 mL pipette with the end cut of the tip. The diameter of the tip opening was approximately 1.5 – 2.0 mm;
23. To wash the DNA, 70% ethanol was added to a final volume of 1.5 mL;
24. Eppendorf tubes were spun at 13,200 rpm for 2 minutes to acquire a DNA pellet in the bottom of the tube;

25. The ethanol was then discarded and DNA left to air-dry;
26. Once dry, 300 µL $T_{10}E_1$ (2.5 mL 1 M Tris HCl, 0.5 mL 0.5 M EDTA, 247 mL dH_2O; pH 8.0) was added and allowed to dissolve at 37°C overnight;

Once dissolved, DNA was quantified using a spectrophotometer (Model ND-1000, NanoDrop Technologies, Delaware, USA) and stored at 4°C; until use. For quantification 2 µL DNA was used and the absorbance was read at 260/280 nm.

11.4.2 DNA isolation from cord tissue

As in methods (section 8.4.3).

11.4.3 Polymorphism detection

Maternal and placental DNA were genotyped for *MTHFR* C677T and A1298C, *MTR* A2756G, *MTRR* A66G, *MTHFD1* G1958A polymorphisms using the Applied Biosystems 7900HT Real Time PCR System and ABI 7300 Sequence Detection System with the SDS Ver. 1.9 software (Applied Biosystems, Foster City, CA). Each reaction was performed in duplicate. In addition a random selection of DNA samples were genotyped using a secondary method, restriction fragment length polymorphism, to confirm the real time data.

11.4.3.1 *Alleleic discrimination primers*

Primers for the *MTHFR* C677T and *MTR* A2756G polymorphisms were ordered from Applied Biosystems (ABI) as Assays-on-Demand (Applied Biosystems, Foster City, CA catalogue number: 1202883 and 12005959 respectively). The sequence information was not available as per company (ABI) guidelines. The primer and reporter sequences for primers ordered as Assays-by-Design were:

*MTHFD1*G1958A

Forward primer: 5'-CATCGCACATGGCAATTCCT-3'

Reverse primer: 5'-TCTGGGCCAACAAGCTTGAG-3'

Reporter 1: 5'-CAGACC**G**GATCGCA-3' VIC (common allele)

Reporter 2: 5'-CAGACC**A**GATCGCA-3' FAM

MTRR A66G

Forward primer: 5'-AGCAGGGACAGGCAAAGG-3'

Reverse primer: 5'-GCAGAAAATCCATGTACCACAGCTT-3'

Reporter 1: 5'-ATCGCAGAAGAAAT**G**TGTGA-3' VIC

Reporter 2: 5'-TCGCAGAAGAAAT**A**TGTGA-3' FAM (common allele)

MTHFR A1298C

Forward primer: 5'-GGAGGAGCTGCTGAAGATGTG-3'

Reverse primer: 5'-CCCGAGAGGTAAAGAACAAAGACTT-3'

Reporter 1: 5'- CCAGTGAAG**C**AAGTGT-3' VIC

Reporter 2: 5'-CCAGTGAAG**A**AAGTGT-3' FAM (common allele)

11.4.3.2 ***Alleleic discrimination reagents and thermal cycling conditions***

The PCR master mix consisted of 12.5 µl 2X TaqMan® Universal PCR Master mix, without AmpErase® UNG (Applied Biosystems, Foster City, CA: catalogue number: 4324018) 1.25 µl 20X Assay-on-Demand™ SNP genotyping assay mix (containing the primers) and 11.25 µl of genomic DNA diluted in ddH_2O. Approximately 10 ng of genomic DNA was used per 25.0 µl reaction.

The thermal cycling conditions for *MTHFR* C677T, *MTR* A2756G were 10 minutes at 95ºC, then 50 cycles with 15 seconds at 92ºC to denature and 1 minute at 60ºC for annealing and extension. Thermal cycling conditions for *MTHFR* A1298C, *MTRR* A66G and *MTHFD1* G1958A included 10 minutes at 95ºC, then 50 cycles with 15 seconds at 96ºC to denature and 90 seconds at 60ºC for annealing and extension.

An example of the scatterplot produced for the *MTHFR* C677T allele distribution is illustrated in Appendix 2.

11.4.4 Micronutrients and genome damage biomarker results

Data for genome damage are outlined in section 9.5.

Data for red cell folate (RCF), serum folate (SF), serum vitamin-B_{12}, RBC vitamin-B_6 and plasma homocysteine are outlined in section 10.5 .

11.4.5 Statistics

The Chi-squared goodness-of-fit test was used to compare the allele proportions and genotype to determine whether the *MTHFR, MTR, MTRR* and *MTHFD1* frequencies were associated with adverse pregnancies and those that developed UPI including PE and IUGR. The Hardy-Weinberg equilibrium equation was performed for all genotype data. The association between maternal and fetal SNPs within the folate metabolic pathway with RCF, SF, serum vitamin-B_{12}, RBC vitamin-B_{6}, plasma Hcy, NDI, genome damage and cell death biomarkers were

determined by one-way ANOVA. Where a significant association was detected, a linear regression was used to test the effects of confounding factors, such as B-vitamin supplement intake and smoking. All data analyses were performed by means of the computer based statistical package of Statistical Product and Service Solution (SPSS) version 14.0 (SPSS, Chicago, IL, USA). Results were reported as mean ± S.E. of the mean and $P < 0.05$ was considered statistically significant.

11.5 Results

Non significant results from this study are located in the appendix.

11.5.1 Maternal polymorphisms

11.5.1.1 Maternal allele distribution

There were no significant differences in allele frequency among women grouped into low risk with normal or adverse pregnancy outcomes or within the high risk pregnancy group with normal or adverse outcomes in all SNPs examined (Appendix 3, Appendix 4). In addition the allele proportions of the four SNPs did not differ significantly when comparing low risk healthy pregnancies (controls) and those who developed UPI, including PE and IUGR (Appendix 5, Appendix 6, Appendix 7).

11.5.1.2 Maternal genotype distribution

Genotype frequencies for all the tested SNPs were calculated and found to fit the Hardy-Weinberg equilibrium and did not deviate markedly from previously reported frequencies for these polymorphisms [213, 293, 437].The maternal *MTHFR, MTR, MTRR* and *MTHFD1* genotype distribution did not differ significantly in women with the low risk pregnancies or high risk pregnancies with normal or adverse outcomes (Appendix 9, Appendix 10). The *MTHFD1* AA genotype was significantly associated with the development of IUGR ($P = 0.047$) as shown in Table 21. The three remaining polymorphisms tested did not show any significant changes in women who developed UPI and PE when compared with control pregnancies (Appendix 11, Appendix 12).

Table 21. Distribution of maternal polymorphisms in low risk healthy pregnancies (control) and women who developed IUGR

Maternal	Control (n = 42)	IUGR (n = 18)	Chi-square P
MTHFR C677T			
CC	15 (35.7%)	10 (55.6%)	
CT	24 (57.1%)	7 (38.9%)	0.560
TT	3 (7.1%)	1 (5.6%)	
MTHFR A1298C			
AA	25 (59.5%)	8 (44.4%)	
AC	15 (35.7%)	9 (50.0%)	0.348
CC	2 (4.8%)	1 (5.6%)	
MTR A2756G			
AA	28 (66.7%)	9 (50.0%)	
AG	10 (23.8%)	8 (44.4%)	0.191
GG	4 (9.5%)	1 (5.6%)	
MTRR A66G			
AA	14 (33.3%)	6 (33.3%)	
AG	18 (42.9%)	9 (50.0%)	0.669
GG	10 (23.8%)	3 (16.7%)	
MTHFD1 G1958A			
GG	17 (40.5%)	6 (33.3%)	
GA	19 (45.2%)	5 (27.8%)	**0.047**
AA	6 (14.3%)	7 (38.9%)	

Data represent number of women for each genotype and percentage (%) from the total study group.

11.5.1.3 Effect of maternal polymorphisms on micronutrient and homocysteine concentrations

The ANOVA results indicate that increased RCF is significantly associated with the *MTHFD1* A1958G variant genotype (P = 0.040). Serum vitamin-B_{12} was significantly associated with the MTRR A66G variant genotype (P = 0.004); however, after controlling for confounding factors such as folic acid and vitamin-B_{12} supplement intake and smoking the significant association was no longer detected. In addition no significant association was found between maternal SNPs on plasma homocysteine concentrations (Table 22). It is possible that these effects are confounded by varying proportions of women taking B-vitamin supplements, between genetic sub groups with a variation in diet or supplement intake.

Table 22. Effect of maternal polymorphisms on circulating micronutrient and plasma homocysteine concentrations

	Hcy μmol/L	RCF nmol/L	SF nmol/L	B_{12} pmol/L	B_6 status*
MTHFR C677T					
CC (n = 61)	4.6 ± 0.1	631.4 ± 29.3	26.6 ± 1.1	236.3 ± 19.3	42.8 ± 2.1
CT (n = 59)	4.6 ± 0.2	648.4 ± 31.4	26.2 ± 1.3	231.4 ± 17.4	42.8 ± 2.0
TT (n = 11)	4.6 ± 0.5	721.3 ± 69.3	25.3 ± 3.9	294.5 ± 54.4	39.6 ± 5.7
ANOVA P	0.993	0.482	0.905	0.388	0.817
MTHFR A1298C					
AA (n = 73)	4.4 ± 0.1	660.3 ± 26.0	26.2 ± 1.1	263.3 ± 18.3	42.9 ± 1.7
AC (n = 49)	4.9 ± 0.3	620.8 ± 37.9	25.7 ± 1.5	201.4 ± 16.5	41.9 ± 2.7
CC (n = 9)	4.3 ± 0.3	673.2 ± 59.7	30.8 ± 2.9	232.7 ± 52.2	42.4 ± 5.7
ANOVA P	0.119	0.631	0.343	0.075	0.942
MTR A2756G					
AA (n = 80)	4.7 ± 0.2	640.7 ± 26.5	26.5 ± 1.1	253.4 ± 18.5	41.2 ± 1.8
AG (n = 43)	4.4 ± 0.2	646.1 ± 36.9	25.5 ± 1.4	216.8 ± 16.7	45.0 ± 2.5
GG (n = 8)	5.0 ± 0.7	710.6 ± 63.9	28.5 ± 2.6	229.4 ± 46.3	41.6 ± 4.2
ANOVA P	0.488	0.701	0.663	0.408	0.450
MTRR A66G					
AA (n = 40)	4.7 ± 0.3	650.7 ± 36.8	25.0 ± 1.5	208.2 ± 13.5	45.9 ± 1.8
AG (n = 68)	4.5 ± 0.2	645.6 ± 30.0	26.9 ± 1.2	227.6 ± 16.1	40.7 ± 2.2
GG (n = 23)	4.6 ± 0.3	646.2 ± 43.5	26.9 ± 2.0	325.2 ± 45.2	41.7 ± 3.5
ANOVA P	0.719	0.994	0.595	**0.004**	0.256
Adjusted P value**				0.696	
MTHFD1 G1958A					
GG (n = 39)	5.0 ± 0.3	546.5 ± 48.1	22.9 ± 2.0	175.8 ± 15.2	45.0 ± 3.5
GA (n = 65)	4.4 ± 0.1	656.6 ± 28.2	26.2 ± 1.1	249.1 ± 17.4	44.2 ± 1.9
AA (n = 27)	4.6 ± 0.3	694.9 ± 35.6	28.6 ± 1.6	261.4 ± 27.6	38.2 ± 2.5
ANOVA P	0.146	**0.040**	0.069	0.056	0.117
Adjusted P value**		0.269			

* Based on PPA activity which is inversely related to vitamin-B_6 concentration in RBCs.
**Controlled for folic acid, vitamin-B_{12} intake and smoking using linear regression analysis.
All data are represented as mean ± SEM.
Hcy, L-Homocysteine in plasma; RCF, Red Cell Folate; SF, Serum Folate; B_{12}, Serum vitamin-B_{12}; B_6, Red Cell vitamin-B_6.

11.5.1.4 Association of maternal polymorphisms on maternal genome damage

Genome damage in peripheral lymphocytes was not associated with SNPs in *MTHFR* 677 and 1298, *MTRR* 66 or *MTHFD1* 1958. The results indicate that the *MTR* 2756G allele is associated with a reduction in the frequency of nucleoplasmic bridges (NPBs), caused by chromosome rearrangement. However, the significance is lost when regression analysis was performed, controlling for Hcy, RCF and smoking (P = 0.097, Table 23). A trend for a reduction in MN-BN and MN-BUD was observed for MTR 2756 homozygous GG genotypes, but these effects were not statistically significant.

Table 23. Association of maternal polymorphisms on maternal genome damage

	‰ MN-BN	‰ NPB-BN	‰ NBUD-BN	NDI	‰ Apoptosis	‰ Necrosis
MTHFR C677T						
CC (n = 59)	19.9 ± 1.3	7.3 ± 0.8	29.3 ± 3.2	2.1 ± 0.03	132.2 ± 6.6	89.8 ± 3.6
CT (n = 60)	21.0 ± 1.5	6.8 ± 0.6	26.0 ± 2.5	2.0 ± 0.03	146.7 ± 7.5	87.4 ± 3.8
TT (n= 12)	22.7 ± 2.3	4.6 ± 0.7	27.7 ± 6.4	1.9 ± 0.09	134.4 ± 18.8	76.1 ± 7.5
ANOVA P	0.679	0.286	0.712	0.067	0.353	0.323
MTHFR A1298C						
AA (n = 76)	19.2 ± 0.9	6.8 ± 0.6	27.1 ± 2.3	2.0 ± 0.03	138.5 ± 6.4	85.9 ± 3.3
AC (n = 46)	23.2 ± 1.9	7.0 ± 0.8	28.1 ± 3.7	2.1 ± 0.03	142.6 ± 8.5	89.9 ± 4.2
CC (n = 9)	20.4 ± 4.1	6.2 ± 1.1	30.2 ± 7.6	2.0 ± 0.08	125.6 ± 15.6	88.5 ± 9.4
ANOVA P	0.129	0.699	0.746	0.322	0.908	0.905
MTR A2756G						
AA (n = 78)	20.4 ± 1.1	7.7 ± 0.7	28.8 ± 2.7	2.0 ± 0.03	140.2 ± 6.3	88.9 ± 3.4
AG (n = 44)	21.9 ± 2.0	5.8 ± 0.5	26.0 ± 2.8	2.0 ± 0.04	138.3 ± 8.6	86.8 ± 4.3
GG (n = 9)	17.5 ± 1.6	4.1 ± 0.7	25.6 ± 6.3	2.2 ± 0.08	132.5 ± 16.6	77.8 ± 4.6
ANOVA P	0.483	**0.050**	0.762	0.281	0.921	0.541
Adjusted P value*		**0.097**				
MTRR A66G						
AA (n = 40)	22.2 ± 2.0	7.2 ± 0.9	28.0 ± 3.4	2.0 ± 0.04	144.7 ± 9.9	85.4 ± 4.5
AG (n = 67)	20.4 ± 1.2	6.8 ± 0.7	27.0 ± 2.9	2.1 ± 0.03	137.6 ± 7.0	89.3 ± 3.7
GG (n = 24)	18.9 ± 1.7	6.1 ± 0.6	28.8 ± 4.0	2.0 ± 0.05	133.6 ± 8.1	85.7 ± 4.7
ANOVA P	0.449	0.732	0.937	0.304	0.709	0.747
MTHFD1 G1958A						
GG (n = 40)	21.1 ± 2.9	5.4 ± 0.7	28.7 ± 4.0	2.0 ± 0.04	146.4 ± 11.3	86.4 ± 5.1
GA (n = 67)	20.2 ± 1.1	6.6 ± 0.6	27.3 ± 2.4	2.1 ± 0.03	129.9 ± 6.6	90.9 ± 3.8
AA (n = 25)	21.1 ± 1.7	8.1 ± 1.1	27.6 ± 4.2	2.0 ± 0.04	149.6 ± 9.1	82.5 ± 4.2
ANOVA P	0.891	0.123	0.966	0.725	0.162	0.334

All data represented as mean ± SEM
‰ represents frequency per 1000 BN cells
*Controlled for RCF, vitamin-B_{12} intake and homocysteine using linear regression analysis

11.5.2 Fetal polymorphisms

11.5.2.1 Fetal allele distribution

The proportion of alleles in babies born from women grouped into low risk or high pregnancy group with normal or adverse outcomes did not differ significantly (Appendix 13, Appendix 14). The fetal allele distribution from low risk healthy pregnancies (controls) compared to those who were born with UPI, including PE and IUGR did not show any marked alterations in allele proportion between groups (Appendix 15, Appendix 16, Appendix 17).

11.5.2.2 Fetal genotype distribution

The fetal genotype frequencies for all tested SNPs were calculated and found to fit Hardy-Weinberg equilibrium equation. When analysing the fetal SNPs within babies born from mothers in the low risk pregnancy group the fetal *MTR* 2756 A > G substitution was

significantly associated with adverse pregnancy outcomes (P = 0.049) compared to clinically normal outcomes. The fetal *MTHFR* C677T and A1298C, *MTRR* A66G and *MTHFD1* G1958A genotypes were not associated with adverse pregnancy outcome compared to clinically normal outcomes (Table 24).

Babies born from mothers in the high risk pregnancy did not show any significant association within SNPs involved in one carbon metabolism in relation to clinically normal outcomes and adverse pregnancy outcomes (Appendix 18).

Table 24. Distribution of various fetal polymorphisms in one carbon metabolism from low risk pregnancies with normal and adverse pregnancy outcomes

Fetal	Normal (n = 41)	Adverse (n = 7)	Chi-square P
MTHFR C677T			
CC	18 (43.9%)	4 (57.1%)	
CT	16 (39.0%)	3 (42.9%)	0.446
TT	7 (17.1%)	0 (0.0%)	
MTHFR A1298C			
AA	19 (46.3%)	5 (71.4%)	
AC	19 (46.3%)	1 (14.3%)	0.276
CC	3 (7.3%)	1 (14.3%)	
MTR A2756G			
AA	23 (56.1%)	3 (42.9%)	
AG	18 (43.9%)	3 (42.9%)	**0.049**
GG	0 (0.0%)	1 (14.35)	
MTRR A66G			
AA	16 (39.0%)	4 (57.1%)	
AG	20 (48.8%)	2 (28.6%)	0.558
GG	5 (12.2%)	1 (14.3%)	
MTHFD1 G1958A			
GG	14 (34.1%)	4 (57.1%)	
GA	18 (43.9%)	2 (28.6%)	0.509
AA	9 (22.0%)	1 (14.3%)	

Data represent number of fetal genotypes and percentage (%) from the total study group.

11.5.2.3 Fetal genotype distribution from low risk healthy pregnancies (controls) and those who developed uteroplacental insufficiency including PE and IUGR.

The results indicate that the fetal *MTR* A2756G variant was associated with the development of UPI (P = 0.022). A significant result was not detected in relation to PE or IUGR (Appendix 19, Appendix 20); however, the group sizes were small and underpowered. The fetal *MTHFR*, *MTRR* and *MTHFD1* variant genotypes did not show any significant relationship with the development of UPI.

Table 25. Distribution of various fetal polymorphisms in one carbon metabolism from low risk healthy (control) pregnancies and those who developed uteroplacental insufficiency

Fetal	Control (n = 41)	UPI (n = 47)	Chi-square P
MTHFR C677T			
CC	18 (43.9%)	23 (48.9%)	
CT	16 (39.0%)	22 (46.8%)	0.132
TT	7 (17.1%)	2 (4.3%)	
MTHFR A1298C			
AA	19 (46.3%)	26 (55.3%)	
AC	19 (46.3%)	16 (34.0%)	0.614
CC	3 (7.3%)	5 (10.6%)	
MTR A2756G			
AA	23 (56.1%)	26 (55.3%)	
AG	18 (43.9%)	14 (29.8%)	**0.022**
GG	0 (0.0%)	7 (14.9%)	
MTRR A66G			
AA	16 (39.0%)	15 (31.9%)	
AG	20 (48.8%)	25 (53.2%)	0.692
GG	5 (12.2%)	7 (14.9%)	
MTHFD1 G1958A			
GG	14 (34.1%)	13 (27.7%)	
GA	18 (43.9%)	24 (51.1%)	0.662
AA	9 (22.0%)	10 (21.3%)	

Data represent number of fetal genotypes and percentage (%) from the total study group.

11.5.2.4 The association of fetal polymorphisms on circulating micronutrients and homocysteine concentrations in the mother

The ANOVA results indicate that the fetal *MTR* A2756G genotype was significantly associated with maternal plasma Hcy concentration (P = 0.022); after applying linear regression the significance improves to P = 0.017. The RCF, SF, vitamin-B_{12} and vitamin-B_6 were not significantly associated to SNPs in *MTHFR, MTRR* or *MTHFD1* (Table 26).

Table 26. Association of fetal polymorphisms with circulating micronutrients and homocysteine concentrations in the mother

	Hcy µmol/L	RCF nmol/L	SF nmol/L	B_{12} pmol/L	B_6 status*
MTHFR C677T					
CC (n = 51)	4.8 ± 0.2	638.4 ± 35.9	26.1 ± 1.4	202.0 ± 15.5	43.5 ± 2.4
CT (n = 50)	4.6 ± 0.2	647.1 ± 31.2	25.9 ± 1.5	259.7 ± 23.7	41.8 ± 2.3
TT (n = 14)	4.7 ± 0.4	716.1 ± 53.4	28.2 ± 2.6	271.5 ± 45.4	39.5 ± 4.3
ANOVA P	0.809	0.541	0.753	0.084	0.703
MTHFR A1298C					
AA (n = 60)	4.9 ± 0.2	651.8 ± 32.3	25.3 ± 1.4	228.5 ± 17.5	40.5 ± 2.0
AC (n = 49)	4.4 ± 0.2	662.4 ± 31.6	27.9 ± 1.4	243.9 ± 22.5	42.4 ± 2.6
CC (n= 9)	4.9 ± 0.3	606.9 ± 74.2	26.9 ± 3.9	220.9 ± 53.6	53.6 ± 4.2
ANOVA P	0.274	0.809	0.412	0.830	0.75
MTR A2756G					
AA (n = 66)	4.5 ± 0.2	665.3 ± 28.8	27.0 ± 1.2	231.1 ± 19.2	42.8 ± 1.9
AG (n = 41)	4.6 ± 0.2	624.7 ± 36.7	25.7 ± 1.7	253.6 ± 22.4	42.6 ± 2.9
GG (n = 11)	5.9 ± 0.5	679.9 ± 74.9	26.3 ± 3.4	180.5 ± 19.5	38.2 ± 4.4
ANOVA P	**0.023**	0.636	0.811	0.325	0.677
Adjusted P value**	**0.017**				
MTRR A66G					
AA (n = 42)	5.0 ± 0.3	637.0 ± 38.0	25.0 ± 1.7	208.5 ± 22.3	43.4 ± 2.6
AG (n = 61)	4.6 ± 0.2	682.7 ± 28.8	28.2 ± 1.2	251.2 ± 17.4	40.9 ± 2.1
GG (n = 14)	4.65± 0.3	566.6 ± 65.1	23.1 ± 2.8	237.9 ± 51.0	44.2 ± 4.4
ANOVA P	0.346	0.220	0.124	0.348	0.679
MTHFD1 G1958A					
GG (n = 38)	4.5 ± 0.3	708.8 ± 35.0	29.2 ± 1.7	228.1 ± 25.6	39.4 ± 2.4
GA (n = 53)	4.7 ± 0.2	655.2 ± 34.0	25.0 ± 1.4	248.4 ± 21.2	42.8 ± 2.6
AA (n = 26)	5.1 ± 0.3	565.9 ± 41.3	25.3 ± 1.8	213.8 ± 18.3	46.1 ± 2.2
ANOVA P	0.318	0.054	0.117	0.590	0.280

* Based on PPA activity which is inversely related to vitamin-B_6 concentration in RBCs

**Controlled for folic acid intake and smoking using linear regression analysis

All data are represented as mean ± SEM

Hcy, L-Homocysteine in plasma; RCF, Red Cell Folate; SF, Serum Folate; B_{12}, Serum vitamin-B_{12}; B_6, Red Cell vitamin-B_6.

11.5.3 Index pregnancy analysis

11.5.3.1 Maternal allele distribution

There were no significant associations detected between maternal allele frequency in the index pregnancies in relation to the development of UPI. However, the A allele frequency in *MTHFD1* 1958 was marginally increased in women who developed UPI (P = 0.061, Appendix 21).

11.5.3.2 Maternal genotype distribution

Analyses based on index pregnancy data revealed that the *MTR* A2756G polymorphism was significantly associated with UPI (P = 0.049, Table 27) suggesting a high risk for those carrying the G allele. The *MTHFR* 677 did show a doubling of the variant homozygote alleles; however with the small sample size these results did not reach significance.

Table 27. Distribution of various maternal genotypes involved in one carbon metabolism genes in low risk healthy normal (control) pregnancies and those with UPI in their index pregnancy

Maternal	Control (n = 41)	UPI (n = 24)	Chi-square P
MTHFR C677T			
CC	15 (36.6%)	10 (41.6%)	
CT	23 (56.1%)	10 (41.6%)	0.377
TT	3 (7.3%)	4 (16.7%)	
MTHFR A1298C			
AA	25 (61.1%)	14 (58.3%)	
AC	14 (34.1%)	8 (33.3%)	0.555
CC	2 (4.9%)	2 (8.3%)	
MTR A2756G			
AA	27 (65.9%)	9 (37.5%)	
AG	10 (24.4%)	13 (54.2%)	**0.049**
GG	4 (9.8%)	2 (8.3%)	
MTRR A66G			
AA	14 (34.1%)	5 (20.8%)	
AG	17 (41.5%)	16 (66.7%)	0.144
GG	10 (24.4%)	3 (12.5%)	
MTHFD1 G1958A			
GG	17 (41.5%)	5 (20.8%)	
GA	19 (46.3%)	13 (54.2%)	0.169
AA	5 (12.2%)	6 (25.0%)	

Data represents number of maternal genotypes and percentage (%) from the total study group.

11.6 Discussion

This study suggests the maternal and fetal *MTR* 2756 G allele to be an important risk factor in the development of UPI. The maternal MTR 2756 polymorphism investigations in the most recent pregnancies (including women treated with high dose B-vitamins) did not reveal an association with UPI; however, in the index pregnancy investigation (including women not treated with high dose B-vitamins) a significant association was detected in relation to UPI. This may suggest that high concentrations of folic acid, vitamin-B_{12} and vitamin-B_6 modify pregnancy outcome. In addition the placental/fetal *MTR* 2756 GG genotype was significantly associated with increased plasma Hcy concentration and with the development of UPI. The *MTR* A2756G substitution results in an amino acid change of an aspartic acid to a glycine (D919G), at the penultimate position in a long helix that leads out of the cobalamin domain [438]. Having the glycine residue at this position could have an effect on the secondary structure of the protein and therefore have functional consequences [439]. Further studies are needed to elucidate if this allele change impacts on enzyme activity and in turn one-carbon metabolism, which may effect placental development and pregnancy outcome.

The other novel finding in this study relates to the maternal *MTHFD1* 1958 AA genotype and the development of IUGR, despite the small cohort. To date there have been no studies investigating the effect of MTHFD1 on PE and/or IUGR. Parle-McDermott et al. investigated *MTHFD1* G1958A in an Irish population and found a significant association with placental abruption [223] and the occurrence of neural tube defects [225]. Studies in relation to MTHFD1 are in the early stages and further investigation into various polymorphisms within the gene and functional tests are required to understand the impact on enzyme activity and the possible risk associated with placental diseases.

There have been a number of previous studies that have investigated polymorphisms within *MTHFR, MTR* and *MTRR* genes and related these with pregnancy diseases in various populations [96, 327, 440-444]. Of these genes the most likely candidate is the *MTHFR* 677 T allele which has been reported as a genetic risk for PE [96, 201, 445]. Using the classification data from the most recent pregnancies the *MTHFR* 677 TT genotype was not related to the development of UPI. However, using the classification data from the index pregnancies the MTHFR 677 TT genotype was more frequent (control: 7.3%, UPI: 16.7%) in those that developed UPI, though due to a small sample size significance was not achieved. These results are in agreement with a review published by Ray and Laskin[109] demonstrating a pooled odds ratio of 2.6 for homozygosity of the MTHFR variant genotype in relation to PE.

Previous studies have shown an association with either *MTHFR* (C677T and A1298C), *MTR* (A2756G), *MTRR* (A66G) genetic polymorphisms and the levels of circulating Hcy concentration [294, 302, 425, 446-451]; however, with the exception of the fetal MTR SNP, an association was not detected in this study. The neutral influence of these polymorphisms on plasma Hcy may be explained by adequate status in folate, vitamin B_{12}, and vitamin-B_6 from supplement intake or dietary sources. Supplementing with folic acid, vitamin-B_{12}, vitamin-B_6 and vitamin-B_2 enhance specific enzyme functions, reducing plasma Hcy concentrations and therefore may have masked the effect of the tested genetic variants. A number of studies [213, 446, 447, 452] have suggested that maternal environmental factors have a much greater influence than genetic factors on the levels of circulating Hcy during pregnancy. In addition the results indicated that the MTHFD1 AA genotype may have been associated with increased RCF; however after controlling for confounding factors such as folic acid and vitamin-B_{12} supplement intake and smoking the relationship was no longer significant. Moreover, the MTRR 66 GG genotype was associated with increased serum vitamin-$B_{12,}$ but again the significance was lost after logistic regression was used to control for vitamin supplement intake with folic acid, vitamin-B_{12} and smoking.

Chromosome breaks, chromosome rearrangement, gene amplification and cell death were not associated with the tested one-carbon metabolism SNPs within the maternal genes. Previous studies[294, 424] have shown that the *MTHFR* 677TT genotype influences DNA instability by increasing the rate of micronuclei (MN, a marker for chromosome breaks and loss) and nucleoplasmic bridges (NPB, a marker of chromosome rearrangement) [453]. In addition, the *MTRR* 66 GG variant genotype has been shown to increase MN frequency [2]; despite previous studies linking polymorphisms involved in one carbon metabolism with genome damage, it is not surprising that in this study the same SNPs are not associated with MN or other genome damage markers as supplementation with folic acid and B-vitamin cofactors are likely to have reduced chromosome breaks, loss and rearrangements. The results indicated that the *MTR* 2756GG polymorphism in maternal lymphocyte DNA was significantly associated with a reduction in nuceloplasmic bridges (NPB); however, after controlling for vitamin supplementation the significance was lost.

Further studies are needed to clarify the need for individualised dietary therapy based on genetic make-up.

12 GLOBAL DNA METHYLATION AND UTEROPLACENTAL INSUFFICIENCY

12.1 Aim

1. To determine the relationship between maternal and placental CpG methylation status.
2. To test whether CpG hypomethylation is associated with increased plasma homocysteine (Hcy) and genome damage.
3. To investigate the relationship between CpG methylation in maternal lymphocytes with circulating B-vitamins, plasma homocysteine (Hcy), genome damage markers and common single nucleotide polymorphisms (SNPs) in enzymes involved in one-carbon metabolism.
4. To determine the effect of maternal concentrations of circulating B-vitamins, plasma Hcy and common SNPs in enzymes involved in one-carbon metabolism on placental CpG methylation.

12.2 Hypotheses

1. Reduced CpG methylation in maternal lymphocyte DNA and placental DNA is a risk factor for developing UPI including PE and/or IUGR
2. Reduced CpG methylation in maternal lymphocytes and placental tissue is correlated with reduced concentrations of red cell folate (RCF), serum folate (SF), serum vitamin-B_{12}, red blood cell (RBC) vitamin-B_6 and/or high plasma Hcy in the mother at 20 weeks gestation.
3. Altered CpG methylation is associated with increased micronuclei, nuceloplasmic bridges, nuclear buds, apoptotic and necrotic cell frequency.
4. Maternal and placental gene polymorphisms involved in one-carbon metabolism enzymes are associated with altered CpG methylation.

12.3 Introduction

Epigenetic modifications of the genome may affect early growth and development through their influence on gene expression[266, 276, 454-456]. Methylation of cytosine residues at CpG dinucleotides is the most extensively characterised epigenetic mark, which is generally associated with transcriptional silencing, imprinting[454, 456] and X chromosome inactivation[457] which may significantly alter embryonic viability[458]. In addition, DNA

methylation contributes to genomic stability and serves as a defence mechanism against transposable elements[271]. Changes in gene expression are achieved through hypermethylation of CpG islands in promoter sequences, modification of histone proteins, and RNA-based silencing of transcripts[271].

The extent of DNA methylation changes in an orchestrated way during development. After fertilisation, during the second phase of large-scale epigenetic reprogramming, a general loss of DNA methylation is observed throughout the preimplantation period of development, but imprinted DNA methylation is maintained[277, 459]. Although traditionally DNA methylation has not been considered to be passed from parents to child, there is increasing evidence that some epigenetic signals may have partial meiotic stability and be transmitted from one generation to the next[460-464]. Once established, genomic methylation patterns are generally stable, but may be subjected to changes induced by nutritional factors, hormones, aging, and stochastic events in the cell[465, 466]. In non-embryonic cells, methylated sites are distributed globally on approximately 80% of the cytosine residues in CpG dinucleotides[270, 271].

Mechanistic insights into the role of DNA methylation and the establishment of methylation patterns during development have come from phenotypic analyses of mice with mutations in the various DNA methyltransferase (Dnmt) genes[467]. Dnmt enzymes attach methyl groups to CpG dinucleotides. These enzymes fit into two general classes based on their preferred DNA substrate. The *de novo* methyltransferases Dnmt3a and Dnmt3b are mainly responsible for introducing cytosine methylation at previously unmethylated CpG sites, whereas the maintenance methyltransferase Dnmt1 copies pre-existing methylation patterns onto the new DNA strand during DNA replication[458] (Figure 24).

Methylation can inhibit gene expression by recruiting proteins, such as methyl CpG binding protein 2 (MeCP2), which in turn recruit transcriptional co-repressors (Figure 24)[468-470]. In addition, DNA methylation can directly repress transcription by blocking transcriptional activators from binding to cognate DNA sequences[471].

NOTE: This figure is included on page 106 in the print copy of the thesis held in the University of Adelaide Library.

Figure 24 Epigenetic silencing of transcription.

When CpG dinucleotides are unmethylated in the promoter, RNA Pol and TF can bind to specific nucleotide sequences and the coding region (exon) is transcribed. Methylation of CpGs by the activity of Dnmt enables recruitment of methyl CpG binding protein-2 (MeCP2), which in turn recruits HDAC/ HMT to form an enzyme complex bound to the gene promoter. The MeCP2/HDAC/HMT complex removes acetyl groups from histones and catalyses methylation of specific lysine residues which causes the DNA to condense. This prevents access of RNA polymerase and transcription factors to DNA. Thus, the overall effect of DNA and histone methylation is to induce long-term silencing of transcription. Abbreviations: Dnmt, DNA methyltransferase; HDAC, histone deacetylase; HMT, histone methyltransferase; PPAR, peroxisome proliferator-activated receptor; RNA Pol, RNA polymerase; TF, transcription factor. Adapted from Godfrey et al., [472].

Both abnormal hypo- and hypermethylation alter epigenetic gene regulatory mechanisms, increase DNA damage and play an important role in the aetiology of human diseases [276, 473]. Immunodeficiency Centromeric region Instability and Facial anomalies (ICF) syndrome and Rett syndrome are inherited disorders caused by altered CpG methylation due to mutations in the DNA methyltransferase 3B gene (*DNMT3B*) and the gene encoding MeCP2, respectively [460]. In addition, altered CpG methylation has been associated with human carcinogenesis, several developmental syndromes [474, 475] and has been implicated in other complex diseases including type-2 diabetes [476], cardiovascular disease [477], obesity [478] and UPI [19].

Folate, vitamin-B_{12} and vitamin-B_6 along with key enzymes are directly involved in DNA methylation via one-carbon metabolism. Within this metabolic pathway is the synthesis of S-adenosylmethionine (SAM), the universal methyl donor for several biological methylation reactions. Methionine is regenerated from homocysteine (Hcy) by methionine synthase (MTR) and methionine synthase reductase (MTRR) in a reaction in which 5-methyltetrahydrofolate (methyl-THF) serves as the substrate[280]. The reduced availability of methyl-THF, the main circulating form of folate, decreases the biosynthesis of SAM, thus limiting the availability of methyl groups for methylation reactions. Therefore, not only can dietary folate depletion decrease genomic DNA methylation in humans[279] and animal models[79] but, as described in the study by Rampersaud and colleagues[279], a folate-replete diet may also restore DNA methylation status. In addition, a study in rats indicated that DNA hypomethylation occurs in relation to uteroplacental insufficiency (UPI) and demonstrated the vulnerability of the fetus in utero, suggesting folate as a key nutritional substrate[19].

Previous global DNA methylation studies thus far have not attempted to address the association with micronutrients and enzymes involved in one-carbon metabolism in the context of UPI. In addition the comparison of global CpG DNA methylation between maternal and placental tissue to date has not been investigated. In this study the measurement of global methylation represents the total amount of hypo- and hypermethylation that has occurred within maternal lymphocytes and placental tissue.

12.4 Methodology

12.4.1 Whole blood lymphocyte isolation

Lymphocytes were isolated from whole blood within four hours of collection. Whole blood was diluted 1:1 with Hanks Balanced Salt Solution (Hanks BSS; Trace Scientific, Melbourne, Australia) and gently inverted to mix. Diluted blood was gently overlayed onto Ficoll-Paque (Amershan BioSciences, Uppsala, Sweden) using a ratio of 1:3, e.g. 2ml Ficoll-Paque: 6ml diluted blood, being careful not to disturb the interface. The tubes were centrifuged at 400 x g for 30 minutes (1770 rpm MSE Mistral 2000R centrifuge) at 18 – 20°C. The buffy lymphocyte layer was removed at the interface of the Ficoll-Paque with the diluted plasma and transferred into a fresh tube using a sterile, plugged Pasteur pipette, taking care not to remove too much Ficoll-Paque. Three times the volume removed was added of Hanks BSS at room temperature, then centrifuged at 180 x g for 10 minutes (1000 rpm MSE Mistral 2000R centrifuge). The supernatant was discarded and the cell pellet (slightly brown colour) was

resuspended in two times the volume removed of Hanks BSS, using a Pasteur pipette, prior to centrifuging at 100 x g for 10 minutes (750 rpm MSE Mistral 2000R centrifuge). The supernatant was discarded and the cells resuspended in 600 µl $T_{10}E_5$ (2.5 mL 1 M Tris HCl, 2.5 mL 0.5 M EDTA, 245 mL dH_2O; pH 8.0) and 40 µl 10% sodium dodecyl sulphate (SDS; pH 7.2) using a Pasteur pipette.

12.4.2 DNA isolation from frozen lymphocytes

DNA was isolated from lymphocytes which had been stored in $T_{10}E_5$ and 10% SDS at -20 ºC. Briefly lymphocytes were defrosted on ice. Then 50 µl of proteinase K (10 mg/ml) was added before incubating overnight at 56 ºC. The tubes were cooled on ice before adding 200 µl of 6M NaCl then shaken vigorously for 15 seconds. A uniform white precipitate was formed. Unwanted cellular matter (white precipitate) was then spun down by centrifugation at 10,000 rpm for 5 minutes at 4ºC. The supernatant was transferred to a clean tube on ice (N.B. This tube should had sufficient capacity to add a further 2 volumes of liquid). Two volumes of cold absolute ethanol (stored at -20°C) was added, immediately tubes were gently inverted to precipitate DNA. The samples were then spun at 16,000 rpm for 1 minute (microfuge) to pellet the DNA. The DNA was washed with 0.5 ml 70% ethanol for a few minutes. If necessary DNA was re-pelleted by spinning at 16,000 rpm for 1 minute (microfuge), then pouring off the supernatant. Alternatively if the DNA was visible it was hooked out to a clean tube. The tubes were then inverted on a clean tissue and left at room temperature in order for the pellet to dry (approximately 10 minutes). The dried DNA was dissolved in 300 µL $T_{10}E_1$ (2.5 mL 1 M Tris HCl, 0.5 mL 0.5 M EDTA, 247 mL dH_2O; pH 8.0) at 37°C overnight. Once dissolved, DNA was quantified using a spectrophotometer (Model ND-1000, NanoDrop Technologies, Delaware, USA); 2 µL DNA was used and the absorbance was read at 260/280 nm. The DNA was stored at -20°C before sending to AmberGen Inc, Waltham, MA, USA on dry ice.

12.4.3 Global CpG methylation assay

Global CpG methylation was measured in maternal lymphocytes and placental DNA using the AmberGen *CpG*lobal™ Protocol (AmberGen Inc, Waltham, MA, USA). In brief**,** 100 ng of genomic DNA was digested with HpaII and MspI (Figure 25), in triplicate in a 96 well microtiter plate for 2 hours at 37°C. In the same wells the 5'overhangs left after digestion were end-filled with biotinylated nucleotides for 30 minutes at 37°C. The biotinylated DNA was adhered to the surface of the 96 well microtiter plate using DNA bind solution and left on

a rotator overnight at room temperature. The wells were washed 4 times with wash solution and the amount of biotin incorporated into the DNA was detected by HRP chemiluminescence. The luminescence was measured using a Wallace Envision 2100 multilabel reader (Perkin Elmer, Shelton, CT, USA). The proportion of unmethylated cytosines was calculated by dividing the values for HpaII luminescence by the average value for MspI luminescence. Therefore, the fraction of methylated cytosine in DNA was:
1 – unmethylated cytosines = methylated cytosines. The full protocol is shown in Appendix 22.

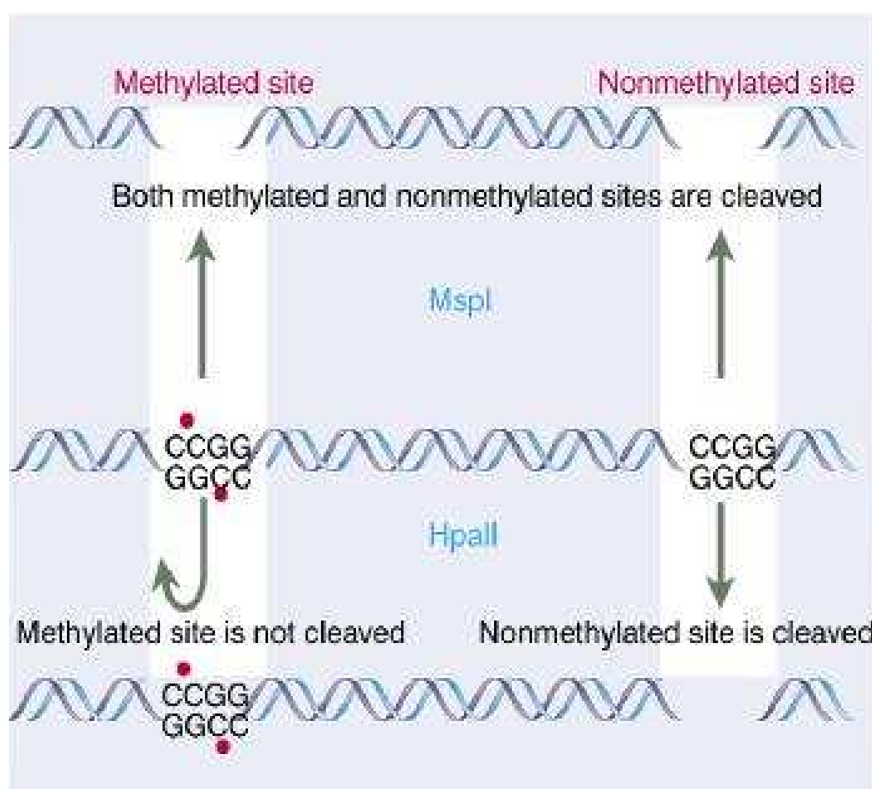

Figure 25 Msp I and Hpa II methylation sensitive restriction enzymes.
Msp I and Hpa II restriction endonucleases both cleave DNA at the C-C-G-G sequence, but Hpa II is blocked by prior methylation of the internal cytidine whereas Msp I is not. The red circles represent methyl groups attached to the CpG dinucleotide.

12.4.4 Statistics

Descriptive statistics analysis was performed to give the spread of the data for both maternal lymphocyte CpG methylation and placental CpG methylation. Pearson’s correlation analysis was used to measure the degree of an association between the CpG methylation in maternal and placenta tissue and variables including supplement intake, age, BMI, smoking, genome

damage markers, micronutrients and homocysteine. Placental DNA CpG methylation was also correlated with circulating maternal folate, vitamin-B_{12}, vitamin-B_6 and homocysteine (Hcy) using Pearson's correlation matrix. One-Way ANOVA was used to investigate the association of maternal and placental polymorphisms in one-carbon metabolism with maternal lymphocyte CpG methylation. In addition placental CpG methylation was compared to placental polymorphisms involved in one-carbon metabolism using One-Way ANOVA. Independent sample t-tests were used to compare CpG methylation in maternal and placental tissue with pregnancy outcome including UPI, PE and IUGR.

12.5 Results

This study included 27 paired maternal and placental DNA samples. The limited number of samples was due to a high number of the placental samples exhibiting DNA degradation. The quality of the samples rendered them unstable for the methylation assay due to artefactual effects of DNA shearing on the methylation assay.

Thirteen matched maternal and placental samples were from women with low risk pregnancies; of these eleven had normal outcomes and were classified into the control group. Fourteen matched maternal and placental samples were from women with high risk pregnancies of whom seven developed UPI, including four cases of PE and one case of IUGR.

12.5.1 Descriptive statistics and Pearson's correlation of maternal and placental CpG methylation index

The descriptive statistics produced for the CpG methylation index in maternal and placental DNA are represented in Table 28.

Table 28 Maternal and placental CpG methylation index descriptive statistics

	N	Mean	Minimum	Maximum	Std. Deviation
Maternal lymphocyte DNA	27	0.842	0.700	0.900	0.040
Placental DNA	27	0.703	0.280	0.880	0.124

The maternal lymphocyte and placental DNA CpG methylation index data did not show a significant correlation $r = 0.149$, $P = 0.458$; however when analysing the data with a paired

sample t-test the results show a significant difference between the maternal lymphocyte and placental tissue methylated cytosines (mCyt) P = P < 0.001.

12.5.2 Pearson's correlation of global CpG methylation with age, BMI and smoking

Global CpG methylation in placental DNA was positively associated with maternal age (r = 0.385, P = 0.047, Table 29). Therefore, increased global CpG methylation was detected in the placentas of older women. No other correlations were detected.

Table 29 Pearson's correlations of CpG global methylation with age, BMI and smoking status in maternal lymphocyte and placental DNA

	Age	BMI	Smokers
Maternal lymphocyte DNA (n = 27)			
r value	0.043	0.332	0.043
P value	0.832	0.091	0.832
Placental DNA (n = 27)			
r value	0.385	0.177	-0.261
*P value	**0.047**	0.377	0.189

* Correlation is significant at the 0.05 level (2-tailed).

12.5.3 Pearson's correlation of global CpG methylation with vitamin supplement intake

The intake of folic acid and B-vitamin supplements did not show a significant association with the global CpG methylation index in maternal and placental DNA (Table 30). Increased vitamin-B_6 supplement intake was marginally associated with higher levels of mCyt in placental tissue.

Table 30 Pearson's correlation of CpG global methylation with B-vitamin supplement intake in maternal and placental DNA

	Folic Acid	Vitamin-B_{12}	Vitamin-B_6
Maternal (n = 27)			
r value	0.046	-0.146	-0.151
P value	0.821	0.497	0.483
Placental (n = 27)			
r value	0.143	0.217	0.402
P value	0.477	0.308	0.051

12.5.4 Pearson's correlation of global CpG methylation with circulating maternal B-vitamins and plasma homocysteine

The maternal circulating concentrations of plasma homocysteine (Hcy), red cell folate (RCF), serum folate (SF), serum vitamin-B_{12} and red cell vitamin-B_6 were not significantly associated with global CpG methylation in the maternal or placental DNA (Table 31). A positive trend was evident between maternal RCF concentrations and global CpG methylation in both maternal lymphocyte and placental tissue DNA, but this did not reach significance.

Table 31 Pearson's correlation of global CpG global methylation with circulating B-vitamins and plasma homocysteine in maternal and placental DNA

	Hcy µmol/L	RCF nmol/L	SF nmol/L	B_{12} pmol/L	B_6 status*
Maternal (n = 27)					
r value	-0.093	0.382	-0.071	-0.112	0.016
P value	0.644	0.081	0.725	0.579	0.935
Placental (n = 27)					
r value	-0.037	0.428	-0.019	0.121	-0.093
P value	0.855	0.077	0.926	0.549	0.644

*Based on PPA activity which is inversely related to vitamin-B_6 concentration in RBCs
Hcy, L-Homocysteine in plasma; RCF, Red Cell Folate; SF, Serum Folate; B_{12}, Serum vitamin-B_{12}; B_6, Red Cell vitamin-B_6.

12.5.5 Pearson's correlation of global CpG methylation with maternal genome damage

The cytokinesis block micronucleus (CBMN) cytome assay genome damage markers in lymphocytes were scored in the 27 subjects whose samples were tested for global CpG methylation. There were no significant correlations observed when comparing maternal or placental CpG methylation with the tested genome damage and cytotoxic biomarkers (Table 32).

Table 32 Pearson correlation of global CpG methylation in maternal lymphocyte and placental DNA with CBMN assay biomarkers in maternal lymphocytes

	MN-BN	NPB-BN	NBUD-BN	NDI	Apoptosis	Necrosis
Maternal (n = 27)						
r value	-0.019	-0.287	-0.133	-0.194	-0.155	0.007
P value	0.924	0.146	0.507	0.333	0.440	0.972
Placental (n = 27)						
r value	-0.161	-0.275	-0.064	0.185	0.060	0.279
P value	0.424	0.165	0.753	0.355	0.766	0.158

MN-BN, Micronucleated binucleate cell; NPB-BN, Binucleated cell with nucleoplasmic bridge; NBUD-BN, Binucleated cell with nuclear bud; NDI, nuclear division index.

12.5.6 CpG methylation and maternal one-carbon metabolism genotypes

The maternal *MTHFR* (C677T and A1298C), *MTR* (A2756G), *MTRR* (A66G) and *MTHFD1* (G1958A) genotypes were determined in each of the 27 samples used for global CpG methylation. The tested genotypes were not significantly associated with global CpG methylation in peripheral lymphocyte DNA and placental DNA, except for the MTHFR 677 TT which was associated with a reduced mCyt in the placental DNA samples (P = 0.026, Table 33).

Table 33. Maternal and placental global CpG methylation in relation to polymorphisms in one-carbon metabolism genes in maternal tissue

Maternal lymphocyte DNA	Maternal mean CpG methylation	Placental mean CpG methylation
MTHFR C677T		
CC (n = 13)	0.832	0.724
CT (n = 11)	0.853	0.726
TT (n = 3)	0.843	0.523
ANOVA P	0.485	**0.026**
MTHFR A1298C		
AA (n = 12)	0.838	0.648
AC (n = 12)	0.849	0.750
CC (n = 3)	0.833	0.767
ANOVA P	0.748	0.082
MTR A2756G		
AA (n = 16)	0.837	0.693
AG (n = 10)	0.844	0.710
GG (n = 1)	0.900	0.790
ANOVA P	0.324	0.746
MTRR A66G		
AA (n = 7)	0.841	0.643
AG (n = 15)	0.836	0.705
GG (n = 5)	0.860	0.780
ANOVA P	0.535	0.168
MTHFD1 G1958A		
GG (n = 8)	0.838	0.705
GA (n = 15)	0.837	0.704
AA (n = 4)	0.865	0.695
ANOVA P	0.480	0.991

12.5.7 Placental global CpG methylation and fetal/placental genotypes involved in one- carbon metabolism

The placental/fetal *MTHFR* A1298C, *MTR* (A2756G), *MTRR* (A66G) and *MTHFD1* (G1958A) genotypes were not significantly related to changes in mean CpG methylation in placental DNA as shown in Table 34. The heterozygous *MTHFR* C677T genotype was marginally significant (P = 0.056) in association with placental global CpG methylation; however there was no obvious trend and these results should be viewed with caution due to small sample size.

Table 34 Placental global CpG methylation in relation to polymorphisms in one-carbon metabolism genes in placental and fetal tissue

Placental tissue DNA	Placental mean CpG methylation*	ANOVA P
MTHFR C677T		
CC (n = 14)	0.729	
CT (n = 11)	0.626	0.056
TT (n = 2)	0.797	
MTHFR A1298C		
AA (n = 7)	0.710	
AC (n = 17)	0.697	0.933
CC (n = 3)	0.723	
MTR A2756G		
AA (n = 11)	0.729	
AG (n = 13)	0.626	0.600
GG (n = 3)	0.797	
MTRR A66G		
AA (n = 6)	0.708	
AG (n = 16)	0.691	0.796
GG (n = 5)	0.732	
MTHFD1 G1958A		
GG (n = 7)	0.670	
GA (n = 14)	0.741	0.260
AA (n = 6)	0.653	

12.5.8 Global CpG methylation and the development of uteroplacental insufficiency

Global CpG methylation in maternal lymphocyte and placental DNA in women who developed uteroplacental insufficiency (UPI) or preeclampsia (PE) or IUGR were not significantly different compared to those with healthy outcomes (controls). The power comparisons were weak due to small sample sizes (Table 35).

Table 35 Mean CpG Methylation status in pregnancy groups

	Maternal mean CpG methylation*	Placental mean CpG methylation*
Low risk (n = 13)	0.837 ± 0.015	0.689 ± 0.043
High risk (n = 14)	0.846 ± 0.007	0.716 ± 0.023
T-test (2-tailed) P	0.553	0.589
Control (n = 11)	0.830 ± 0.018	0.678 ± 0.053
UPI (n = 7)	0.849 ± 0.007	0.694 ± 0.038
T-test (2-tailed) P	0.514	0.671
Control (n = 11)	0.830 ± 0.018	0.678 ± 0.053
PE (n = 4)	0.863 ± 0.006	0.745 ± 0.041
T-test (2-tailed) P	0.302	0.466
Control (n = 11)	0.830 ± 0.012	0.678 ± 0.053
IUGR (n = 1)	0.860 ± 0.000	0.760 ± 0.000
T-test (2-tailed) P	0.635	0.651

All data are represented as mean ± SEM

12.6 Discussion

The present study indicates that global CpG methylation (the level of 5-methylcytosine (mCyt)) in maternal lymphocyte DNA at 20 weeks gestation is unlikely to be a useful diagnostic for the prediction of uteroplacental insufficiency (UPI); however, these results do not exclude a change in specific genomic DNA methylation patterns in important subsets of maternal and/or placental tissues and/or genes in relation to UPI. It is well recognised that methylation of genomic DNA is involved in a wide range of pathological cellular phenomena[461] and it is plausible that small changes in gene specific methylation patterns may impact on placental development and subsequently UPI.

The results show that increased maternal age was significantly associated with an increased level of mCyt in the placenta. Previous findings have consistently shown an age-dependent decrease of global methylation levels in human tissues[270, 461, 479, 480]; however, to date no studies have compared maternal age with global methylation in placental tissue. The results in the previous chapter demonstrate that older women had a higher folic acid supplement intake than younger women, which may account for this rise in mCyt.

Maternal single nucleotide polymorphisms (SNPs) within genes involved in one-carbon metabolism and methyl group synthesis were not significantly related to changes in mCyt levels in maternal lymphocyte DNA. Previous studies have shown that homozygotes for the

MTHFR 677 T exhibit a significantly lower level of mCyt in DNA, but only under conditions of low folate status[282, 481]. At increased levels of folate, the amount of mCyt in DNA from *MTHFR* TT carriers does not differ from that among the *MTHFR* 677 CC individuals[282, 481]. This finding may explain the non significant results observed in this study as nutritional supplementation with high doses of folic acid can increase methylation and normalise gene expression at specific loci[482]. Hence, supplementation with methyl donors, such as folic acid, may correct for genetically based epigenetic deficiencies[265]. In contrast the maternal *MTHFR* 677 TT genotype was associated with reduced mCyt in placental DNA suggesting a greater susceptibility to genetic effects in this rapidly growing tissue. MTHFR is considered a key enzyme in one-carbon metabolism, since it is responsible for the irreversible conversion of 5, 10-methyleneTHF to 5-methylTHF, which serves as methyl donor for methionine, the precursor of SAM.

A positive trend was observed in relation to mCyt status in both maternal and placenta DNA with increased maternal red cell folate. These findings are not as strong as in previous studies[102, 103, 278, 483, 484]; however this may be due to small sample size. A study by Jacob et al.[278] clearly demonstrated the significant effects of folate on methylation. This study showed more than 100% increase in DNA hypomethylation after 9 weeks on low folate (56–111 μg/day) and a subsequent increase in DNA methylation after a further 3 weeks on a high folate diet (286–516 μg/day). Moreover, folate and B-vitamin deficiency increased Hcy levels, which causes DNA hypomethylation, and affects the expression of genes such as methyl CpG binding protein 2[485-487]. In addition a study by Park et al.[488] showed that the maternal folate and Hcy concentration along with the MTHFR 677 genetic polymorphism during pregnancy affected DNA methylation in the placenta.

Both hypo- or hyper-gene-specific methylation have been described in relation to impaired folate status. Previous animal studies have shown folate deficiency induces hypomethylation [489] although, under dietary folate/methyl depletion, DNA methyltransferase activity in rats has been described to be up-regulated[489, 490], leading to hypermethylation at specific loci[491]. Hypomethylation occurs mostly outside of promoters in CpG depleted areas as well as in repetitive elements[473] and has been shown to increase chromosome loss and breaks in the form of micronuclei (MN)[270, 326, 492].

The results from this study do not indicate that global CpG methylation is associated with CBMN genome damage and cytotoxic biomarkers in this cohort; however, this study had limited samples and therefore we can not discard the role of methylation and genome damage until further larger studies are performed. A study in young Australian adults indicated that

supplementation with folic acid and vitamin-B_{12} did not alter methylation in lymphocytes, but did reduce MN frequency[231] suggesting there are other mechanisms besides CpG methylation that may account for the genome protective effects of folate and vitamin-B_{12}, which include prevention of uracil incorporation into DNA which leads to chromosome breakage.

The ICF immunodeficiency syndrome, is caused by hypomethylation which leads to despiralisation of centromeric heterochromatin of chromosomes 1, 9 and 16, which consequently gives rise to MN [493, 494]. In addition increased genome damage can also be caused by hypermethylation of CpG islands within or adjacent to the promoter regions of housekeeping genes involved in cell cycle check points and DNA repair[266, 333]. Hypermethylation of the mitotic spindle check-point genes, such as APC, BUB1 and HCDC4, can reduce their expression and increase genome damage in the form of MN[333, 495-498].

The undetected change in global CpG methylation among women with healthy pregnancy and women who developed UPI suggests that other mechanisms may be operating. Alternatively, this study suggests that methylation differences are very subtle occurring at specific sites or genes. Placental and fetal development is dependent upon imprinted genes regulated by methylation[296, 499, 500]. In addition, the placenta exhibits unique features of epigenetic DNA regulation including X inactivation[501, 502] and the expression of retrotransposons[503-505]. Therefore, gene specific methylation targeted at genes involved in placentation should be investigated in future studies.

13 GENERAL DISCUSSION

Accumulating evidence shows that trophoblastic invasiveness is regulated by a balance between the local expression of molecules which promote invasion and those that inhibit invasion[331]. For these signalling molecules to be expressed and accurate angiogenesis to occur, genomic stability is crucial as gene dosage may alter gene expression resulting in altered cellular phenotypes and possibly reduced proliferative potential of cells. Angiogenesis, defined as the formation of new vessels sprouting from pre-existing endothelium, is essential to allow uteroplacental circulation which is necessary for the growing needs of the fetus[332]. Trophoblast invasion and angiogenesis may be altered if cells experience abnormal DNA damage rates, given that increased DNA damage often results in altered gene expression, cell cycle delay, reduced nuclear division rate and cell death[333].

The major finding in this study is that increased micronuclei (MN) in maternal peripheral lymphocytes (at 20 weeks gestation) is associated prospectively with UPI. MN can result from chromosome loss or chromosome breakage. Therefore, MN can be used to classify DNA damage into the aneugenic category, which refers to the loss or gain of whole chromosomes, thereby inducing an aneuploidy, or clastogenic category referring to chromosome breakage. Of particular interest is that the additional CBMN genome damage (NPB, NBUD and NDI) and cytotoxic biomarkers (apoptosis and necrosis) were not significantly increased in association with UPI. Nucleoplasmic bridges (NPB) are caused by telomere end fusions or mis-repair of chromosome breaks and are often detected with an adjacent micronucleus. Both MN and NPB can occur in cells exposed to clastogens (DNA-breaking agents)[506]. In addition to MN and NPB the CBMN assay allows for the detection of nuclear buds (NB), which represent a mechanism by which cells remove amplified DNA and are therefore considered a marker of possible gene amplification[506]. As MN was the only genome damage marker to be significantly increased in UPI relative to healthy pregnancy controls, it is plausible that MN have resulted from chromosome loss, not chromosome breakage, resulting in possible aneuploidy. Chromosome loss has been shown to produce permanent genomic changes in cells, and such changes can alter gene expression[507]. Altered gene expression may affect trophoblast cell phenotype impacting on invasion and modification of maternal spiral arteries ultimately reducing uteroplacental circulation. In addition, aneuploidy has been associated with infertility[508], developmental defects in the fetus[509] and leukaemia[510].

In this study folate, vitamin-B_{12} and vitamin-B_6 deficiency did not appear to be the cause of increased genome damage, which may be explained by the high B-vitamin consumption of many women in this study; however, the need for folate in fetal growth was emphasised as maternal red cell folate (RCF) < 400 nmol/L measured in the mother at 20 weeks gestation was prospectively associated with the IUGR.

Circulating Hcy was correlated with increased chromosome damage (MN and NPB), as indicated in previous studies[231, 511]. In addition, past studies have associated Hcy with UPI[134], but despite several pathophysiological hypotheses including impaired cell proliferation[303], increased oxidative stress[512], apoptosis[246, 513], reduced extra-embryonic vascular development[514] , augmenting inflammatory responses, and hypomethylation[515] it has not been clear whether increased Hcy is causally related to UPI or whether it is just a marker of the increased risk for UPI. This study suggests a causal relationship as increased Hcy at 20 weeks was associated prospectively with the subsequent development of UPI in later pregnancy.

Our results are of particular interest because, despite high supplementation with folic acid, vitamin-B_{12} and vitamin-B_6 in women with high risk pregnancies, their plasma Hcy levels were not significantly lower compared to those with low risk pregnancies and reduced supplement intakes. There is general agreement that Hcy concentrations are lower during pregnancy[107, 118, 384]; however, the reason for this is unknown. Steegers-Theunissen et al.[516] have suggested that the low levels are due to active remethylation of Hcy to methionine in the fetal tissues, based on the observation of low Hcy compared to high methionine concentrations in amniotic fluid in early pregnancy. It is likely that a higher rate of Hcy is consumed in the mother to produce methionine for methylation reactions via S-adenosyl methionine (SAM) and for polyamine and protein synthesis to support placental and fetal development[118, 385, 514].

Of interest is that the fetal *MTR* 2756 GG genotype was significantly associated with increased plasma Hcy concentration and UPI; however, the maternal *MTR* 2756 polymorphisms (women treated with high dose B-vitamins) did not reveal an association with UPI in the current pregnancies. In an attempt to depict a better understanding of the genetic impact without the interference of increased B-vitamin consumption the index pregnancies (prior pregnancies without increased B-vitamins) were analysed. The additional analyses

revealed a significant association with the *MTR* 2756 GG maternal genotype and UPI, suggesting that B-vitamins may have improved pregnancy outcome.

The novel *MTHFD1* 1958 single nucleotide polymorphism (SNP) was associated with a risk for IUGR, warranting the need for functional studies associated with this polymorphism. A strong association was not detected in SNPs within the *MTHFR* gene and other one-carbon metabolism genes; however, these results are not surprising due to the high supplementation levels which would have altered kinetics in the one – carbon metabolic cycle and compensated for underlying metabolic defects. The index pregnancy (prior pregnancies without increased B-vitamins) analysis revealed a stronger association with the *MTHFR* 677 T allele and UPI; however, larger studies are needed to clarify this.

The analysis of global CpG methylation in relation to genome damage, one – carbon metabolism and UPI did not reveal and significant associations. This suggests that DNA hypomethylation, which has been shown to cause chromosomes loss[366], was not the cause of increased MN in this study.

13.1 Future directions

This study has resulted in exciting preliminary data on the association of MN and Hcy with UPI. The next step is to perform a larger cohort study that does not discriminate between high risk and low risk pregnancies, in order to prospectively confirm and quantify the association between MN frequency and UPI and, in particular, also the different types of UPI, i.e. preeclampsia, versus IUGR, versus placental abruption and placental pathology-associated preterm labour. In addition studies are required to verify that the increased MN is induced by chromosome loss, and importantly if it is a specific chromosome that is continually eliminated during mitosis. This can be done using the CBMN cytome assay, combined with FISH or other cytological methods such as differential staining of chromosomes or with the use of centromeric DNA probes, which allow distinction between clastogenic events, chromosome loss and chromosome non-disjunction[517].

Structures that are thought to be involved in chromosomal segregation should be the major targets in future studies to identify the mechanism behind the increase in chromosome loss. Chromosome segregation machinery components that are implicated with chromosome loss include deficiencies in the cell cycle controlling genes (e.g. MAD[518] and BUB[368]), defects in mitotic spindle assembly[319, 517, 519], kinetochore proteins[310], histone phosphorylation and topoisomerase II.

Proliferating cells have check points that ensure that cell division does not proceed if DNA damage has not been repaired or if chromosome alignment is imprecise[520-522]. Progression through the cell division cycle is discontinuous, in the sense that the cell pauses prior to each cell cycle transition to check that everything necessary is in place before committing itself to proceeding further[523-525]. These cell cycle checkpoints are essential for preserving the normal chromosome number and act synergistically with homologous recombination functions to ensure that chromosomes are segregated correctly to daughter cells[526]. The spindle checkpoint is a highly conserved signal transduction pathway that links the initiation of anaphase to spindle assembly and the completion of chromosome – microtubule attachment [527, 528]. The presence of even a single misaligned or unattached chromosome is sufficient to activate the checkpoint and arrest a cell at the metaphase to anaphase transition [529, 530]. Arrest caused by an unattached chromosome is overcome by laser ablation of the kinetochore, the structure that mediates chromosome–microtubule attachment[531, 532]. Thus, the signal for checkpoint-dependent arrest arises from, or is transduced through, kinetochores[58].

Genes involved in the spindle assembly checkpoint were first identified in the yeast *S. cerevisiae* and include the mitotic arrest defective genes *MAD* 1–3 [518] and the budding uninhibited by benzimidazole genes *BUB* 1–3[527]. Mad1–3p, Bub1p, and Bub3p are proteins that link anaphase to the completion of spindle assembly[533]; however, Bub2p appears to be part of a second pathway that acts later in the cell cycle to link spindle assembly to mitotic exit and cytokinesis[533, 534]. The addition of antimicrotubule drugs to yeast cells lacking any single *MAD* or *BUB* gene causes the cells to proceed through mitosis without having established chromosome – microtubule attachments. This causes extensive chromosome loss and cell death[518, 527]. In animal cells, several Mad and Bub proteins localize to kinetochores unattached to microtubules, consistent with the observation that kinetochores are involved in generating the checkpoint signal[528, 535].

Abnormal phosphorylation patterns of the histones during metaphase could lead to abnormal chromosome segregation and extensive chromosome loss during mitosis[536]. Alternatively, chromosome loss can be induced by perturbing anaphase segregation with topoisomerase II inhibitors[537]. Topoisomerase II is an important enzyme associated with many aspects of chromosome dynamics including DNA replication, transcription, recombination, chromosome condensation and segregation[538]. Of these, chromosome segregation is the most seriously affected by loss of topoisomerase II expression or activity[537], hence topoisomerase II activity could be a possible target for future studies.

Furthermore, Visintin et al.[539] suggested a specific connection between the spindle checkpoint and programmed cell death. It is believed that apoptosis is triggered after a cell has experienced a prolonged mitotic arrest, thereby reducing the chance that the cell can escape the checkpoint and become aneuploid[540]. In this study reduced apoptosis was observed in association with UPI. This suggests that cells with DNA damage may have escaped apoptosis allowing an unattached chromosome to progress in a micronucleus, which may explain the increase in MN frequency in women who developed UPI.

In addition to B-vitamins other micronutrients have been identified in maintenance of genome stability[1, 102, 229, 541, 542], and it is plausible that women with increased MN were deficient in micronutrients that affect chromosome segregation. There are approximately 40 micronutrients required in the human diet[543], and many are crucial in determining genomic stability[372]. For example, calcium has been associated with checkpoint-related cell cycle transitions[544-546]. In addition, zinc, selenium, and vitamins C and E, as well as a variety of classes of nonessential nutrients and bioactive components have been shown to influence cellular processes associated with cell cycle control, apoptosis, and angiogenesis[547, 548]. A recent report determined the association between dietary intake and genome damage in lymphocytes[366] measured using the CBMN cytome assay, which highlighted increased intake of vitamin E, retinol, folic acid, nicotinic acid and calcium with significant reductions in MN frequency. This illustrates the strong impact of a variety of micronutrients on genome health and the need for further studies incorporating other dietary factors when investigating the relationship between MN and UPI. In addition, studies have suggested that regular use of a multivitamin supplement in the periconceptional period may help to prevent severe preterm births[100], extreme SGA[100] and PE[99].

Aside from investigations into the mechanism behind increased MN future studies are required to validate the prospective relationship between Hcy and UPI. In addition, factors that can cause increased Hcy should be considered: these include smoking (including the amount of cigarettes)[394], metabolic deficiencies or defects, such as reduced riboflavin [101] and macronutrient intake as reports have shown that high fat[549-551] and high sucrose[551] increase circulating Hcy concentrations. Of particular interest is that this study may indicate that high risk patients need increased B-vitamin consumption to achieve normal Hcy concentrations that the low risk (healthy) population readily maintain.

This study has shown that reduced folate and high Hcy are significantly associated with IUGR. In order to understand the mechanism behind reduced growth it would be beneficial in future studies to have knowledge on methionine concentrations. In addition, it is plausible that

the *MTR* 2756 G allele may slow the conversion of Hcy to methionine increasing the risk for UPI. Therefore, these data may suggest that high concentrations of folic acid, vitamin-B_{12} and vitamin-B_6 are beneficial in modifying pregnancy outcome in *MTR* 2756 G allele carriers. Moreover, a recent study with 10,601 adults indicated that individuals with the *MTHFR* TT genotype were particularly sensitive to the status of several B-vitamins[552], indicating the need to design investigations which account for vitamin/mineral cofactors that interact with associated enzymes when assessing polymorphisms.

It is plausible that single nucleotide polymorphisms that were tested in genes involved in one - carbon metabolism may harbour undiscovered potential variation; therefore there is a need to explore polymorphisms that have not been published by sequencing or a comparable method. Furthermore, not all potential folate genes have been studied, in particular the many methyltransferases that catalyze the transfer of methyl groups for DNA, RNA and proteins, have not, so far, been investigated. The ICF immunodeficiency syndrome, shows that a mutation in the DNA methyltransferase gene, causes despiralisation of heterochromatin and loss of chromosomes 1, 9 and 16 into MN[553, 554]. The use of dedicated SNP arrays that allow high-throughput genotyping, in combination with haplotype analyses[376, 548], may help elucidate a wider range of SNPs associated with genome damage and UPI.

The majority of publications related to DNA damage provide evidence that higher frequencies of MN are found in the populations exposed to genotoxic agents[310]. Therefore, we can speculate that women with an abnormally high frequency of MN within the high risk and pathologic groups may have been chronically exposed to a mutagen or aneugen. Furthermore, there is emerging evidence that air pollution is associated with aneuploidy and elevated risk of adverse pregnancy outcomes[555-557]. Carcinogenic polycyclic aromatic hydrocarbons (PAHs) are widely present in the environment, as products of the combustion of fossil fuels and of cigarette smoking, in ambient indoor air, and in grilled and smoked foods[558-563]. Polymorphisms in metabolic and DNA repair genes identify subjects susceptible to DNA damage by exposure to carcinogenic PAHs. Susceptible polymorphisms in metabolic and DNA repair genes that may be associated with increased aneuploidy and MN include cytochrome P450 (*CYP1A1*)[564], glutathione *S*-transferase (*GST*)[565] , and *N*-acetyltransferases *(NAT*)[566]. In addition CYP1A1, NAT2, GSTM1 and GSTT1 polymorphisms have been associated with low birth weight and specific cancers[564, 567].

This study indicated that global CpG methylation in maternal lymphocyte DNA is unlikely to be a useful diagnostic for the prediction of uteroplacental insufficiency (UPI); however, these results do not exclude a change in specific genomic DNA methylation patterns in important

subsets of maternal and/or placental tissues and/or genes in relation to UPI. Future studies should target gene specific methylation focusing on genes involved in placentation, such as *IGF* and mitotic spindle check point genes such as *MAD* and *BUB*. Furthermore, messenger RNA and proteomic array technology may be useful to identify differences in gene expression that are indicative of increased risk for UPI.

13.2 Conclusion

To the best of our knowledge this is the first study to measure maternal genome damage in relation to uteroplacental insufficiency (UPI) including preeclampsia (PE) and intrauterine growth restriction (IUGR). In addition, individual factors involved in one - carbon metabolism that have been related to UPI in previous studies have been combined to provide a more comprehensive investigation into the pathophysiology of UPI. These preliminary results may lead to the development of diagnostic indices for increased risk for UPI, using the MN index along with metabolic and genetic factors associated with one-carbon metabolism (Figure 26).

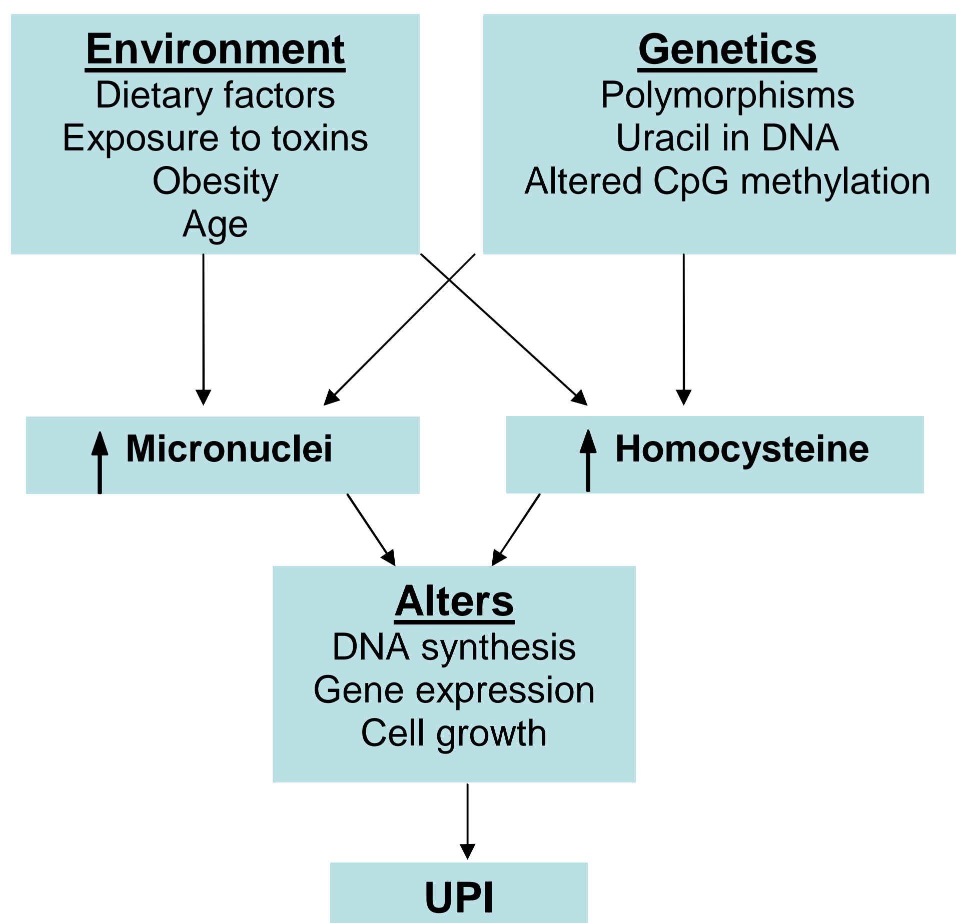

Figure 26 Schematic mechanism for development of UPI

Understanding the impact of specific micronutrients, their related metabolic pathways and genome stability in relation to pregnancy outcomes is an important area in reproductive research. Advances in our knowledge have opened up a new opportunity in the prevention of pregnancy diseases based on the concepts that (a) excessive genome damage is related to infertility, UPI and developmental defects, (b) genome damage caused by micronutrient deficiency is preventable, (c) accurate diagnosis of genome instability using DNA damage biomarkers that are sensitive to micronutrient deficiency is technically feasible and (d) it is possible to optimise nutritional status and verify efficacy by confirmation of a reduction in genome damage rate after intervention[284].

Given the fundamental importance of the nutritional genomic affects of dietary factors, and the pace of development in molecular diagnostics, it is not hard to envisage a new era in preventive medicine that has even greater emphasis on diet as a means to a healthy pregnancy and long and healthy life[298]—indeed, a return to Hippocrates' famous "Let food be thy medicine and medicine be thy food" philosophy; however, in this era we have the advantage of technology to diagnose the effect of dietary choice at the genomic level.

14 REFERENCES

1. Fenech, M. and L.R. Ferguson, *Vitamins/minerals and genomic stability in humans.* Mutat Res, 2001. **475**(1-2): p. 1-6.
2. Zijno, A., et al., *Folate status, metabolic genotype, and biomarkers of genotoxicity in healthy subjects.* Carcinogenesis, 2003. **24**(6): p. 1097-103.
3. Wilcox, A.J., et al., *Incidence of early loss of pregnancy.* N Engl J Med, 1988. **319**(4): p. 189-94.
4. Hertig, A.T., *Human trophoblast: normal and abnormal. A plea for the study of the normal so as to understand the abnormal.* American Journal of Clinical Pathology, 1967.
5. Kliman, H., *Trophoblast infiltration.* Reproductive Medicine Review, 1994. **3**: p. 137-57.
6. Pijnenborg, R., et al., *Interaction of interstitial trophoblast with placental bed capillaries and venules of normotensive and pre-eclamptic pregnancies.* Placenta, 1998. **19**(8): p. 569-75.
7. Robertson, W.B., I. Brosens, and W.N. Landells, *Abnormal placentation.* Obstet Gynecol Annu, 1985. **14**: p. 411-26.
8. Pijnenborg, R., et al., *Placental bed spiral arteries in the hypertensive disorders of pregnancy.* Br J Obstet Gynaecol, 1991. **98**(7): p. 648-55.
9. Sheppard, B.L. and J. Bonnar, *The ultrastructure of the arterial supply of the human placenta in pregnancy complicated by fetal growth retardation.* Br J Obstet Gynaecol, 1976. **83**(12): p. 948-59.
10. Sheppard, B.L. and J. Bonnar, *An ultrastructural study of utero-placental spiral arteries in hypertensive and normotensive pregnancy and fetal growth retardation.* Br J Obstet Gynaecol, 1981. **88**(7): p. 695-705.
11. Gerretsen, G., et al., *Trophoblast alterations in the placental bed in relation to physiological changes in spiral arteries.* Br J Obstet Gynaecol, 1983. **90**(1): p. 34-9.
12. Kliman, H.J., *Uteroplacental blood flow. The story of decidualization, menstruation, and trophoblast invasion.* Am J Pathol, 2000. **157**(6): p. 1759-68.
13. Hirano, H., Y. Imai, and H. Ito, *Spiral artery of placenta: development and pathology-immunohistochemical, microscopical, and electron-microscopic study.* Kobe J Med Sci, 2002. **48**(1-2): p. 13-23.

14. Brosens, I., H.G. Dixon, and W.B. Robertson, *Fetal growth retardation and the arteries of the placental bed.* Br J Obstet Gynaecol, 1977. **84**(9): p. 656-63.
15. Khong, T.Y., et al., *Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants.* Br J Obstet Gynaecol, 1986. **93**(10): p. 1049-59.
16. Welsh, A.W., P.R. Bennett, and N.M. Fisk, *Quantitative digital analysis of regional placental perfusion using power Doppler in placental abruption.* Ultrasound Obstet Gynecol, 2001. **17**(3): p. 203-8.
17. Degani, S., et al., *Fetal and uteroplacental flow velocity waveforms in the expectant management of placental abruption.* Gynecol Obstet Invest, 1990. **30**(1): p. 59-60.
18. Defoort, P., M. Thiery, and P. Vanhaesebrouck, *Uteroplacental and umbilical artery blood velocity waveforms in placental abruption assessed by Doppler ultrasound.* Br J Obstet Gynaecol, 1989. **96**(2): p. 254-5.
19. MacLennan, N.K., et al., *Uteroplacental insufficiency alters DNA methylation, one-carbon metabolism, and histone acetylation in IUGR rats.* Physiol Genomics, 2004. **18**(1): p. 43-50.
20. Brosens, I., W.B. Robertson, and H.G. Dixon, *The physiological response of the vessels of the placental bed to normal pregnancy.* J Pathol Bacteriol, 1967. **93**(2): p. 569-79.
21. Marais, W.D., *Human decidual spiral arteries. Ultrastructure of the intima in normal vessels.* J Obstet Gynaecol Br Commonw, 1968. **75**(5): p. 552-67.
22. Logan, S.K., S.J. Fisher, and C.H. Damsky, *Human placental cells transformed with temperature-sensitive simian virus 40 are immortalized and mimic the phenotype of invasive cytotrophoblasts at both permissive and nonpermissive temperatures.* Cancer Res, 1992. **52**(21): p. 6001-9.
23. De Wolf, F., I. Brosens, and W.B. Robertson, *Ultrastructure of uteroplacental arteries.* Contrib Gynecol Obstet, 1982. **9**: p. 86-99.
24. Strickland, S. and W.G. Richards, *Invasion of the trophoblasts.* Cell, 1992. **71**(3): p. 355-7.
25. Adams, E.C., A.T. Hertig, and J. Rock, *A description of 34 human ova within the first 17 days of development.* Am J Anat, 1956. **98**(3): p. 435-93.
26. Cross, J.C., Z. Werb, and S.J. Fisher, *Implantation and the placenta: key pieces of the development puzzle.* Science, 1994. **266**(5190): p. 1508-18.
27. Moffett-King, A., *Natural killer cells and pregnancy.* Nat Rev Immunol, 2002. **2**(9): p. 656-63.

28. Silver, M., et al., *Placental blood flow: some fetal and maternal cardiovascular adjustments during gestation.* J Reprod Fertil Suppl, 1982. **31**: p. 139-60.
29. Ueland, K., *Maternal cardiovascular dynamics. VII. Intrapartum blood volume changes.* Am J Obstet Gynecol, 1976. **126**(6): p. 671-7.
30. Gowland, P., *Placental MRI.* Semin Fetal Neonatal Med, 2005. **10**(5): p. 485-90.
31. Redman, C.W. and I.L. Sargent, *Pre-eclampsia, the placenta and the maternal systemic inflammatory response--a review.* Placenta, 2003. **24 Suppl A**: p. S21-7.
32. Lyall, F., et al., *Hemeoxygenase expression in human placenta and placental bed implies a role in regulation of trophoblast invasion and placental function.* Faseb J, 2000. **14**(1): p. 208-19.
33. Lyall, F., et al., *Human trophoblast invasion and spiral artery transformation: the role of PECAM-1 in normal pregnancy, preeclampsia, and fetal growth restriction.* Am J Pathol, 2001. **158**(5): p. 1713-21.
34. Sibai, B., G. Dekker, and M. Kupferminc, *Pre-eclampsia.* Lancet, 2005. **365**(9461): p. 785-99.
35. Newstead, J., P. von Dadelszen, and L.A. Magee, *Preeclampsia and future cardiovascular risk.* Expert Rev Cardiovasc Ther, 2007. **5**(2): p. 283-94.
36. Douglas, K.A. and C.W. Redman, *Eclampsia in the United Kingdom.* Bmj, 1994. **309**(6966): p. 1395-400.
37. Brown, M.A., J. Wang, and J.A. Whitworth, *The renin-angiotensin-aldosterone system in pre-eclampsia.* Clin Exp Hypertens, 1997. **19**(5-6): p. 713-26.
38. Brown, M.A., et al., *The detection, investigation and management of hypertension in pregnancy: full consensus statement.* Aust N Z J Obstet Gynaecol, 2000. **40**(2): p. 139-55.
39. Zhou, Y., C.H. Damsky, and S.J. Fisher, *Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype. One cause of defective endovascular invasion in this syndrome?* J Clin Invest, 1997. **99**(9): p. 2152-64.
40. Bulmer, J.N., *Immune aspects of pathology of the placental bed contributing to pregnancy pathology.* Baillieres Clin Obstet Gynaecol, 1992. **6**(3): p. 461-88.
41. Redman, C.W., G.P. Sacks, and I.L. Sargent, *Preeclampsia: an excessive maternal inflammatory response to pregnancy.* Am J Obstet Gynecol, 1999. **180**(2 Pt 1): p. 499-506.
42. Brown, D.W., *Pre-eclampsia: a mistake of trophoblastic cells for tumour cells?* Med Hypotheses, 1999. **53**(2): p. 124-6.

43. Widschwendter, M., H. Schrocksnadel, and M.G. Mortl, *Pre-eclampsia: a disorder of placental mitochondria?* Mol Med Today, 1998. **4**(7): p. 286-91.
44. van Beck, E. and L.L. Peeters, *Pathogenesis of preeclampsia: a comprehensive model.* Obstet Gynecol Surv, 1998. **53**(4): p. 233-9.
45. Dekker, G.A., P.Y. Robillard, and T.C. Hulsey, *Immune maladaptation in the etiology of preeclampsia: a review of corroborative epidemiologic studies.* Obstet Gynecol Surv, 1998. **53**(6): p. 377-82.
46. Dekker, G.A. and B.M. Sibai, *Etiology and pathogenesis of preeclampsia: current concepts.* Am J Obstet Gynecol, 1998. **179**(5): p. 1359-75.
47. Salas, S.P., *What causes pre-eclampsia?* Baillieres Best Pract Res Clin Obstet Gynaecol, 1999. **13**(1): p. 41-57.
48. Soma, H., et al., *Morphologic changes in the hypertensive placenta.* Contrib Gynecol Obstet, 1982. **9**: p. 58-75.
49. Rodrigues, F.A., et al., *Hypothesis: a central role for platelet activating factor in the pathogenesis of pre-eclampsia.* Med Hypotheses, 1989. **28**(4): p. 285-6.
50. Venkatesha, S., et al., *Soluble endoglin contributes to the pathogenesis of preeclampsia.* Nat Med, 2006. **12**(6): p. 642-9.
51. Germain, A.M., et al., *Endothelial dysfunction: a link among preeclampsia, recurrent pregnancy loss, and future cardiovascular events?* Hypertension, 2007. **49**(1): p. 90-5.
52. Maynard, S.E., et al., *Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia.* J Clin Invest, 2003. **111**(5): p. 649-58.
53. Smith, G.C., et al., *Circulating angiogenic factors in early pregnancy and the risk of preeclampsia, intrauterine growth restriction, spontaneous preterm birth, and stillbirth.* Obstet Gynecol, 2007. **109**(6): p. 1316-24.
54. Petraglia, F., et al., *Hypertension in pregnancy: changes in activin A maternal serum concentration.* Placenta, 1995. **16**(5): p. 447-54.
55. Sud, S.S., et al., *Serial plasma fibronectin levels in pre-eclamptic and normotensive women.* Int J Gynaecol Obstet, 1999. **66**(2): p. 123-8.
56. Roberts, J.M. and C.W. Redman, *Pre-eclampsia: more than pregnancy-induced hypertension.* Lancet, 1993. **341**(8858): p. 1447-51.
57. Al-Mufti, R., et al., *Increased fetal erythroblasts in women who subsequently develop pre-eclampsia.* Hum Reprod, 2000. **15**(7): p. 1624-8.
58. Holzgreve, W., et al., *Elevation in erythroblast count in maternal blood before the onset of preeclampsia.* Am J Obstet Gynecol, 2001. **184**(2): p. 165-8.

59. Leung, T.N., et al., *Increased maternal plasma fetal DNA concentrations in women who eventually develop preeclampsia.* Clin Chem, 2001. **47**(1): p. 137-9.
60. Welfare, A.I.o.H.a., *Australian babies: their health and wellbeing*. 2004: Sydney.
61. Mick, E., et al., *Impact of low birth weight on attention-deficit hyperactivity disorder.* J Dev Behav Pediatr, 2002. **23**(1): p. 16-22.
62. Leeson, C.P., et al., *Impact of low birth weight and cardiovascular risk factors on endothelial function in early adult life.* Circulation, 2001. **103**(9): p. 1264-8.
63. Barker, D.J., *The developmental origins of well-being.* Philos Trans R Soc Lond B Biol Sci, 2004. **359**(1449): p. 1359-66.
64. Baserga, M., et al., *Uteroplacental insufficiency alters nephrogenesis and downregulates cyclooxygenase-2 expression in a model of IUGR with adult-onset hypertension.* Am J Physiol Regul Integr Comp Physiol, 2007. **292**(5): p. R1943-55.
65. Barker, D.J., *Fetal programming of coronary heart disease.* Trends Endocrinol Metab, 2002. **13**(9): p. 364-8.
66. Godfrey, K.M. and D.J. Barker, *Fetal programming and adult health.* Public Health Nutr, 2001. **4**(2B): p. 611-24.
67. Lubchenco, L.O., C. Hansman, and E. Boyd, *Intrauterine growth in length and head circumference as estimated from live births at gestational ages from 26 to 42 weeks.* Pediatrics, 1966. **37**(3): p. 403-8.
68. Usher, R. and F. McLean, *Intrauterine growth of live-born Caucasian infants at sea level: standards obtained from measurements in 7 dimensions of infants born between 25 and 44 weeks of gestation.* J Pediatr, 1969. **74**(6): p. 901-10.
69. Battaglia, F.C. and L.O. Lubchenco, *A practical classification of newborn infants by weight and gestational age.* J Pediatr, 1967. **71**(2): p. 159-63.
70. Lubchenco, L.O., D.T. Searls, and J.V. Brazie, *Neonatal mortality rate: relationship to birth weight and gestational age.* J Pediatr, 1972. **81**(4): p. 814-22.
71. Thomas, P., et al., *A new look at intrauterine growth and the impact of race, altitude, and gender.* Pediatrics, 2000. **106**(2): p. E21.
72. Bernstein, I.M., et al., *Morbidity and mortality among very-low-birth-weight neonates with intrauterine growth restriction. The Vermont Oxford Network.* Am J Obstet Gynecol, 2000. **182**(1 Pt 1): p. 198-206.
73. Pain, S., et al., *Customised birthweight: coefficients for an Australian population and validation of the model.* Aust N Z J Obstet Gynaecol, 2006. **46**(5): p. 388-94.
74. McCowan, L.M., J.E. Harding, and A.W. Stewart, *Customized birthweight centiles predict SGA pregnancies with perinatal morbidity.* Bjog, 2005. **112**(8): p. 1026-33.

75. Selling, K.E., et al., *Intergenerational effects of preterm birth and reduced intrauterine growth: a population-based study of Swedish mother-offspring pairs.* Bjog, 2006. **113**(4): p. 430-40.
76. Catov, J.M., et al., *Association between infant birth weight and maternal cardiovascular risk factors in the health, aging, and body composition study.* Ann Epidemiol, 2007. **17**(1): p. 36-43.
77. Phillips, D.I., A. Jones, and P.A. Goulden, *Birth weight, stress, and the metabolic syndrome in adult life.* Ann N Y Acad Sci, 2006. **1083**: p. 28-36.
78. Bada, H.S., et al., *Low birth weight and preterm births: etiologic fraction attributable to prenatal drug exposure.* J Perinatol, 2005. **25**(10): p. 631-7.
79. Balaghi, M. and C. Wagner, *DNA methylation in folate deficiency: use of CpG methylase.* Biochem Biophys Res Commun, 1993. **193**(3): p. 1184-90.
80. Chiolero, A., P. Bovet, and F. Paccaud, *Association between maternal smoking and low birth weight in Switzerland: the EDEN study.* Swiss Med Wkly, 2005. **135**(35-36): p. 525-30.
81. Bernstein, I.M., et al., *Maternal smoking and its association with birth weight.* Obstet Gynecol, 2005. **106**(5 Pt 1): p. 986-91.
82. Mariscal, M., et al., *Pattern of alcohol consumption during pregnancy and risk for low birth weight.* Ann Epidemiol, 2006. **16**(6): p. 432-8.
83. Frederick, I.O., et al., *Adult weight change, weight cycling, and prepregnancy obesity in relation to risk of preeclampsia.* Epidemiology, 2006. **17**(4): p. 428-34.
84. Moran, C., et al., *Increased insulin levels independent of gestational overweight in women with preeclampsia.* Arch Med Res, 2006. **37**(6): p. 749-54.
85. Sibai, B.M., *Imitators of severe preeclampsia.* Obstet Gynecol, 2007. **109**(4): p. 956-66.
86. Makedos, G., et al., *Homocysteine, folic acid and B12 serum levels in pregnancy complicated with preeclampsia.* Arch Gynecol Obstet, 2007. **275**(2): p. 121-4.
87. Kramer, M.S., et al., *Etiologic determinants of abruptio placentae.* Obstet Gynecol, 1997. **89**(2): p. 221-6.
88. Ananth, C.V., K. Demissie, and M.L. Hanley, *Birth weight discordancy and adverse perinatal outcomes among twin gestations in the United States: the effect of placental abruption.* Am J Obstet Gynecol, 2003. **188**(4): p. 954-60.
89. Kyrklund-Blomberg, N.B., G. Gennser, and S. Cnattingius, *Placental abruption and perinatal death.* Paediatr Perinat Epidemiol, 2001. **15**(3): p. 290-7.
90. Rasmussen, S., et al., *Perinatal mortality and case fatality after placental abruption in Norway 1967-1991.* Acta Obstet Gynecol Scand, 1996. **75**(3): p. 229-34.

91. Raymond, E.G. and J.L. Mills, *Placental abruption. Maternal risk factors and associated fetal conditions.* Acta Obstet Gynecol Scand, 1993. **72**(8): p. 633-9.

92. Prochazka, M., et al., *Frequency of selected thrombophilias in women with placental abruption.* Aust N Z J Obstet Gynaecol, 2007. **47**(4): p. 297-301.

93. Ananth, C.V., et al., *Preterm premature rupture of membranes, intrauterine infection, and oligohydramnios: risk factors for placental abruption.* Obstet Gynecol, 2004. **104**(1): p. 71-7.

94. Lockwood, C.J., et al., *Mechanisms of abruption-induced premature rupture of the fetal membranes: thrombin-enhanced interleukin-8 expression in term decidua.* Am J Pathol, 2005. **167**(5): p. 1443-9.

95. Williams, M.A., R. Mittendorf, and R.R. Monson, *Chronic hypertension, cigarette smoking, and abruptio placentae.* Epidemiology, 1991. **2**(6): p. 450-3.

96. Kupferminc, M.J., et al., *Increased frequency of genetic thrombophilia in women with complications of pregnancy.* N Engl J Med, 1999. **340**(1): p. 9-13.

97. Maloney, C.A. and W.D. Rees, *Gene-nutrient interactions during fetal development.* Reproduction, 2005. **130**(4): p. 401-10.

98. Selhub, J., *Folate, vitamin B12 and vitamin B6 and one carbon metabolism.* J Nutr Health Aging, 2002. **6**(1): p. 39-42.

99. Bodnar, L.M., et al., *Periconceptional multivitamin use reduces the risk of preeclampsia.* Am J Epidemiol, 2006. **164**(5): p. 470-7.

100. Catov, J.M., et al., *Association of periconceptional multivitamin use and risk of preterm or small-for-gestational-age births.* Am J Epidemiol, 2007. **166**(3): p. 296-303.

101. Forges, T., et al., *Impact of folate and homocysteine metabolism on human reproductive health.* Hum Reprod Update, 2007.

102. Fenech, M., *The role of folic acid and Vitamin B12 in genomic stability of human cells.* Mutat Res, 2001. **475**(1-2): p. 57-67.

103. Cravo, M., et al., *DNA methylation as an intermediate biomarker in colorectal cancer: modulation by folic acid supplementation.* Eur J Cancer Prev, 1994. **3**(6): p. 473-9.

104. Wagner, C., *Folate in Health and Disease: Biochemical role of folate in cellular metabolism*, ed. L. Bailey. 1995: Marcel Dekker. 23–42.

105. Field, C.J., et al., *Dietary folate improves age-related decreases in lymphocyte function.* J Nutr Biochem, 2006. **17**(1): p. 37-44.

106. Miller, A., Kelly, G., *Methionine and Homocysteine Metabolism and the Nutritional Prevention of Certain Birth Defects and Complications of Pregnancy.* Alternative medicine review, 1996. **1**(4): p. 220-235.

107. Tamura, T. and M.F. Picciano, *Folate and human reproduction.* Am J Clin Nutr, 2006. **83**(5): p. 993-1016.
108. Szymanski, W. and A. Kazdepka-Zieminska, *[Effect of homocysteine concentration in follicular fluid on a degree of oocyte maturity].* Ginekol Pol, 2003. **74**(10): p. 1392-6.
109. Ray, J.G. and C.A. Laskin, *Folic acid and homocyst(e)ine metabolic defects and the risk of placental abruption, pre-eclampsia and spontaneous pregnancy loss: A systematic review.* Placenta, 1999. **20**(7): p. 519-29.
110. Ray, J.G., *Folic acid food fortification in Canada.* Nutr Rev, 2004. **62**(6 Pt 2): p. S35-9.
111. Hertrampf, E. and F. Cortes, *Folic acid fortification of wheat flour: Chile.* Nutr Rev, 2004. **62**(6 Pt 2): p. S44-8; discussion S49.
112. Chen, L.T. and M.A. Rivera, *The Costa Rican experience: reduction of neural tube defects following food fortification programs.* Nutr Rev, 2004. **62**(6 Pt 2): p. S40-3.
113. Chanarin, I., D.L. Mollin, and B.B. Anderson, *Folic acid deficiency and the megaloblastic anaemias.* Proc R Soc Med, 1958. **51**(9): p. 757-63.
114. Landon, M.J. and F.E. Hytten, *The excretion of folate in pregnancy.* J Obstet Gynaecol Br Commonw, 1971. **78**(9): p. 769-75.
115. Bruinse, H.W., H. van der Berg, and A.A. Haspels, *Maternal serum folacin levels during and after normal pregnancy.* Eur J Obstet Gynecol Reprod Biol, 1985. **20**(3): p. 153-8.
116. Cikot, R.J., et al., *Longitudinal vitamin and homocysteine levels in normal pregnancy.* Br J Nutr, 2001. **85**(1): p. 49-58.
117. *Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. MRC Vitamin Study Research Group.* Lancet, 1991. **338**(8760): p. 131-7.
118. Hague, W.M., *Homocysteine and pregnancy.* Best Pract Res Clin Obstet Gynaecol, 2003. **17**(3): p. 459-69.
119. Togawa, T., et al., *Measurement of homocysteine thiolactone hydrolase activity using high-performance liquid chromatography with fluorescence detection and polymorphisms of paraoxonase in normal human serum.* J Chromatogr B Analyt Technol Biomed Life Sci, 2005. **819**(1): p. 67-72.
120. Jakubowski, H., *Molecular basis of homocysteine toxicity in humans.* Cell Mol Life Sci, 2004. **61**(4): p. 470-87.
121. Frosst, P., et al., *A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase.* Nat Genet, 1995. **10**(1): p. 111-3.

122. Boushey, C.J., et al., *A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes.* Jama, 1995. **274**(13): p. 1049-57.
123. Eskes, T.K., *Homocysteine and human reproduction.* Clin Exp Obstet Gynecol, 2000. **27**(3-4): p. 157-67.
124. Zhao, W., et al., *Neural tube defects and maternal biomarkers of folate, homocysteine, and glutathione metabolism.* Birth Defects Res A Clin Mol Teratol, 2006. **76**(4): p. 230-6.
125. Shen, L.P. and X.Q. Qiu, *[Methylenetetrahydrofolate polymorphism, folic acid, homocysteine associated with neural tube defects].* Zhonghua Liu Xing Bing Xue Za Zhi, 2004. **25**(10): p. 908-10.
126. Powers, R.W., et al., *Plasma homocysteine concentration is increased in preeclampsia and is associated with evidence of endothelial activation.* Am J Obstet Gynecol, 1998. **179**(6 Pt 1): p. 1605-11.
127. Rajkovic, A., et al., *Methylenetetrahydrofolate reductase 677 C --> T polymorphism, plasma folate, vitamin B(12) concentrations, and risk of preeclampsia among black African women from Zimbabwe.* Mol Genet Metab, 2000. **69**(1): p. 33-9.
128. Dekker, G.A., et al., *Underlying disorders associated with severe early-onset preeclampsia.* Am J Obstet Gynecol, 1995. **173**(4): p. 1042-8.
129. Lopez-Quesada, E., M.A. Vilaseca, and J.M. Lailla, *Plasma total homocysteine in uncomplicated pregnancy and in preeclampsia.* Eur J Obstet Gynecol Reprod Biol, 2003. **108**(1): p. 45-9.
130. Hague, W.M. and M. Whiting. *2nd International Congress on Homocysteine*. in *2nd International Congress on Homocysteine*. 1997. Nijmegen.
131. Budde, M.P., et al., *Risk factors for placental abruption in a socio-economically disadvantaged region.* J Matern Fetal Neonatal Med, 2007. **20**(9): p. 687-93.
132. Unfried, G., et al., *The C677T polymorphism of the methylenetetrahydrofolate reductase gene and idiopathic recurrent miscarriage.* Obstet Gynecol, 2002. **99**(4): p. 614-9.
133. Khong, T.Y. and W.M. Hague, *The placenta in maternal hyperhomocysteinaemia.* Br J Obstet Gynaecol, 1999. **106**(3): p. 273-8.
134. Vollset, S.E., et al., *Plasma total homocysteine, pregnancy complications, and adverse pregnancy outcomes: the Hordaland Homocysteine study.* Am J Clin Nutr, 2000. **71**(4): p. 962-8.

135. Wenstrom, K.D., et al., *Association of the C677T methylenetetrahydrofolate reductase mutation and elevated homocysteine levels with congenital cardiac malformations.* Am J Obstet Gynecol, 2001. **184**(5): p. 806-12; discussion 812-7.

136. Refsum, H., *Folate, vitamin B12 and homocysteine in relation to birth defects and pregnancy outcome.* Br J Nutr, 2001. **85 Suppl 2**: p. S109-13.

137. Refsum, H., et al., *Homocysteine and cardiovascular disease.* Annu Rev Med, 1998. **49**: p. 31-62.

138. Poddar, R., et al., *Homocysteine induces expression and secretion of monocyte chemoattractant protein-1 and interleukin-8 in human aortic endothelial cells: implications for vascular disease.* Circulation, 2001. **103**(22): p. 2717-23.

139. Romerio, S.C., et al., *Acute hyperhomocysteinemia decreases NO bioavailability in healthy adults.* Atherosclerosis, 2004. **176**(2): p. 337-44.

140. Stuhlinger, M.C. and O. Stanger, *Asymmetric dimethyl-L-arginine (ADMA): a possible link between homocyst(e)ine and endothelial dysfunction.* Curr Drug Metab, 2005. **6**(1): p. 3-14.

141. Chambers, J.C., et al., *Association of maternal endothelial dysfunction with preeclampsia.* Jama, 2001. **285**(12): p. 1607-12.

142. Beattie, D.K., et al., *Influence of systemic factors on pre-existing intimal hyperplasia and their effect on the outcome of infrainguinal arterial reconstruction with vein.* Br J Surg, 1999. **86**(11): p. 1441-7.

143. Davidge, S.T., *Oxidative stress and altered endothelial cell function in preeclampsia.* Semin Reprod Endocrinol, 1998. **16**(1): p. 65-73.

144. Starkebaum, G. and J.M. Harlan, *Endothelial cell injury due to copper-catalyzed hydrogen peroxide generation from homocysteine.* J Clin Invest, 1986. **77**(4): p. 1370-6.

145. Weiss, N., *Mechanisms of increased vascular oxidant stress in hyperhomocys-teinemia and its impact on endothelial function.* Curr Drug Metab, 2005. **6**(1): p. 27-36.

146. Johnson, M.R., et al., *Does endothelial cell activation occur with intrauterine growth restriction?* Bjog, 2002. **109**(7): p. 836-9.

147. Jaouen, G., *Bioorganometallics: Biomolecules, Labeling, Medicine*. 3 ed. Bioorganometallics. Vol. 527. 2006, Weinheim: Wiley-VCH.

148. Oh, R. and D.L. Brown, *Vitamin B12 deficiency.* Am Fam Physician, 2003. **67**(5): p. 979-86.

149. Woodward, J.C. and P.M. Newberne, *The pathogenesis of hydrocephalus in newborn rats deficient in vitamin B12.* J Embryol Exp Morphol, 1967. **17**(1): p. 177-87.

150. Miller, A., et al., *Vitamin B12, demyelination, remyelination and repair in multiple sclerosis.* J Neurol Sci, 2005. **233**(1-2): p. 93-7.

151. Strauss, E.W., R.M. Donaldson, Jr., and F.H. Gardner, *A relationship between intestinal bacteria and the absorption of vitamin B12 in rats with diverticula of the small bowel.* Lancet, 1961. **2**: p. 736-8.

152. Albert, M.J., V.I. Mathan, and S.J. Baker, *Vitamin B12 synthesis by human small intestinal bacteria.* Nature, 1980. **283**(5749): p. 781-2.

153. Metz, J., et al., *Biochemical indices of vitamin B12 nutrition in pregnant patients with subnormal serum vitamin B12 levels.* Am J Hematol, 1995. **48**(4): p. 251-5.

154. Pardo, J., L. Gindes, and R. Orvieto, *Cobalamin (vitamin B12) metabolism during pregnancy.* Int J Gynaecol Obstet, 2004. **84**(1): p. 77-8.

155. Koebnick, C., et al., *Longitudinal concentrations of vitamin B(12) and vitamin B(12)-binding proteins during uncomplicated pregnancy.* Clin Chem, 2002. **48**(6 Pt 1): p. 928-33.

156. Shojania, A.M., *Folic acid and vitamin B12 deficiency in pregnancy and in the neonatal period.* Clin Perinatol, 1984. **11**(2): p. 433-59.

157. Carretti, N., et al., *Pattern of vitamin B12 and folic acid during pregnancy.* Gynecol Obstet Invest, 1994. **38**(2): p. 78-81.

158. Roessner, C.A. and A.I. Scott, *Fine-tuning our knowledge of the anaerobic route to cobalamin (vitamin B12).* J Bacteriol, 2006. **188**(21): p. 7331-4.

159. Croft, M.T., et al., *Algae acquire vitamin B12 through a symbiotic relationship with bacteria.* Nature, 2005. **438**(7064): p. 90-3.

160. Loferer, M.J., H.H. Loeffler, and K.R. Liedl, *A QM-MM interface between CHARMM and TURBOMOLE: implementation and application to systems in bulk phase and biologically active systems.* J Comput Chem, 2003. **24**(10): p. 1240-9.

161. Bondevik, G.T., et al., *Homocysteine and methylmalonic acid levels in pregnant Nepali women. Should cobalamin supplementation be considered?* Eur J Clin Nutr, 2001. **55**(10): p. 856-64.

162. Koebnick, C., et al., *Long-term ovo-lacto vegetarian diet impairs vitamin B-12 status in pregnant women.* J Nutr, 2004. **134**(12): p. 3319-26.

163. Sharma, A.K., et al., *Incidence of neural tube defects of Lucknow over a 10 year period from 1982-1991.* Indian J Med Res, 1994. **99**: p. 223-6.

164. Botto, L.D., et al., *Neural-tube defects.* N Engl J Med, 1999. **341**(20): p. 1509-19.

165. Williams, L.J., et al., *Decline in the prevalence of spina bifida and anencephaly by race/ethnicity: 1995-2002.* Pediatrics, 2005. **116**(3): p. 580-6.

166. Ray, J.G., et al., *Vitamin B12 and the risk of neural tube defects in a folic-acid-fortified population.* Epidemiology, 2007. **18**(3): p. 362-6.
167. Muthayya, S., et al., *Low maternal vitamin B12 status is associated with intrauterine growth retardation in urban South Indians.* Eur J Clin Nutr, 2006. **60**(6): p. 791-801.
168. Schneede, J., et al., *Methylmalonic acid and homocysteine in plasma as indicators of functional cobalamin deficiency in infants on macrobiotic diets.* Pediatr Res, 1994. **36**(2): p. 194-201.
169. Savage, D.G., et al., *Sensitivity of serum methylmalonic acid and total homocysteine determinations for diagnosing cobalamin and folate deficiencies.* Am J Med, 1994. **96**(3): p. 239-46.
170. Kirke, P.N., et al., *Maternal plasma folate and vitamin B12 are independent risk factors for neural tube defects.* Q J Med, 1993. **86**(11): p. 703-8.
171. Murphy, M.M., et al., *Longitudinal Study of the Effect of Pregnancy on Maternal and Fetal Cobalamin Status in Healthy Women and Their Offspring.* J Nutr, 2007. **137**(8): p. 1863-1867.
172. Groenen, P.M., et al., *Marginal maternal vitamin B12 status increases the risk of offspring with spina bifida.* Am J Obstet Gynecol, 2004. **191**(1): p. 11-7.
173. Suarez, L., et al., *Maternal serum B12 levels and risk for neural tube defects in a Texas-Mexico border population.* Ann Epidemiol, 2003. **13**(2): p. 81-8.
174. Ronnenberg, A.G., et al., *Preconception homocysteine and B vitamin status and birth outcomes in Chinese women.* Am J Clin Nutr, 2002. **76**(6): p. 1385-91.
175. Lindblad, B., et al., *Folate, vitamin B12, and homocysteine levels in South Asian women with growth-retarded fetuses.* Acta Obstet Gynecol Scand, 2005. **84**(11): p. 1055-61.
176. Reznikoff-Etievant, M.F., et al., *Low Vitamin B(12) level as a risk factor for very early recurrent abortion.* Eur J Obstet Gynecol Reprod Biol, 2002. **104**(2): p. 156-9.
177. Jain, S.K., *Vitamin B6 (pyridoxamine) supplementation and complications of diabetes.* Metabolism, 2007. **56**(2): p. 168-71.
178. Leklem, J.E., *Vitamin B6. Reservoirs, receptors, and red-cell reactions.* Ann N Y Acad Sci, 1992. **669**: p. 34-41; discussion 41-3.
179. Spinneker, A., et al., *Vitamin B6 status, deficiency and its consequences--an overview.* Nutr Hosp, 2007. **22**(1): p. 7-24.
180. Ramakrishna, T., *Vitamins and brain development.* Physiol Res, 1999. **48**(3): p. 175-87.
181. Gregory, J.F., 3rd, *Nutritional Properties and significance of vitamin glycosides.* Annu Rev Nutr, 1998. **18**: p. 277-96.

182. Cotter, A.M., et al., *Elevated plasma homocysteine in early pregnancy: a risk factor for the development of severe preeclampsia.* Am J Obstet Gynecol, 2001. **185**(4): p. 781-5.
183. de Vries, J.I., et al., *Hyperhomocysteinaemia and protein S deficiency in complicated pregnancies.* Br J Obstet Gynaecol, 1997. **104**(11): p. 1248-54.
184. Middleton, H.M., 3rd, *Uptake of pyridoxine by in vivo perfused segments of rat small intestine: a possible role for intracellular vitamin metabolism.* J Nutr, 1985. **115**(8): p. 1079-88.
185. Thaver, D., M.A. Saeed, and Z.A. Bhutta, *Pyridoxine (vitamin B6) supplementation in pregnancy.* Cochrane Database Syst Rev, 2006(2): p. CD000179.
186. Chang, S.J., *Adequacy of maternal pyridoxine supplementation during pregnancy in relation to the vitamin B6 status and growth of neonates at birth.* J Nutr Sci Vitaminol (Tokyo), 1999. **45**(4): p. 449-58.
187. de la Calle, M., et al., *Homocysteine, folic acid and B-group vitamins in obstetrics and gynaecology.* Eur J Obstet Gynecol Reprod Biol, 2003. **107**(2): p. 125-34.
188. Krapels, I.P., et al., *Maternal dietary B vitamin intake, other than folate, and the association with orofacial cleft in the offspring.* Eur J Nutr, 2004. **43**(1): p. 7-14.
189. Czeizel, A.E., et al., *Oral pyridoxine during pregnancy : potential protective effect for cardiovascular malformations.* Drugs R D, 2004. **5**(5): p. 259-69.
190. Wouters, M.G., et al., *Hyperhomocysteinemia: a risk factor in women with unexplained recurrent early pregnancy loss.* Fertil Steril, 1993. **60**(5): p. 820-5.
191. Goddijn-Wessel, T.A., et al., *Hyperhomocysteinemia: a risk factor for placental abruption or infarction.* Eur J Obstet Gynecol Reprod Biol, 1996. **66**(1): p. 23-9.
192. Choi, S.W. and S. Friso, *Interactions between folate and aging for carcinogenesis.* Clin Chem Lab Med, 2005. **43**(10): p. 1151-7.
193. Bailey, L.B. and J.F. Gregory, 3rd, *Folate metabolism and requirements.* J Nutr, 1999. **129**(4): p. 779-82.
194. Appling, D.R., *Compartmentation of folate-mediated one-carbon metabolism in eukaryotes.* Faseb J, 1991. **5**(12): p. 2645-51.
195. Herbert, V. and R. Zalusky, *Interrelations of vitamin B12 and folic acid metabolism: folic acid clearance studies.* J Clin Invest, 1962. **41**: p. 1263-76.
196. Scott, J. and D. Weir, *Folate/vitamin B12 inter-relationships.* Essays Biochem, 1994. **28**: p. 63-72.
197. Lissak, A., et al., *Polymorphism for mutation of cytosine to thymine at location 677 in the methylenetetrahydrofolate reductase gene is associated with recurrent early fetal loss.* Am J Obstet Gynecol, 1999. **181**(1): p. 126-30.

198. Kang, S.S., et al., *Intermediate homocysteinemia: a thermolabile variant of methylenetetrahydrofolate reductase.* Am J Hum Genet, 1988. **43**(4): p. 414-21.

199. Fowler, B., *Homocysteine: overview of biochemistry, molecular biology, and role in disease processes.* Semin Vasc Med, 2005. **5**(2): p. 77-86.

200. Shields, D.C., et al., *The "thermolabile" variant of methylenetetrahydrofolate reductase and neural tube defects: An evaluation of genetic risk and the relative importance of the genotypes of the embryo and the mother.* Am J Hum Genet, 1999. **64**(4): p. 1045-55.

201. Sohda, S., et al., *Methylenetetrahydrofolate reductase polymorphism and pre-eclampsia.* J Med Genet, 1997. **34**(6): p. 525-6.

202. van der Put, N.M., et al., *A second common mutation in the methylenetetrahydrofolate reductase gene: an additional risk factor for neural-tube defects?* Am J Hum Genet, 1998. **62**(5): p. 1044-51.

203. Weisberg, I., et al., *A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity.* Mol Genet Metab, 1998. **64**(3): p. 169-72.

204. Rozen, R., *Genetic Variation in Folate Metabolism*, in *Folate and Human Development*, E.J. Massaro and J.M. Rogers, Editors. 2002, Humana Press Inc. p. 27-42.

205. Khong, T.Y. and W.M. Hague, *Biparental contribution to fetal thrombophilia in discordant twin intrauterine growth restriction.* Am J Obstet Gynecol, 2001. **185**(1): p. 244-5.

206. Bagley, P.J. and J. Selhub, *A common mutation in the methylenetetrahydrofolate reductase gene is associated with an accumulation of formylated tetrahydrofolates in red blood cells.* Proc Natl Acad Sci U S A, 1998. **95**(22): p. 13217-20.

207. Jacques, P.F., et al., *Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations.* Circulation, 1996. **93**(1): p. 7-9.

208. Weisberg, I.S., et al., *The 1298A-->C polymorphism in methylenetetrahydrofolate reductase (MTHFR): in vitro expression and association with homocysteine.* Atherosclerosis, 2001. **156**(2): p. 409-15.

209. Bosco, P., et al., *Methionine synthase (MTR) 2756 (A --> G) polymorphism, double heterozygosity methionine synthase 2756 AG/methionine synthase reductase (MTRR) 66 AG, and elevated homocysteinemia are three risk factors for having a child with Down syndrome.* Am J Med Genet A, 2003. **121**(3): p. 219-24.

210. Also-Rallo, E., et al., *Polymorphisms of genes involved in homocysteine metabolism in preeclampsia and in uncomplicated pregnancies.* Eur J Obstet Gynecol Reprod Biol, 2005. **120**(1): p. 45-52.

211. Leclerc, D., et al., *Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria.* Proc Natl Acad Sci U S A, 1998. **95**(6): p. 3059-64.

212. Fujii, K., J.H. Galivan, and F.M. Huennekens, *Activation of methionine synthase: further characterization of flavoprotein system.* Arch Biochem Biophys, 1977. **178**(2): p. 662-70.

213. Gaughan, D.J., et al., *The methionine synthase reductase (MTRR) A66G polymorphism is a novel genetic determinant of plasma homocysteine concentrations.* Atherosclerosis, 2001. **157**(2): p. 451-6.

214. Wilson, A., et al., *A common variant in methionine synthase reductase combined with low cobalamin (vitamin B12) increases risk for spina bifida.* Mol Genet Metab, 1999. **67**(4): p. 317-23.

215. Brilakis, E.S., et al., *Methylenetetrahydrofolate reductase (MTHFR) 677C>T and methionine synthase reductase (MTRR) 66A>G polymorphisms: association with serum homocysteine and angiographic coronary artery disease in the era of flour products fortified with folic acid.* Atherosclerosis, 2003. **168**(2): p. 315-22.

216. Zhu, H., et al., *Homocysteine remethylation enzyme polymorphisms and increased risks for neural tube defects.* Mol Genet Metab, 2003. **78**(3): p. 216-21.

217. Gueant-Rodriguez, R.M., et al., *Transcobalamin and methionine synthase reductase mutated polymorphisms aggravate the risk of neural tube defects in humans.* Neurosci Lett, 2003. **344**(3): p. 189-92.

218. Ananth, C.V., et al., *Polymorphisms in methionine synthase reductase and betaine-homocysteine S-methyltransferase genes: Risk of placental abruption.* Mol Genet Metab, 2007. **91**(1): p. 104-10.

219. O'Leary, V.B., et al., *MTRR and MTHFR polymorphism: link to Down syndrome?* Am J Med Genet, 2002. **107**(2): p. 151-5.

220. Eskes, T.K., *Abnormal folate metabolism in mothers with Down syndrome offspring: review of the literature.* Eur J Obstet Gynecol Reprod Biol, 2006. **124**(2): p. 130-3.

221. Doolin, M.T., et al., *Maternal genetic effects, exerted by genes involved in homocysteine remethylation, influence the risk of spina bifida.* Am J Hum Genet, 2002. **71**(5): p. 1222-6.

222. Tan, L.U., E.J. Drury, and R.E. MacKenzie, *Methylenetetrahydrofolate dehydrogenase-methenyltetrahydrofolate cyclohydrolase-formyltetrahydrofolate*

synthetase. A multifunctional protein from porcine liver. J Biol Chem, 1977. **252**(3): p. 1117-22.

223. Parle-McDermott, A., et al., *MTHFD1 R653Q polymorphism is a maternal genetic risk factor for severe abruptio placentae.* Am J Med Genet A, 2005. **132**(4): p. 365-8.
224. Brody, L.C., et al., *A polymorphism, R653Q, in the trifunctional enzyme methylenetetrahydrofolate dehydrogenase/methenyltetrahydrofolate cyclohydrolase/formyltetrahydrofolate synthetase is a maternal genetic risk factor for neural tube defects: report of the Birth Defects Research Group.* Am J Hum Genet, 2002. **71**(5): p. 1207-15.
225. Parle-McDermott, A., et al., *Confirmation of the R653Q polymorphism of the trifunctional C1-synthase enzyme as a maternal risk for neural tube defects in the Irish population.* Eur J Hum Genet, 2006. **14**(6): p. 768-72.
226. Hol, F.A., et al., *Molecular genetic analysis of the gene encoding the trifunctional enzyme MTHFD (methylenetetrahydrofolate-dehydrogenase, methenyltetrahydrofolate-cyclohydrolase, formyltetrahydrofolate synthetase) in patients with neural tube defects.* Clin Genet, 1998. **53**(2): p. 119-25.
227. Swarthmore, *The human placenta: an overview and some highlights*.
228. Cruz Suarez, R., M. Gustafsson, and K. Mrabit, *IAEA Occupational Radiation Protection Programme.* Radiat Prot Dosimetry, 2001. **96**(1-3): p. 17-20.
229. Fenech, M., *Nutritional treatment of genome instability: a paradigm shift in disease prevention and in the setting of recommended dietary allowances.* Nutrition Research Reviews, 2003. **16**: p. 109-122.
230. Crott, J.W., et al., *The effect of folic acid deficiency and MTHFR C677T polymorphism on chromosome damage in human lymphocytes in vitro.* Cancer Epidemiol Biomarkers Prev, 2001. **10**(10): p. 1089-96.
231. Fenech, M., C. Aitken, and J. Rinaldi, *Folate, vitamin B12, homocysteine status and DNA damage in young Australian adults.* Carcinogenesis, 1998. **19**(7): p. 1163-71.
232. Das, K.C. and V. Herbert, *In vitro DNA synthesis by megaloblastic bone marrow: effect of folates and cobalamins on thymidine incorporation and de novo thymidylate synthesis.* Am J Hematol, 1989. **31**(1): p. 11-20.
233. Wickramasinghe, S.N. and S. Fida, *Bone marrow cells from vitamin B12- and folate-deficient patients misincorporate uracil into DNA.* Blood, 1994. **83**(6): p. 1656-61.
234. Blount, B.C., et al., *Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: implications for cancer and neuronal damage.* Proc Natl Acad Sci U S A, 1997. **94**(7): p. 3290-5.

235. Dianov, G.L., et al., *Repair of uracil residues closely spaced on the opposite strands of plasmid DNA results in double-strand break and deletion formation.* Mol Gen Genet, 1991. **225**(3): p. 448-52.
236. Chern, C.L., et al., *Folate deficiency-induced oxidative stress and apoptosis are mediated via homocysteine-dependent overproduction of hydrogen peroxide and enhanced activation of NF-kappaB in human Hep G2 cells.* Biomed Pharmacother, 2001. **55**(8): p. 434-42.
237. Kitchin, K.T. and S. Ahmad, *Oxidative stress as a possible mode of action for arsenic carcinogenesis.* Toxicol Lett, 2003. **137**(1-2): p. 3-13.
238. Halliwell, B., *Oxidative stress and cancer: have we moved forward?* Biochem J, 2007. **401**(1): p. 1-11.
239. Matxain, J.M., et al., *Theoretical Study of the Reaction of Vitamin B(6) with (1)O(2).* Chemistry, 2007.
240. Jauniaux, E., L. Poston, and G.J. Burton, *Placental-related diseases of pregnancy: Involvement of oxidative stress and implications in human evolution.* Hum Reprod Update, 2006. **12**(6): p. 747-55.
241. Hung, T.H. and G.J. Burton, *Hypoxia and reoxygenation: a possible mechanism for placental oxidative stress in preeclampsia.* Taiwan J Obstet Gynecol, 2006. **45**(3): p. 189-200.
242. Sankaralingam, S., et al., *Preeclampsia: current understanding of the molecular basis of vascular dysfunction.* Expert Rev Mol Med, 2006. **8**(3): p. 1-20.
243. Bainbridge, S.A., et al., *Carbon monoxide inhibits hypoxia/reoxygenation-induced apoptosis and secondary necrosis in syncytiotrophoblast.* Am J Pathol, 2006. **169**(3): p. 774-83.
244. Buemi, M., et al., *Effects of homocysteine on proliferation, necrosis, and apoptosis of vascular smooth muscle cells in culture and influence of folic acid.* Thromb Res, 2001. **104**(3): p. 207-13.
245. Ebisch, I.M., et al., *The importance of folate, zinc and antioxidants in the pathogenesis and prevention of subfertility.* Hum Reprod Update, 2007. **13**(2): p. 163-74.
246. Steegers-Theunissen, R.P., et al., *Folate affects apoptosis in human trophoblastic cells.* Bjog, 2000. **107**(12): p. 1513-5.
247. James, S.J., A.G. Basnakian, and B.J. Miller, *In vitro folate deficiency induces deoxynucleotide pool imbalance, apoptosis, and mutagenesis in Chinese hamster ovary cells.* Cancer Res, 1994. **54**(19): p. 5075-80.
248. Courtemanche, C., et al., *Folate deficiency inhibits the proliferation of primary human CD8+ T lymphocytes in vitro.* J Immunol, 2004. **173**(5): p. 3186-92.

249. Koury, M.J. and P. Ponka, *New insights into erythropoiesis: the roles of folate, vitamin B12, and iron.* Annu Rev Nutr, 2004. **24**: p. 105-31.

250. Bagislar, S., et al., *Extremely skewed X-chromosome inactivation patterns in women with recurrent spontaneous abortion.* Aust N Z J Obstet Gynaecol, 2006. **46**(5): p. 384-7.

251. Tsukishiro, S., et al., *Paternal uniparental disomy of chromosome 14 and unique exchange of chromosome 7 in cases of spontaneous abortion.* J Hum Genet, 2005. **50**(3): p. 112-7.

252. Rives, N., et al., *Assessment of sex chromosome aneuploidy in sperm nuclei from 47,XXY and 46,XY/47,XXY males: comparison with fertile and infertile males with normal karyotype.* Mol Hum Reprod, 2000. **6**(2): p. 107-12.

253. Al-Hassan, S., et al., *Sperm chromosomal abnormalities in patients with unexplained recurrent abortions.* Arch Androl, 2005. **51**(1): p. 69-76.

254. Azim, M., et al., *Chromosomal abnormalities as a cause of recurrent abortions: a hospital experience.* J Pak Med Assoc, 2003. **53**(3): p. 117-9.

255. Fenech, M., *The in vitro micronucleus technique.* Mutat Res, 2000. **455**(1-2): p. 81-95.

256. Trkova, M., et al., *Increased micronuclei frequencies in couples with reproductive failure.* Reprod Toxicol, 2000. **14**(4): p. 331-5.

257. Levario-Carrillo, M., et al., *Micronucleus frequency in human umbilical cord lymphocytes.* Mutat Res, 2005. **586**(1): p. 68-75.

258. Aitken, R.J., P. Koopman, and S.E. Lewis, *Seeds of concern.* Nature, 2004. **432**(7013): p. 48-52.

259. Barroso, G., M. Morshedi, and S. Oehninger, *Analysis of DNA fragmentation, plasma membrane translocation of phosphatidylserine and oxidative stress in human spermatozoa.* Hum Reprod, 2000. **15**(6): p. 1338-44.

260. Aitken, R.J. and G.N. De Iuliis, *Origins and consequences of DNA damage in male germ cells.* Reprod Biomed Online, 2007. **14**(6): p. 727-33.

261. Wong, W.Y., et al., *The impact of calcium, magnesium, zinc, and copper in blood and seminal plasma on semen parameters in men.* Reprod Toxicol, 2001. **15**(2): p. 131-6.

262. Razin, A. and A.D. Riggs, *DNA methylation and gene function.* Science, 1980. **210**(4470): p. 604-10.

263. Robertson, K.D. and A.P. Wolffe, *DNA methylation in health and disease.* Nat Rev Genet, 2000. **1**(1): p. 11-9.

264. Klimasauskas, S., et al., *HhaI methyltransferase flips its target base out of the DNA helix.* Cell, 1994. **76**(2): p. 357-69.

265. Waterland, R.A., *Assessing the effects of high methionine intake on DNA methylation.* J Nutr, 2006. **136**(6 Suppl): p. 1706S-1710S.
266. Jaenisch, R. and A. Bird, *Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals.* Nat Genet, 2003. **33 Suppl**: p. 245-54.
267. Laird, P.W., *The power and the promise of DNA methylation markers.* Nat Rev Cancer, 2003. **3**(4): p. 253-66.
268. Razin, S.V., et al., *Non-clonability correlates with genomic instability: a case study of a unique DNA region.* J Mol Biol, 2001. **307**(2): p. 481-6.
269. Kim, Y.I., et al., *Folate deficiency in rats induces DNA strand breaks and hypomethylation within the p53 tumor suppressor gene.* Am J Clin Nutr, 1997. **65**(1): p. 46-52.
270. Suzuki, T., M. Fujii, and D. Ayusawa, *Demethylation of classical satellite 2 and 3 DNA with chromosomal instability in senescent human fibroblasts.* Exp Gerontol, 2002. **37**(8-9): p. 1005-14.
271. van Vliet, J., N.A. Oates, and E. Whitelaw, *Epigenetic mechanisms in the context of complex diseases.* Cell Mol Life Sci, 2007.
272. Henikoff, S., *Heterochromatin function in complex genomes.* Biochim Biophys Acta, 2000. **1470**(1): p. O1-8.
273. Razin, A., *CpG methylation, chromatin structure and gene silencing-a three-way connection.* Embo J, 1998. **17**(17): p. 4905-8.
274. Walsh, C.P., J.R. Chaillet, and T.H. Bestor, *Transcription of IAP endogenous retroviruses is constrained by cytosine methylation.* Nat Genet, 1998. **20**(2): p. 116-7.
275. Li, E., *Chromatin modification and epigenetic reprogramming in mammalian development.* Nat Rev Genet, 2002. **3**(9): p. 662-73.
276. Jones, P.A., *Overview of cancer epigenetics.* Semin Hematol, 2005. **42**(3 Suppl 2): p. S3-8.
277. Reik, W., F. Santos, and W. Dean, *Mammalian epigenomics: reprogramming the genome for development and therapy.* Theriogenology, 2003. **59**(1): p. 21-32.
278. Jacob, R.A., et al., *Moderate folate depletion increases plasma homocysteine and decreases lymphocyte DNA methylation in postmenopausal women.* J Nutr, 1998. **128**(7): p. 1204-12.
279. Rampersaud, G.C., et al., *Genomic DNA methylation decreases in response to moderate folate depletion in elderly women.* Am J Clin Nutr, 2000. **72**(4): p. 998-1003.
280. Friso, S. and S.W. Choi, *Gene-nutrient interactions in one-carbon metabolism.* Curr Drug Metab, 2005. **6**(1): p. 37-46.

281. Duerre, J.A. and M. Briske-Anderson, *Effect of adenosine metabolites on methyltransferase reactions in isolated rat livers.* Biochim Biophys Acta, 1981. **678**(2): p. 275-82.

282. Friso, S., et al., *A common mutation in the 5,10-methylenetetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status.* Proc Natl Acad Sci U S A, 2002. **99**(8): p. 5606-11.

283. Waterland, R.A. and R.L. Jirtle, *Transposable elements: targets for early nutritional effects on epigenetic gene regulation.* Mol Cell Biol, 2003. **23**(15): p. 5293-300.

284. Fenech, M., *Genome health nutrigenomics and nutrigenetics - diagnosis and nutritional treatment of genome damage on an individual basis.* Food Chem Toxicol, 2007.

285. Sankoff, D., et al., *Chromosomal distributions of breakpoints in cancer, infertility, and evolution.* Theor Popul Biol, 2002. **61**(4): p. 497-501.

286. Narula, A., et al., *Smad4 overexpression causes germ cell ablation and leydig cell hyperplasia in transgenic mice.* Am J Pathol, 2002. **161**(5): p. 1723-34.

287. Hsia, K.T., et al., *DNA repair gene Ercc1 is essential for normal spermatogenesis and oogenesis and for functional integrity of germ cell DNA in the mouse.* Development, 2003. **130**(2): p. 369-78.

288. Liu, L., et al., *An essential role for functional telomeres in mouse germ cells during fertilization and early development.* Dev Biol, 2002. **249**(1): p. 74-84.

289. Vinson, R.K. and B.F. Hales, *DNA repair during organogenesis.* Mutat Res, 2002. **509**(1-2): p. 79-91.

290. Aitken, R.J., N.E. Skakkebaek, and S.D. Roman, *Male reproductive health and the environment.* Med J Aust, 2006. **185**(8): p. 414-5.

291. Agarwal, A., S. Gupta, and R. Sharma, *Oxidative stress and its implications in female infertility - a clinician's perspective.* Reprod Biomed Online, 2005. **11**(5): p. 641-50.

292. Kapiszewska, M., et al., *Uracil misincorporation into DNA of leukocytes of young women with positive folate balance depends on plasma vitamin B12 concentrations and methylenetetrahydrofolate reductase polymorphisms. A pilot study.* J Nutr Biochem, 2005. **16**(8): p. 467-78.

293. Ishikawa, H., et al., *A polymorphism of the methionine synthase reductase gene increases chromosomal damage in peripheral lymphocytes in smokers.* Mutat Res, 2006. **599**(1-2): p. 135-43.

294. Botto, N., et al., *Genetic polymorphisms in folate and homocysteine metabolism as risk factors for DNA damage.* Eur J Hum Genet, 2003. **11**(9): p. 671-8.

295. Gupta, S., et al., *The role of oxidative stress in spontaneous abortion and recurrent pregnancy loss: a systematic review.* Obstet Gynecol Surv, 2007. **62**(5): p. 335-47; quiz 353-4.
296. Paoloni-Giacobino, A., *Epigenetics in reproductive medicine.* Pediatr Res, 2007. **61**(5 Pt 2): p. 51R-57R.
297. Thaler, C.D. and D. Epel, *Nitric oxide in oocyte maturation, ovulation, fertilization, cleavage and implantation: a little dab'll do ya.* Curr Pharm Des, 2003. **9**(5): p. 399-409.
298. Lucock, M., *Is folic acid the ultimate functional food component for disease prevention?* Bmj, 2004. **328**(7433): p. 211-4.
299. Calhaz-Jorge, C., et al., *Peritoneal fluid concentrations of interleukin-8 in patients with endometriosis depend on the severity of the disorder and are higher in the luteal phase.* Hum Reprod, 2003. **18**(3): p. 593-7.
300. Gmyrek, G.B., et al., *Evaluation of monocyte chemotactic protein-1 levels in peripheral blood of infertile women with endometriosis.* Eur J Obstet Gynecol Reprod Biol, 2005. **122**(2): p. 199-205.
301. Pinto, X., et al., *Homocysteine and the MTHFR 677C-->T allele in premature coronary artery disease. Case control and family studies.* Eur J Clin Invest, 2001. **31**(1): p. 24-30.
302. Feix, A., et al., *Increased prevalence of combined MTR and MTHFR genotypes among individuals with severely elevated total homocysteine plasma levels.* Am J Kidney Dis, 2001. **38**(5): p. 956-64.
303. Picerno, I., et al., *Homocysteine induces DNA damage and alterations in proliferative capacity of T-lymphocytes: a model for immunosenescence?* Biogerontology, 2007. **8**(2): p. 111-9.
304. Makedos, G., et al., *Homocysteine, folic acid and B12 serum levels in pregnancy complicated with preeclampsia.* Arch Gynecol Obstet, 2006.
305. Cotter, A.M., et al., *Elevated plasma homocysteine in early pregnancy: a risk factor for the development of nonsevere preeclampsia.* Am J Obstet Gynecol, 2003. **189**(2): p. 391-4; discussion 394-6.
306. Steegers-Theunissen, R.P., et al., *Hyperhomocysteinaemia and recurrent spontaneous abortion or abruptio placentae.* Lancet, 1992. **339**(8801): p. 1122-3.
307. Daly, S., et al., *Homocysteine and folic acid: implications for pregnancy.* Semin Vasc Med, 2005. **5**(2): p. 190-200.
308. Srb, V. and V. Puza, *[Methods for the analysis of human chromosome aberrations (author's transl)].* Cas Lek Cesk, 1975. **114**(8): p. 231-6.

309. Agency, I.A.E., *Biological dosimetry: chromosomal aberrations analysis for dose assessment, TRS-405*. 2001, International Atomic Energy Agency.

310. Albertini, R.J., et al., *IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans. International Programme on Chemical Safety.* Mutat Res, 2000. **463**(2): p. 111-72.

311. Fenech, M., et al., *HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures.* Mutat Res, 2003. **534**(1-2): p. 65-75.

312. Bonassi, S., et al., *HUman MicroNucleus project: international database comparison for results with the cytokinesis-block micronucleus assay in human lymphocytes: I. Effect of laboratory protocol, scoring criteria, and host factors on the frequency of micronuclei.* Environ Mol Mutagen, 2001. **37**(1): p. 31-45.

313. Bonassi, S., et al., *Chromosomal aberrations in lymphocytes predict human cancer independently of exposure to carcinogens. European Study Group on Cytogenetic Biomarkers and Health.* Cancer Res, 2000. **60**(6): p. 1619-25.

314. Bonassi, S., et al., *Human population studies with cytogenetic biomarkers: review of the literature and future prospectives.* Environ Mol Mutagen, 2005. **45**(2-3): p. 258-70.

315. Rossner, P., et al., *Chromosomal aberrations in lymphocytes of healthy subjects and risk of cancer.* Environ Health Perspect, 2005. **113**(5): p. 517-20.

316. Migliore, L., et al., *Preferential occurrence of chromosome 21 malsegregation in peripheral blood lymphocytes of Alzheimer disease patients.* Cytogenet Cell Genet, 1999. **87**(1-2): p. 41-6.

317. Migliore, L., et al., *Chromosome and oxidative damage biomarkers in lymphocytes of Parkinson's disease patients.* Int J Hyg Environ Health, 2001. **204**(1): p. 61-6.

318. Fenech, M. and A.A. Morley, *Cytokinesis-block micronucleus method in human lymphocytes: effect of in vivo ageing and low dose X-irradiation.* Mutat Res, 1986. **161**(2): p. 193-8.

319. Fenech, M., *Chromosomal biomarkers of genomic instability relevant to cancer.* Drug Discov Today, 2002. **7**(22): p. 1128-37.

320. Zhou, Y., et al., *Preeclampsia is associated with abnormal expression of adhesion molecules by invasive cytotrophoblasts.* J Clin Invest, 1993. **91**(3): p. 950-60.

321. DiFederico, E., O. Genbacev, and S.J. Fisher, *Preeclampsia is associated with widespread apoptosis of placental cytotrophoblasts within the uterine wall.* Am J Pathol, 1999. **155**(1): p. 293-301.

322. Feinberg, R.F., H.J. Kliman, and A.W. Cohen, *Preeclampsia, trisomy 13, and the placental bed.* Obstet Gynecol, 1991. **78**(3 Pt 2): p. 505-8.
323. Adamsons, K. and R.C. Wallach, *Single cause for initiation of labor and toxemia: a hypothesis.* Am J Perinatol, 1989. **6**(2): p. 133-7.
324. Cockell, A.P. and L. Poston, *Flow-mediated vasodilatation is enhanced in normal pregnancy but reduced in preeclampsia.* Hypertension, 1997. **30**(2 Pt 1): p. 247-51.
325. Elliott, R. and T.J. Ong, *Nutritional genomics.* Bmj, 2002. **324**(7351): p. 1438-42.
326. Guttenbach, M. and M. Schmid, *Exclusion of specific human chromosomes into micronuclei by 5-azacytidine treatment of lymphocyte cultures.* Exp Cell Res, 1994. **211**(1): p. 127-32.
327. Nurk, E., et al., *Associations between maternal methylenetetrahydrofolate reductase polymorphisms and adverse outcomes of pregnancy: the Hordaland Homocysteine Study.* Am J Med, 2004. **117**(1): p. 26-31.
328. Institute, P., *Birthweight centile calculator*, Perinatal Institute.
329. Roberts, C.L. and P.A. Lancaster, *Australian national birthweight percentiles by gestational age.* Med J Aust, 1999. **170**(3): p. 114-8.
330. Huppertz, B., et al., *Placental morphology: from molecule to mother -- a dedication to Peter Kaufmann -- a review.* Placenta, 2006. **27 Suppl A**: p. S3-8.
331. Damsky, C.H., et al., *Integrin switching regulates normal trophoblast invasion.* Development, 1994. **120**(12): p. 3657-66.
332. Brosens, I.A., W.B. Robertson, and H.G. Dixon, *The role of the spiral arteries in the pathogenesis of preeclampsia.* Obstet Gynecol Annu, 1972. **1**: p. 177-91.
333. Fenech, M., *The Genome Health Clinic and Genome Health Nutrigenomics concepts: diagnosis and nutritional treatment of genome and epigenome damage on an individual basis.* Mutagenesis, 2005. **20**(4): p. 255-69.
334. Hustin, J., J.M. Foidart, and R. Lambotte, *Maternal vascular lesions in pre-eclampsia and intrauterine growth retardation: light microscopy and immunofluorescence.* Placenta, 1983. **4 Spec No**: p. 489-98.
335. Sargent, I.L., A.M. Borzychowski, and C.W. Redman, *Immunoregulation in normal pregnancy and pre-eclampsia: an overview.* Reprod Biomed Online, 2006. **13**(5): p. 680-6.
336. Branch, D.W., et al., *The association of antiphospholipid antibodies with severe preeclampsia.* Obstet Gynecol, 1989. **73**(4): p. 541-5.
337. Stark, J.M., *Pre-eclampsia and cytokine induced oxidative stress.* Br J Obstet Gynaecol, 1993. **100**(2): p. 105-9.

338. Sharma, J.B., et al., *Oxidative stress markers and antioxidant levels in normal pregnancy and pre-eclampsia.* Int J Gynaecol Obstet, 2006. **94**(1): p. 23-7.
339. Sizonenko, S.V., et al., *Impact of intrauterine growth restriction and glucocorticoids on brain development: insights using advanced magnetic resonance imaging.* Mol Cell Endocrinol, 2006. **254-255**: p. 163-71.
340. Prada, J.A. and R.C. Tsang, *Biological mechanisms of environmentally induced causes of IUGR.* Eur J Clin Nutr, 1998. **52 Suppl 1**: p. S21-7; discussion S27-8.
341. Kleijer, M.E., G.A. Dekker, and A.R. Heard, *Risk factors for intrauterine growth restriction in a socio-economically disadvantaged region.* J Matern Fetal Neonatal Med, 2005. **18**(1): p. 23-30.
342. Di Renzo, G.C., et al., *Potential effects of nutrients on placental function and fetal growth.* Nestle Nutr Workshop Ser Pediatr Program, 2005(55): p. 73-81; discussion 81-2.
343. Fenech, M., *Cytokinesis-block micronucleus assay evolves into a "cytome" assay of chromosomal instability, mitotic dysfunction and cell death.* Mutat Res, 2006. **600**(1-2): p. 58-66.
344. Kassie, F., et al., *Khat (Catha edulis) consumption causes genotoxic effects in humans.* Int J Cancer, 2001. **92**(3): p. 329-32.
345. IAEA, *Manual in biodosimetry, TRS-405*. 2001, International Atomic Energy Agency.
346. El-Zein, R.A., et al., *Cytokinesis-blocked micronucleus assay as a novel biomarker for lung cancer risk.* Cancer Res, 2006. **66**(12): p. 6449-56.
347. Eastmond, D.A. and J.D. Tucker, *Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody.* Environ Mol Mutagen, 1989. **13**(1): p. 34-43.
348. Shimizu, N., et al., *Selective entrapment of extrachromosomally amplified DNA by nuclear budding and micronucleation during S phase.* J Cell Biol, 1998. **140**(6): p. 1307-20.
349. Shimizu, N., T. Shimura, and T. Tanaka, *Selective elimination of acentric double minutes from cancer cells through the extrusion of micronuclei.* Mutat Res, 2000. **448**(1): p. 81-90.
350. Leeners, B., et al., *Smoking and the risk of developing hypertensive diseases in pregnancy: what is the effect on HELLP syndrome?* Acta Obstet Gynecol Scand, 2006. **85**(10): p. 1217-24.
351. Delpisheh, A., et al., *Socio-economic status, smoking during pregnancy and birth outcomes: an analysis of cross-sectional community studies in Liverpool (1993-2001).* J Child Health Care, 2006. **10**(2): p. 140-8.

352. Dekker, G. and B. Sibai, *Primary, secondary, and tertiary prevention of pre-eclampsia.* Lancet, 2001. **357**(9251): p. 209-15.

353. O'Brien, T.E., J.G. Ray, and W.S. Chan, *Maternal body mass index and the risk of preeclampsia: a systematic overview.* Epidemiology, 2003. **14**(3): p. 368-74.

354. Neggers, Y. and R.L. Goldenberg, *Some thoughts on body mass index, micronutrient intakes and pregnancy outcome.* J Nutr, 2003. **133**(5 Suppl 2): p. 1737S-1740S.

355. Micheletti, F., et al., *The lifespan of major histocompatibility complex class I/peptide complexes determines the efficiency of cytotoxic T-lymphocyte responses.* Immunology, 1999. **96**(3): p. 411-5.

356. Huber, R., H. Braselmann, and M. Bauchinger, *Intra- and inter-individual variation of background and radiation-induced micronucleus frequencies in human lymphocytes.* Int J Radiat Biol, 1992. **61**(5): p. 655-61.

357. Migliore, L., et al., *Micronucleated lymphocytes in people occupationally exposed to potential environmental contaminants: the age effect.* Mutat Res, 1991. **256**(1): p. 13-20.

358. Di Giorgio, C., et al., *The micronucleus assay in human lymphocytes: screening for inter-individual variability and application to biomonitoring.* Carcinogenesis, 1994. **15**(2): p. 313-7.

359. Fenech, M., *Important variables that influence base-line micronucleus frequency in cytokinesis-blocked lymphocytes-a biomarker for DNA damage in human populations.* Mutat Res, 1998. **404**(1-2): p. 155-65.

360. Milosevic-Ethordevic, O., et al., *Effect of various doses of gestogens on micronuclei frequency in human peripheral blood lymphocytes of pregnant women.* Hum Reprod, 2003. **18**(2): p. 433-6.

361. Fenech, M., S. Neville, and J. Rinaldi, *Sex is an important variable affecting spontaneous micronucleus frequency in cytokinesis-blocked lymphocytes.* Mutat Res, 1994. **313**(2-3): p. 203-7.

362. Hando, J.C., J. Nath, and J.D. Tucker, *Sex chromosomes, micronuclei and aging in women.* Chromosoma, 1994. **103**(3): p. 186-92.

363. Norppa, H. and G.C. Falck, *What do human micronuclei contain?* Mutagenesis, 2003. **18**(3): p. 221-33.

364. Lindberg, H.K., et al., *Origin of nuclear buds and micronuclei in normal and folate-deprived human lymphocytes.* Mutat Res, 2007. **617**(1-2): p. 33-45.

365. Hochwagen, A. and A. Amon, *Checking your breaks: surveillance mechanisms of meiotic recombination.* Curr Biol, 2006. **16**(6): p. R217-28.

366. Fenech, M., et al., *Low intake of calcium, folate, nicotinic acid, vitamin E, retinol, beta-carotene and high intake of pantothenic acid, biotin and riboflavin are significantly associated with increased genome instability--results from a dietary intake and micronucleus index survey in South Australia.* Carcinogenesis, 2005. **26**(5): p. 991-9.
367. Thomas, P., K. Umegaki, and M. Fenech, *Nucleoplasmic bridges are a sensitive measure of chromosome rearrangement in the cytokinesis-block micronucleus assay.* Mutagenesis, 2003. **18**(2): p. 187-94.
368. Rajagopalan, H. and C. Lengauer, *Aneuploidy and cancer.* Nature, 2004. **432**(7015): p. 338-41.
369. Bianca, S., et al., *Multiple aneuploidy recurrence risk.* Am J Med Genet A, 2006. **140**(17): p. 1888-9; author reply 1890-1.
370. Scala, I., et al., *Analysis of seven maternal polymorphisms of genes involved in homocysteine/folate metabolism and risk of Down syndrome offspring.* Genet Med, 2006. **8**(7): p. 409-16.
371. Fenech, M. and J.W. Crott, *Micronuclei, nucleoplasmic bridges and nuclear buds induced in folic acid deficient human lymphocytes-evidence for breakage-fusion-bridge cycles in the cytokinesis-block micronucleus assay.* Mutat Res, 2002. **504**(1-2): p. 131-6.
372. Fenech, M., *Recommended dietary allowances (RDAs) for genomic stability.* Mutat Res, 2001. **480-481**: p. 51-4.
373. Depeint, F., et al., *Mitochondrial function and toxicity: role of B vitamins on the one-carbon transfer pathways.* Chem Biol Interact, 2006. **163**(1-2): p. 113-32.
374. Burdge, G.C., *Homocysteine: a role in fetal programming?* Br J Nutr, 2006. **96**(3): p. 415-7.
375. Jacobs, P., L. Wood, and R. Bick, *Homocysteine in vascular disease: an emerging clinical perspective.* Cardiovasc J S Afr, 2006. **17**(3): p. 135-9.
376. Blom, H.J., et al., *Neural tube defects and folate: case far from closed.* Nat Rev Neurosci, 2006. **7**(9): p. 724-31.
377. Di Simone, N., et al., *Effect of folic acid on homocysteine-induced trophoblast apoptosis.* Mol Hum Reprod, 2004. **10**(9): p. 665-9.
378. Kamudhamas, A., et al., *Homocysteine thiolactone induces apoptosis in cultured human trophoblasts: a mechanism for homocysteine-mediated placental dysfunction?* Am J Obstet Gynecol, 2004. **191**(2): p. 563-71.

379. Guerra-Shinohara, E.M., et al., *Relationship between total homocysteine and folate levels in pregnant women and their newborn babies according to maternal serum levels of vitamin B12.* Bjog, 2002. **109**(7): p. 784-91.
380. Stroes, E.S., et al., *Folic acid reverts dysfunction of endothelial nitric oxide synthase.* Circ Res, 2000. **86**(11): p. 1129-34.
381. Di Simone, N., et al., *Homocysteine induces trophoblast cell death with apoptotic features.* Biol Reprod, 2003. **69**(4): p. 1129-34.
382. *Lowering blood homocysteine with folic acid based supplements: meta-analysis of randomised trials. Homocysteine Lowering Trialists' Collaboration.* Bmj, 1998. **316**(7135): p. 894-8.
383. McPartlin, J., et al., *Accelerated folate breakdown in pregnancy.* Lancet, 1993. **341**(8838): p. 148-9.
384. Murphy, M.M., et al., *The pregnancy-related decrease in fasting plasma homocysteine is not explained by folic acid supplementation, hemodilution, or a decrease in albumin in a longitudinal study.* Am J Clin Nutr, 2002. **76**(3): p. 614-9.
385. Brosnan, J.T. and M.E. Brosnan, *The sulfur-containing amino acids: an overview.* J Nutr, 2006. **136**(6 Suppl): p. 1636S-1640S.
386. Mount, J.N., et al., *Adaptation of coenzyme stimulation assays for the nutritional assessment of vitamins B1, B2 and B6 using the Cobas Bio centrifugal analyser.* Ann Clin Biochem, 1987. **24 (Pt 1)**: p. 41-6.
387. NHMRC, *Nutrient Reference Values for Australia and New Zealand Including Recommended Dietary Intakes*. 2006, nhmrc.publications@nhmrc.gov.au.
388. Mignini, L.E., et al., *Mapping the theories of preeclampsia: the role of homocysteine.* Obstet Gynecol, 2005. **105**(2): p. 411-25.
389. Sorensen, T.K., et al., *Elevated second-trimester serum homocyst(e)ine levels and subsequent risk of preeclampsia.* Gynecol Obstet Invest, 1999. **48**(2): p. 98-103.
390. Hogg, B.B., et al., *Second-trimester plasma homocysteine levels and pregnancy-induced hypertension, preeclampsia, and intrauterine growth restriction.* Am J Obstet Gynecol, 2000. **183**(4): p. 805-9.
391. Hietala, R., U. Turpeinen, and T. Laatikainen, *Serum homocysteine at 16 weeks and subsequent preeclampsia.* Obstet Gynecol, 2001. **97**(4): p. 527-9.
392. Zeeman, G.G., et al., *Homocysteine plasma concentration levels for the prediction of preeclampsia in women with chronic hypertension.* Am J Obstet Gynecol, 2003. **189**(2): p. 574-6.

393. Holmes, V.A., et al., *Homocysteine is lower in the third trimester of pregnancy in women with enhanced folate status from continued folic acid supplementation.* Clin Chem, 2005. **51**(3): p. 629-34.

394. Baccarelli, A., et al., *Air pollution, smoking, and plasma homocysteine.* Environ Health Perspect, 2007. **115**(2): p. 176-81.

395. van Wersch, J.W., Y. Janssens, and J.A. Zandvoort, *Folic acid, Vitamin B(12), and homocysteine in smoking and non-smoking pregnant women.* Eur J Obstet Gynecol Reprod Biol, 2002. **103**(1): p. 18-21.

396. Uysal, O., E. Arikan, and B. Cakir, *Plasma total homocysteine level and its association with carotid intima-media thickness in obesity.* J Endocrinol Invest, 2005. **28**(10): p. 928-34.

397. Fonseca, V.A., L.M. Fink, and P.A. Kern, *Insulin sensitivity and plasma homocysteine concentrations in non-diabetic obese and normal weight subjects.* Atherosclerosis, 2003. **167**(1): p. 105-9.

398. Konukoglu, D., et al., *Plasma homocysteine levels in obese and non-obese subjects with or without hypertension; its relationship with oxidative stress and copper.* Clin Biochem, 2003. **36**(5): p. 405-8.

399. El-Khairy, L., et al., *Lifestyle and cardiovascular disease risk factors as determinants of total cysteine in plasma: the Hordaland Homocysteine Study.* Am J Clin Nutr, 1999. **70**(6): p. 1016-24.

400. McDonald, S.D., et al., *Folate levels in pregnant women who smoke: an important gene/environment interaction.* Am J Obstet Gynecol, 2002. **187**(3): p. 620-5.

401. Cogswell, M.E., P. Weisberg, and C. Spong, *Cigarette smoking, alcohol use and adverse pregnancy outcomes: implications for micronutrient supplementation.* J Nutr, 2003. **133**(5 Suppl 2): p. 1722S-1731S.

402. Mannino, D.M., et al., *Tobacco smoke exposure and decreased serum and red blood cell folate levels: data from the Third National Health and Nutrition Examination Survey.* Nicotine Tob Res, 2003. **5**(3): p. 357-62.

403. Alberg, A., *The influence of cigarette smoking on circulating concentrations of antioxidant micronutrients.* Toxicology, 2002. **180**(2): p. 121-37.

404. Relton, C.L., M.S. Pearce, and L. Parker, *The influence of erythrocyte folate and serum vitamin B12 status on birth weight.* Br J Nutr, 2005. **93**(5): p. 593-9.

405. Neggers, Y.H., et al., *The relationship between maternal dietary intake and infant birthweight.* Acta Obstet Gynecol Scand Suppl, 1997. **165**: p. 71-5.

406. Scholl, T.O. and W.G. Johnson, *Folic acid: influence on the outcome of pregnancy.* Am J Clin Nutr, 2000. **71**(5 Suppl): p. 1295S-303S.

407. de Weerd, S., et al., *Maternal periconceptional biochemical and hematological parameters, vitamin profiles and pregnancy outcome.* Eur J Clin Nutr, 2003. **57**(9): p. 1128-34.
408. Finnell, R.H., A. Gould, and O. Spiegelstein, *Pathobiology and genetics of neural tube defects.* Epilepsia, 2003. **44 Suppl 3**: p. 14-23.
409. Angius, A., et al., *Methylenetetrahydrofolate reductase gene polymorphisms in Burkina Faso: impact on plasma fasting homocysteine and after methionine loading test.* Clin Lab, 2007. **53**(1-2): p. 29-33.
410. Bower, C. and F.J. Stanley, *Dietary folate as a risk factor for neural-tube defects: evidence from a case-control study in Western Australia.* Med J Aust, 1989. **150**(11): p. 613-9.
411. Bower, C., et al., *Absorption of pteroylpolyglutamates in mothers of infants with neural tube defects.* Br J Nutr, 1993. **69**(3): p. 827-34.
412. Schorah, C.J., et al., *Possible abnormalities of folate and vitamin B12 metabolism associated with neural tube defects.* Ann N Y Acad Sci, 1993. **678**: p. 81-91.
413. Davis, B.A., et al., *Folic acid absorption in women with a history of pregnancy with neural tube defect.* Am J Clin Nutr, 1995. **62**(4): p. 782-4.
414. Lucock, M., et al., *Biotransformation of pteroylmonoglutamic acid during absorption: implications of Michaelis-Menten kinetics.* Eur J Clin Nutr, 1989. **43**(9): p. 631-5.
415. Kelly, P., et al., *Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements.* Am J Clin Nutr, 1997. **65**(6): p. 1790-5.
416. Landon, M.J., D.H. Eyre, and F.E. Hytten, *Transfer of folate to the fetus.* Br J Obstet Gynaecol, 1975. **82**(1): p. 12-19.
417. Baker, H., et al., *Role of placenta in maternal-fetal vitamin transfer in humans.* Am J Obstet Gynecol, 1981. **141**(7): p. 792-6.
418. Henderson, G.I., et al., *Maternal-to-fetal transfer of 5-methyltetrahydrofolate by the perfused human placental cotyledon: evidence for a concentrative role by placental folate receptors in fetal folate delivery.* J Lab Clin Med, 1995. **126**(2): p. 184-203.
419. Bisseling, T.M., et al., *Placental folate transport and binding are not impaired in pregnancies complicated by fetal growth restriction.* Placenta, 2004. **25**(6): p. 588-93.
420. Campbell, N.R., *How safe are folic acid supplements?* Arch Intern Med, 1996. **156**(15): p. 1638-44.
421. Munoz-Moran, E., et al., *Genetic selection and folate intake during pregnancy.* Lancet, 1998. **352**(9134): p. 1120-1.
422. Lucock, M. and Z. Yates, *Folic acid - vitamin and panacea or genetic time bomb?* Nat Rev Genet, 2005. **6**(3): p. 235-40.

423. Santos, L.M. and M.Z. Pereira, *[The effect of folic acid fortification on the reduction of neural tube defects].* Cad Saude Publica, 2007. **23**(1): p. 17-24.
424. Kimura, M., et al., *Methylenetetrahydrofolate reductase C677T polymorphism, folic acid and riboflavin are important determinants of genome stability in cultured human lymphocytes.* J Nutr, 2004. **134**(1): p. 48-56.
425. Fredriksen, A., et al., *Large-scale population-based metabolic phenotyping of thirteen genetic polymorphisms related to one-carbon metabolism.* Hum Mutat, 2007.
426. Yamada, K., et al., *Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase.* Proc Natl Acad Sci U S A, 2001. **98**(26): p. 14853-8.
427. Christensen, B., et al., *Correlation of a common mutation in the methylenetetrahydrofolate reductase gene with plasma homocysteine in patients with premature coronary artery disease.* Arterioscler Thromb Vasc Biol, 1997. **17**(3): p. 569-73.
428. Motulsky, A.G., *Nutritional ecogenetics: homocysteine-related arteriosclerotic vascular disease, neural tube defects, and folic acid.* Am J Hum Genet, 1996. **58**(1): p. 17-20.
429. van der Molen, E.F., et al., *A common mutation in the 5,10-methylenetetrahydrofolate reductase gene as a new risk factor for placental vasculopathy.* Am J Obstet Gynecol, 2000. **182**(5): p. 1258-63.
430. Lachmeijer, A.M., et al., *Mutations in the gene for methylenetetrahydrofolate reductase, homocysteine levels, and vitamin status in women with a history of preeclampsia.* Am J Obstet Gynecol, 2001. **184**(3): p. 394-402.
431. Gibson, C.S., et al., *Associations between inherited thrombophilias, gestational age, and cerebral palsy.* Am J Obstet Gynecol, 2005. **193**(4): p. 1437.
432. Finkelstein, J.D., *The metabolism of homocysteine: pathways and regulation.* Eur J Pediatr, 1998. **157 Suppl 2**: p. S40-4.
433. Mudd, S.H., et al., *Methyl balance and transmethylation fluxes in humans.* Am J Clin Nutr, 2007. **85**(1): p. 19-25.
434. Ludwig, M.L. and R.G. Matthews, *Structure-based perspectives on B12-dependent enzymes.* Annu Rev Biochem, 1997. **66**: p. 269-313.
435. Brown, C.A., et al., *A common polymorphism in methionine synthase reductase increases risk of premature coronary artery disease.* J Cardiovasc Risk, 2000. **7**(3): p. 197-200.
436. Olteanu, H., T. Munson, and R. Banerjee, *Differences in the efficiency of reductive activation of methionine synthase and exogenous electron acceptors between the*

common polymorphic variants of human methionine synthase reductase. Biochemistry, 2002. **41**(45): p. 13378-85.

437. Linnebank, M., et al., *The methionine synthase polymorphism c.2756Aright curved arrow G (D919G) is relevant for disease-free longevity.* Int J Mol Med, 2005. **16**(4): p. 759-61.

438. Drennan, C.L., et al., *How a protein binds B12: A 3.0 A X-ray structure of B12-binding domains of methionine synthase.* Science, 1994. **266**(5191): p. 1669-74.

439. van der Put, N.M., et al., *Sequence analysis of the coding region of human methionine synthase: relevance to hyperhomocysteinaemia in neural-tube defects and vascular disease.* Qjm, 1997. **90**(8): p. 511-7.

440. Martinelli, P., et al., *Familial thrombophilia and the occurrence of fetal growth restriction.* Haematologica, 2001. **86**(4): p. 428-31.

441. Grandone, E., et al., *Homocysteine metabolism in families from southern Italy with neural tube defects: role of genetic and nutritional determinants.* Prenat Diagn, 2006. **26**(1): p. 1-5.

442. Infante-Rivard, C., et al., *Thrombophilic polymorphisms and intrauterine growth restriction.* Epidemiology, 2005. **16**(3): p. 281-7.

443. Murphy, R.P., et al., *Prospective evaluation of the risk conferred by factor V Leiden and thermolabile methylenetetrahydrofolate reductase polymorphisms in pregnancy.* Arterioscler Thromb Vasc Biol, 2000. **20**(1): p. 266-70.

444. Kaiser, T., S.P. Brennecke, and E.K. Moses, *C677T methylenetetrahydrofolate reductase polymorphism is not a risk factor for pre-eclampsia/eclampsia among Australian women.* Hum Hered, 2001. **51**(1-2): p. 20-2.

445. Grandone, E., et al., *Factor V Leiden, C > T MTHFR polymorphism and genetic susceptibility to preeclampsia.* Thromb Haemost, 1997. **77**(6): p. 1052-4.

446. Klerk, M., et al., *The 2756A>G variant in the gene encoding methionine synthase: its relation with plasma homocysteine levels and risk of coronary heart disease in a Dutch case-control study.* Thromb Res, 2003. **110**(2-3): p. 87-91.

447. Friedman, G., et al., *A common mutation A1298C in human methylenetetrahydrofolate reductase gene: association with plasma total homocysteine and folate concentrations.* J Nutr, 1999. **129**(9): p. 1656-61.

448. Hanson, N.Q., et al., *C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase gene: incidence and effect of combined genotypes on plasma fasting and post-methionine load homocysteine in vascular disease.* Clin Chem, 2001. **47**(4): p. 661-6.

449. Feix, A., et al., *Methionine synthase reductase MTRR 66A > G has no effect on total homocysteine, folate, and Vitamin B12 concentrations in renal transplant patients.* Atherosclerosis, 2004. **174**(1): p. 43-8.

450. Vaughn, J.D., et al., *Methionine synthase reductase 66A->G polymorphism is associated with increased plasma homocysteine concentration when combined with the homozygous methylenetetrahydrofolate reductase 677C->T variant.* J Nutr, 2004. **134**(11): p. 2985-90.

451. Laraqui, A., et al., *Influence of methionine synthase (A2756G) and methionine synthase reductase (A66G) polymorphisms on plasma homocysteine levels and relation to risk of coronary artery disease.* Acta Cardiol, 2006. **61**(1): p. 51-61.

452. Alessio, A.C., et al., *Polymorphisms in the methylenetetrahydrofolate reductase and methionine synthase reductase genes and homocysteine levels in Brazilian children.* Am J Med Genet A, 2004. **128**(3): p. 256-60.

453. Leopardi, P., et al., *Effects of folic acid deficiency and MTHFR C677T polymorphism on spontaneous and radiation-induced micronuclei in human lymphocytes.* Mutagenesis, 2006. **21**(5): p. 327-33.

454. Hiendleder, S., et al., *Tissue-specific elevated genomic cytosine methylation levels are associated with an overgrowth phenotype of bovine fetuses derived by in vitro techniques.* Biol Reprod, 2004. **71**(1): p. 217-23.

455. Grewal, S.I. and D. Moazed, *Heterochromatin and epigenetic control of gene expression.* Science, 2003. **301**(5634): p. 798-802.

456. Meehan, R.R., *DNA methylation in animal development.* Semin Cell Dev Biol, 2003. **14**(1): p. 53-65.

457. Constancia, M., et al., *Imprinting mechanisms.* Genome Res, 1998. **8**(9): p. 881-900.

458. Klose, R.J. and A.P. Bird, *Genomic DNA methylation: the mark and its mediators.* Trends Biochem Sci, 2006. **31**(2): p. 89-97.

459. Jaenisch, R., *DNA methylation and imprinting: why bother?* Trends Genet, 1997. **13**(8): p. 323-9.

460. Shimabukuro, M., et al., *Global hypomethylation of peripheral leukocyte DNA in male patients with schizophrenia: A potential link between epigenetics and schizophrenia.* J Psychiatr Res, 2006.

461. Fuke, C., et al., *Age related changes in 5-methylcytosine content in human peripheral leukocytes and placentas: an HPLC-based study.* Ann Hum Genet, 2004. **68**(Pt 3): p. 196-204.

462. Roemer, I., et al., *Epigenetic inheritance in the mouse.* Curr Biol, 1997. **7**(4): p. 277-80.

463. Sutherland, H.G., et al., *Reactivation of heritably silenced gene expression in mice.* Mamm Genome, 2000. **11**(5): p. 347-55.
464. Rakyan, V.K., et al., *Metastable epialleles in mammals.* Trends Genet, 2002. **18**(7): p. 348-51.
465. Wolff, G.L., et al., *Maternal epigenetics and methyl supplements affect agouti gene expression in Avy/a mice.* Faseb J, 1998. **12**(11): p. 949-57.
466. Hsieh, C.L., *Dynamics of DNA methylation pattern.* Curr Opin Genet Dev, 2000. **10**(2): p. 224-8.
467. Bestor, T.H., *The DNA methyltransferases of mammals.* Hum Mol Genet, 2000. **9**(16): p. 2395-402.
468. Boyes, J. and A. Bird, *DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein.* Cell, 1991. **64**(6): p. 1123-34.
469. Hendrich, B. and A. Bird, *Identification and characterization of a family of mammalian methyl-CpG binding proteins.* Mol Cell Biol, 1998. **18**(11): p. 6538-47.
470. Nan, X., et al., *Transcriptional repression by the methyl-CpG-binding protein MeCP2 involves a histone deacetylase complex.* Nature, 1998. **393**(6683): p. 386-9.
471. Watt, F. and P.L. Molloy, *Cytosine methylation prevents binding to DNA of a HeLa cell transcription factor required for optimal expression of the adenovirus major late promoter.* Genes Dev, 1988. **2**(9): p. 1136-43.
472. Godfrey, K.M., et al., *Epigenetic mechanisms and the mismatch concept of the developmental origins of health and disease.* Pediatr Res, 2007. **61**(5 Pt 2): p. 5R-10R.
473. Wilson, A.S., B.E. Power, and P.L. Molloy, *DNA hypomethylation and human diseases.* Biochim Biophys Acta, 2007. **1775**(1): p. 138-62.
474. Egger, G., et al., *Epigenetics in human disease and prospects for epigenetic therapy.* Nature, 2004. **429**(6990): p. 457-63.
475. Jiang, Y.H., J. Bressler, and A.L. Beaudet, *Epigenetics and human disease.* Annu Rev Genomics Hum Genet, 2004. **5**: p. 479-510.
476. Maier, S. and A. Olek, *Diabetes: a candidate disease for efficient DNA methylation profiling.* J Nutr, 2002. **132**(8 Suppl): p. 2440S-2443S.
477. Corwin, E.J., *The concept of epigenetics and its role in the development of cardiovascular disease: commentary on "new and emerging theories of cardiovascular disease".* Biol Res Nurs, 2004. **6**(1): p. 11-6; discussion 21-3.
478. Waterland, R.A., *Does nutrition during infancy and early childhood contribute to later obesity via metabolic imprinting of epigenetic gene regulatory mechanisms?* Nestle Nutr Workshop Ser Pediatr Program, 2005. **56**: p. 157-71; discussion 171-4.

479. Drinkwater, R.D., et al., *Human lymphocytes aged in vivo have reduced levels of methylation in transcriptionally active and inactive DNA.* Mutat Res, 1989. **219**(1): p. 29-37.

480. Tawa, R., et al., *Changes of DNA methylation level during pre- and postnatal periods in mice.* Differentiation, 1990. **45**(1): p. 44-8.

481. Ueland, P.M., et al., *Biological and clinical implications of the MTHFR C677T polymorphism.* Trends Pharmacol Sci, 2001. **22**(4): p. 195-201.

482. Ingrosso, D., et al., *Folate treatment and unbalanced methylation and changes of allelic expression induced by hyperhomocysteinaemia in patients with uraemia.* Lancet, 2003. **361**(9370): p. 1693-9.

483. Fowler, B.M., et al., *Hypomethylation in cervical tissue: is there a correlation with folate status?* Cancer Epidemiol Biomarkers Prev, 1998. **7**(10): p. 901-6.

484. Piyathilake, C.J., et al., *Localized folate and vitamin B-12 deficiency in squamous cell lung cancer is associated with global DNA hypomethylation.* Nutr Cancer, 2000. **37**(1): p. 99-107.

485. Esfandiari, F., et al., *Methyl deficiency causes reduction of the methyl-CpG-binding protein, MeCP2, in rat liver.* Carcinogenesis, 2003. **24**(12): p. 1935-40.

486. Halsted, C.H., et al., *Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig.* Proc Natl Acad Sci U S A, 2002. **99**(15): p. 10072-7.

487. Yi, P., et al., *Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation.* J Biol Chem, 2000. **275**(38): p. 29318-23.

488. Park, B.H., et al., *[Folate and homocysteine levels during pregnancy affect DNA methylation in human placenta].* J Prev Med Pub Health, 2005. **38**(4): p. 437-42.

489. Kim, Y.I., et al., *Exon-specific DNA hypomethylation of the p53 gene of rat colon induced by dimethylhydrazine. Modulation by dietary folate.* Am J Pathol, 1996. **149**(4): p. 1129-37.

490. Pogribny, I.P., L.A. Poirier, and S.J. James, *Differential sensitivity to loss of cytosine methyl groups within the hepatic p53 gene of folate/methyl deficient rats.* Carcinogenesis, 1995. **16**(11): p. 2863-7.

491. Pogribny, I.P., B.J. Miller, and S.J. James, *Alterations in hepatic p53 gene methylation patterns during tumor progression with folate/methyl deficiency in the rat.* Cancer Lett, 1997. **115**(1): p. 31-8.

492. James, S.J., et al., *Mechanisms of DNA damage, DNA hypomethylation, and tumor progression in the folate/methyl-deficient rat model of hepatocarcinogenesis.* J Nutr, 2003. **133**(11 Suppl 1): p. 3740S-3747S.
493. Gisselsson, D., et al., *Interphase chromosomal abnormalities and mitotic missegregation of hypomethylated sequences in ICF syndrome cells.* Chromosoma, 2005. **114**(2): p. 118-26.
494. Tuck-Muller, C.M., et al., *DNA hypomethylation and unusual chromosome instability in cell lines from ICF syndrome patients.* Cytogenet Cell Genet, 2000. **89**(1-2): p. 121-8.
495. Jallepalli, P.V. and C. Lengauer, *Chromosome segregation and cancer: cutting through the mystery.* Nat Rev Cancer, 2001. **1**(2): p. 109-17.
496. Kaplan, K.B., et al., *A role for the Adenomatous Polyposis Coli protein in chromosome segregation.* Nat Cell Biol, 2001. **3**(4): p. 429-32.
497. Rajagopalan, H., et al., *Inactivation of hCDC4 can cause chromosomal instability.* Nature, 2004. **428**(6978): p. 77-81.
498. Shichiri, M., et al., *Genetic and epigenetic inactivation of mitotic checkpoint genes hBUB1 and hBUBR1 and their relationship to survival.* Cancer Res, 2002. **62**(1): p. 13-7.
499. Kierszenbaum, A.L., *Genomic imprinting and epigenetic reprogramming: unearthing the garden of forking paths.* Mol Reprod Dev, 2002. **63**(3): p. 269-72.
500. Devriendt, K., *Hydatidiform mole and triploidy: the role of genomic imprinting in placental development.* Hum Reprod Update, 2005. **11**(2): p. 137-42.
501. Looijenga, L.H., et al., *Heterogeneous X inactivation in trophoblastic cells of human full-term female placentas.* Am J Hum Genet, 1999. **64**(5): p. 1445-52.
502. Uehara, S., et al., *Preferential X-chromosome inactivation in women with idiopathic recurrent pregnancy loss.* Fertil Steril, 2001. **76**(5): p. 908-14.
503. Kjellman, C., et al., *HERV-F (XA34) is a full-length human endogenous retrovirus expressed in placental and fetal tissues.* Gene, 1999. **239**(1): p. 99-107.
504. Kriegs, J.O., et al., *Retroposed elements as archives for the evolutionary history of placental mammals.* PLoS Biol, 2006. **4**(4): p. e91.
505. Malassine, A., et al., *Expression of the fusogenic HERV-FRD Env glycoprotein (syncytin 2) in human placenta is restricted to villous cytotrophoblastic cells.* Placenta, 2007. **28**(2-3): p. 185-91.
506. Fenech, M., *Biomarkers of genetic damage for cancer epidemiology.* Toxicology, 2002. **181-182**: p. 411-6.

507. Kaushal, D., et al., *Alteration of gene expression by chromosome loss in the postnatal mouse brain.* J Neurosci, 2003. **23**(13): p. 5599-606.

508. Rubio, C., et al., *Embryo aneuploidy screening for unexplained recurrent miscarriage: a minireview.* Am J Reprod Immunol, 2005. **53**(4): p. 159-65.

509. Bianco, K., et al., *History of miscarriage and increased incidence of fetal aneuploidy in subsequent pregnancy.* Obstet Gynecol, 2006. **107**(5): p. 1098-102.

510. Izraeli, S., *Perspective: chromosomal aneuploidy in leukemia--lessons from down syndrome.* Hematol Oncol, 2006. **24**(1): p. 3-6.

511. Fenech, M.F., I.E. Dreosti, and J.R. Rinaldi, *Folate, vitamin B12, homocysteine status and chromosome damage rate in lymphocytes of older men.* Carcinogenesis, 1997. **18**(7): p. 1329-36.

512. Herrmann, W., et al., *Alteration of homocysteine catabolism in pre-eclampsia, HELLP syndrome and placental insufficiency.* Clin Chem Lab Med, 2004. **42**(10): p. 1109-16.

513. Shastry, S., et al., *Homocysteine induces mesangial cell apoptosis via activation of p38-mitogen-activated protein kinase.* Kidney Int, 2007. **71**(4): p. 304-11.

514. Steegers-Theunissen, R.P. and E.A. Steegers, *Nutrient-gene interactions in early pregnancy: a vascular hypothesis.* Eur J Obstet Gynecol Reprod Biol, 2003. **106**(2): p. 115-7.

515. James, S.J., et al., *Elevation in S-adenosylhomocysteine and DNA hypomethylation: potential epigenetic mechanism for homocysteine-related pathology.* J Nutr, 2002. **132**(8 Suppl): p. 2361S-2366S.

516. Steegers-Theunissen, R.P., et al., *Maternal and fetal levels of methionine and homocysteine in early human pregnancy.* Br J Obstet Gynaecol, 1997. **104**(1): p. 20-4.

517. Touil, N., et al., *Analysis of chromosome loss and chromosome segregation in cytokinesis-blocked human lymphocytes: non-disjunction is the prevalent mistake in chromosome segregation produced by low dose exposure to ionizing radiation.* Mutagenesis, 2000. **15**(1): p. 1-7.

518. Li, R. and A.W. Murray, *Feedback control of mitosis in budding yeast.* Cell, 1991. **66**(3): p. 519-31.

519. Fenech, M., et al., *The HUman MicroNucleus Project--An international collaborative study on the use of the micronucleus technique for measuring DNA damage in humans.* Mutat Res, 1999. **428**(1-2): p. 271-83.

520. Minshull, J., et al., *A MAP kinase-dependent spindle assembly checkpoint in Xenopus egg extracts.* Cell, 1994. **79**(3): p. 475-86.

521. Wang, J.Y., *Cellular responses to DNA damage.* Curr Opin Cell Biol, 1998. **10**(2): p. 240-7.

522. Yang, X.H. and L. Zou, *Checkpoint and coordinated cellular responses to DNA damage.* Results Probl Cell Differ, 2006. **42**: p. 65-92.

523. Whitaker, M., *Calcium microdomains and cell cycle control.* Cell Calcium, 2006. **40**(5-6): p. 585-92.

524. Chang, J.H. and H.Y. Kwon, *Expression of 14-3-3delta, cdc2 and cyclin B proteins related to exotoxin A-induced apoptosis in HeLa S3 cells.* Int Immunopharmacol, 2007. **7**(9): p. 1185-91.

525. Fang, F. and J.W. Newport, *Evidence that the G1-S and G2-M transitions are controlled by different cdc2 proteins in higher eukaryotes.* Cell, 1991. **66**(4): p. 731-42.

526. Klein, H.L., *Spontaneous chromosome loss in Saccharomyces cerevisiae is suppressed by DNA damage checkpoint functions.* Genetics, 2001. **159**(4): p. 1501-9.

527. Hoyt, M.A., L. Totis, and B.T. Roberts, *S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function.* Cell, 1991. **66**(3): p. 507-17.

528. Taylor, S.S. and F. McKeon, *Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage.* Cell, 1997. **89**(5): p. 727-35.

529. Lacefield, S. and A.W. Murray, *The spindle checkpoint rescues the meiotic segregation of chromosomes whose crossovers are far from the centromere.* Nat Genet, 2007.

530. Clemenson, C. and M.C. Marsolier-Kergoat, *The spindle assembly checkpoint regulates the phosphorylation state of a subset of DNA checkpoint proteins in Saccharomyces cerevisiae.* Mol Cell Biol, 2006. **26**(24): p. 9149-61.

531. Rieder, C.L., et al., *The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores.* J Cell Biol, 1995. **130**(4): p. 941-8.

532. Matsumoto, T., *[Spindle checkpoint: cell cycle regulation by a signal emitted from kinetochores].* Tanpakushitsu Kakusan Koso, 2004. **49**(12): p. 1998-2004.

533. Alexandru, G., et al., *Sister chromatid separation and chromosome re-duplication are regulated by different mechanisms in response to spindle damage.* Embo J, 1999. **18**(10): p. 2707-21.

534. Pereira, G., et al., *The Bub2p spindle checkpoint links nuclear migration with mitotic exit.* Mol Cell, 2000. **6**(1): p. 1-10.

535. Dobles, M., et al., *Chromosome missegregation and apoptosis in mice lacking the mitotic checkpoint protein Mad2.* Cell, 2000. **101**(6): p. 635-45.

536. Giraldo, A.M., et al., *Proliferative characteristics and chromosomal stability of bovine donor cells for nuclear transfer.* Mol Reprod Dev, 2006. **73**(10): p. 1230-8.

537. Porter, A.C. and C.J. Farr, *Topoisomerase II: untangling its contribution at the centromere.* Chromosome Res, 2004. **12**(6): p. 569-83.

538. Forterre, P., et al., *Origin and evolution of DNA topoisomerases.* Biochimie, 2007. **89**(4): p. 427-46.

539. Visintin, R., S. Prinz, and A. Amon, *CDC20 and CDH1: a family of substrate-specific activators of APC-dependent proteolysis.* Science, 1997. **278**(5337): p. 460-3.

540. Castedo, M., et al., *Mitotic catastrophe constitutes a special case of apoptosis whose suppression entails aneuploidy.* Oncogene, 2004. **23**(25): p. 4362-70.

541. Ames, B.N., *Low micronutrient intake may accelerate the degenerative diseases of aging through allocation of scarce micronutrients by triage.* Proc Natl Acad Sci U S A, 2006. **103**(47): p. 17589-94.

542. Fenech, M., *Micronutrients and genomic stability: a new paradigm for recommended dietary allowances (RDAs).* Food Chem Toxicol, 2002. **40**(8): p. 1113-7.

543. Fletcher, R.H. and K.M. Fairfield, *Vitamins for chronic disease prevention in adults: clinical applications.* Jama, 2002. **287**(23): p. 3127-9.

544. Whitaker, M., *Calcium at fertilization and in early development.* Physiol Rev, 2006. **86**(1): p. 25-88.

545. Hepler, P.K., *Calcium and mitosis.* Int Rev Cytol, 1992. **138**: p. 239-68.

546. Whitaker, M. and M.G. Larman, *Calcium and mitosis.* Semin Cell Dev Biol, 2001. **12**(1): p. 53-8.

547. Davis, C.D. and E.O. Uthus, *DNA methylation, cancer susceptibility, and nutrient interactions.* Exp Biol Med (Maywood), 2004. **229**(10): p. 988-95.

548. Trujillo, E., C. Davis, and J. Milner, *Nutrigenomics, proteomics, metabolomics, and the practice of dietetics.* J Am Diet Assoc, 2006. **106**(3): p. 403-13.

549. Appel, L.J., et al., *Effect of dietary patterns on serum homocysteine: results of a randomized, controlled feeding study.* Circulation, 2000. **102**(8): p. 852-7.

550. Berstad, P., et al., *Dietary fat and plasma total homocysteine concentrations in 2 adult age groups: the Hordaland Homocysteine Study.* Am J Clin Nutr, 2007. **85**(6): p. 1598-605.

551. Fonseca, V., et al., *Effects of a high-fat-sucrose diet on enzymes in homocysteine metabolism in the rat.* Metabolism, 2000. **49**(6): p. 736-41.

552. Hustad, S., et al., *The methylenetetrahydrofolate reductase 677C-->T polymorphism as a modulator of a B vitamin network with major effects on homocysteine metabolism.* Am J Hum Genet, 2007. **80**(5): p. 846-55.

553. Xu, G.L., et al., *Chromosome instability and immunodeficiency syndrome caused by mutations in a DNA methyltransferase gene.* Nature, 1999. **402**(6758): p. 187-91.
554. Kondo, T., et al., *Whole-genome methylation scan in ICF syndrome: hypomethylation of non-satellite DNA repeats D4Z4 and NBL2.* Hum Mol Genet, 2000. **9**(4): p. 597-604.
555. Esplugues, A., et al., *[Air pollutant exposure during pregnancy and fetal and early childhood development. Research protocol of the INMA (Childhood and Environment Project)].* Gac Sanit, 2007. **21**(2): p. 162-71.
556. Huynh, M., et al., *Relationships between air pollution and preterm birth in California.* Paediatr Perinat Epidemiol, 2006. **20**(6): p. 454-61.
557. Hansen, C., et al., *Low levels of ambient air pollution during pregnancy and fetal growth among term neonates in Brisbane, Australia.* Environ Res, 2007. **103**(3): p. 383-9.
558. Hung, H.S., et al., *Association of cooking oil fumes exposure with lung cancer: involvement of inhibitor of apoptosis proteins in cell survival and proliferation in vitro.* Mutat Res, 2007. **628**(2): p. 107-16.
559. Ruchirawat, M., et al., *Assessment of potential cancer risk in children exposed to urban air pollution in Bangkok, Thailand.* Toxicol Lett, 2007. **168**(3): p. 200-9.
560. Hien, T.T., et al., *Comparison of particle-phase polycyclic aromatic hydrocarbons and their variability causes in the ambient air in Ho Chi Minh City, Vietnam and in Osaka, Japan, during 2005-2006.* Sci Total Environ, 2007. **382**(1): p. 70-81.
561. Manuel, J., *Secondhand smoke: displaced enthusiasm?* Environ Health Perspect, 2007. **115**(3): p. A129.
562. Rossner, P., Jr., et al., *Air pollution by carcinogenic PAHs and plasma levels of p53 and p21(WAF1) proteins.* Mutat Res, 2007. **620**(1-2): p. 34-40.
563. Raimondi, S., et al., *Effects of diet on biomarkers of exposure and effects, and on oxidative damage.* Mutat Res, 2007. **620**(1-2): p. 93-102.
564. Wang, X., et al., *Maternal cigarette smoking, metabolic gene polymorphism, and infant birth weight.* Jama, 2002. **287**(2): p. 195-202.
565. Singh, R., et al., *The relationship between biomarkers of oxidative DNA damage, polycyclic aromatic hydrocarbon DNA adducts, antioxidant status and genetic susceptibility following exposure to environmental air pollution in humans.* Mutat Res, 2007. **620**(1-2): p. 83-92.
566. Binkova, B., et al., *PAH-DNA adducts in environmentally exposed population in relation to metabolic and DNA repair gene polymorphisms.* Mutat Res, 2007. **620**(1-2): p. 49-61.

567. Sram, R.J., et al., *Association of DNA adducts and genotypes with birth weight.* Mutat Res, 2006. **608**(2): p. 121-8.

15 APPENDIX

Appendix 1 Pearsons correlation of genome damage markers age, BMI and smoking

		NPB-BN	NBUD-BN	NDI	Apoptosis	Necrosis	Age	BMI	Smoker
MN-BN	Pearson Correlation	0.247	0.422	-0.277	0.069	0.056	0.253	0.067	0.241
N = 136	Sig. (2-tailed)	**0.004**	**> 0.001**	**0.001**	0.427	0.515	**0.003**	0.442	**0.005**
NPBs	Pearson Correlation		0.437	-0.207	0.184	0.043	-0.126	0.144	0.017
N = 136	Sig. (2-tailed)		**> 0.001**	**0.016**	**0.032**	0.622	0.144	0.094	0.843
NBUDs	Pearson Correlation			-0.401	0.237	0.090	-0.030	0.043	-0.024
N = 136	Sig. (2-tailed)			**0.001**	**0.006**	0.295	0.731	0.616	0.783
NDI	Pearson Correlation				-0.007	0.149	-0.082	0.029	0.077
N = 136	Sig. (2-tailed)				0.933	0.082	0.341	0.735	0.372
Apoptosis	Pearson Correlation					0.625	-0.145	0.024	0.020
N = 136	Sig. (2-tailed)					**> 0.001**	0.092	0.785	0.821
Necrosis	Pearson Correlation						-0.078	-0.069	0.021
N = 136	Sig. (2-tailed)						0.369	0.424	0.806
Age	Pearson Correlation							0.007	-0.184
N = 143	Sig. (2-tailed)							0.931	**0.028**
BMI	Pearson Correlation								0.132
N = 143	Sig. (2-tailed)								0.115

Appendix 2: Alleleic discrimination output

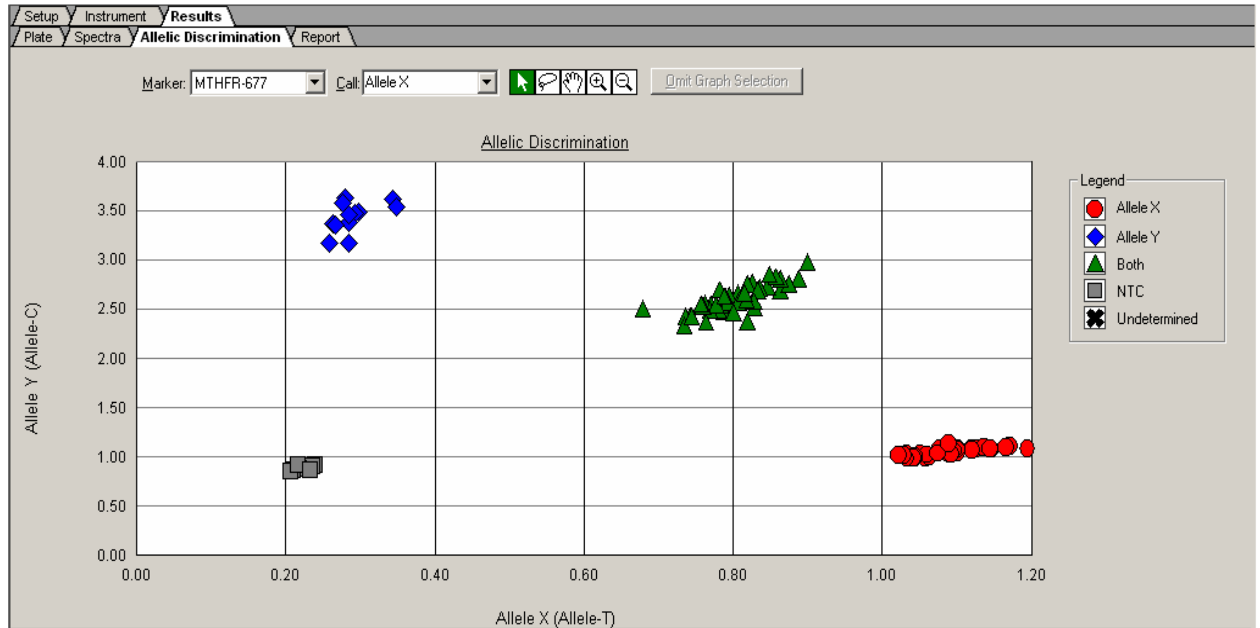

Figure 27. Allelic discrimination output

This figure represents the Applied Biosystems ABI output for alleleic discrimination. The red circles represent the homozygous common alleles, the blue diamond represents the homozygous variant alleles, the green triangle represents the heterozygotes and the grey squares represent the non template controls.

Appendix 3 Distribution of various maternal alleles involved in one carbon metabolism genes in low risk pregnancies with normal and adverse outcomes

Maternal	Normal (n = 42)	Adverse (n = 7)	Chi-square P
MTHFR C677T			
C	54 (64.3%)	10 (71.4%)	0.603
T	30 (35.7%)	4 (28.6%)	
MTHFR A1298C			
A	65 (77.4%)	11 (78.6%)	0.921
C	19 (22.6%)	3 (21.4%)	
MTR A2756G			
A	66 (78.6%)	9 (64.3%)	0.243
G	18 (21.4%)	5 (35.7%)	
MTRR A66G			
A	46 (54.8%)	8 (57.1%)	0.868
G	38 (45.2%)	6 (42.9%)	
MTHFD1 G1958A			
G	53 (63.1%)	8 (57.1%)	0.670
A	31 (36.9%)	6 (42.9%)	

Data represents number of alleles for each group and percentage (%) within each group.

Appendix 4 Distribution of various maternal alleles involved in one carbon metabolism genes in high risk pregnancies with normal and adverse outcomes

Maternal	Normal (n = 28)	Adverse (n = 61)	Chi-square P
MTHFR C677T			
C	35 (62.5%)	88 (72.1%)	0.196
T	21 (37.5%)	34 (27.9%)	
MTHFR A1298C			
A	43 (76.8%)	89 (72.9%)	0.587
C	13 (23.2%)	33 (27.1%)	
MTR A2756G			
A	45 (80.4%)	93 (76.2%)	0.540
G	11 (19.6%)	29 (23.8%)	
MTRR A66G			
A	36 (64.3%)	64 (52.5%)	0.139
G	20 (35.7%)	58 (47.5%)	
MTHFD1 G1958A			
G	28 (50.0%)	57 (46.7%)	0.684
A	28 (50.0%	65 (53.3%)	

Data represents number of alleles for each group and percentage (%) within each group.

Appendix 5 Distribution of various maternal alleles involved in one carbon metabolism genes in low risk healthy pregnancies and UPI

Maternal	Control (n = 42)	UPI (n = 48)	Chi-square P
MTHFR C677T			
C	54 (64.3%)	67 (69.8%)	0.432
T	30 (35.7%)	29 (30.2%)	
MTHFR A1298C			
A	65 (77.4%)	63 (65.6%)	0.533
C	19 (22.6%)	23 (24.0%)	
MTR A2756G			
A	66 (78.6%)	70 (72.9%)	0.378
G	18 (21.4%)	26 (27.1%)	
MTRR A66G			
A	46 (54.8%)	52 (54.2%)	0.936
G	38 (45.2%)	44 (45.8%)	
MTHFD1 G1958A			
G	53 (63.1%)	50 (52.8%)	0.136
A	31 (36.9%)	46 (47.9%)	

Data represents number of alleles for each group and percentage (%) within each group.

Appendix 6 Distribution of various maternal alleles involved in one carbon metabolism genes in low risk healthy pregnancies and PE

Maternal	Control (n = 42)	PE (n = 15)	Chi-square P
MTHFR C677T			
C	54 (64.3%)	21 (70.0%)	0.571
T	30 (35.7%)	9 (30.0%)	
MTHFR A1298C			
A	65 (77.4%)	20 (66.7%)	0.247
C	19 (22.6%)	10 (33.3%)	
MTR A2756G			
A	66 (78.6%)	25 (83.3%)	0.577
G	18 (21.4%)	5 (16.7%)	
MTRR A66G			
A	46 (54.8%)	15 (50.0%)	0.653
G	38 (45.2%)	15 (50.0%)	
MTHFD1 G1958A			
G	53 (63.1%)	18 (60.0%)	0.764
A	31 (36.9%)	12 (40.0%)	

Data represents number of alleles for each group and percentage (%) within each group.

Appendix 7 Distribution of various maternal alleles involved in one carbon metabolism genes in low risk healthy pregnancies and IUGR

Maternal	Control (n = 42)	IUGR (n = 18)	Chi-square P
MTHFR C677T			
C	54 (64.3%)	27 (75.0%)	0.251
T	30 (35.7%)	9 (25.0%)	
MTHFR A1298C			
A	65 (77.4%)	25 (69.4%)	0.357
C	19 (22.6%)	11 (30.6%)	
MTR A2756G			
A	66 (78.6%)	26 (72.2%)	0.568
G	18 (21.4%)	10 (27.8%)	
MTRR A66G			
A	46 (54.8%)	21 (58.3%)	0.718
G	38 (45.2%)	15 (41.7%)	
MTHFD1 G1958A			
G	53 (63.1%)	17 (47.2%)	0.214
A	31 (36.9%)	19 (52.8%)	

Data represents number of alleles for each group and percentage (%) within each group.

Appendix 8 Distribution of various maternal genotypes involved in one carbon metabolism genes in low risk pregnancies and pregnancies at high risk of developing UPI

Maternal	Low Risk	High Risk	Chi-square P
MTHFR C677T			
CC	18 (36.7%)	44 (49.5%)	
CT	28 (57.1%)	35 (39.3%)	0.122
TT	3 (6.1%)	10 (11.2%)	
MTHFR A1298C			
AA	30 (61.2%)	49 (55.1%)	
AC	16 (32.7%)	34 (38.2%)	0.780
CC	3 (6.7%)	6(6.7%)	
MTR A2756G			
AA	31 (63.3%)	53 (59.6%)	
AG	13 (26.5%)	32 (36.0%)	0.285
GG	5 (10.2%)	4 (4.5%)	
MTRR A66G			
AA	16 (32.7%)	26 (29.2%)	
AG	22 (44.9%)	48 (53.9%)	0.561
GG	11 (22.4%)	15 (16.9%)	
MTHFD1 G1958A			
GG	19 (38.8%)	24 (27.0%)	
GA	23 (46.9%)	45 (50.6%)	0.275
AA	7 (14.3%)	20 (22.5%)	

Data represents number of women for each genotype and percentage (%) from the total study group.

Appendix 9: Distribution of various maternal genotypes involved in one carbon metabolism genes in low risk pregnancies with normal and adverse outcomes

Maternal	Normal (n = 42)	Adverse (n = 7)	Chi-square P
MTHFR C677T			
CC	15 (35.7%)	3 (42.9%)	
CT	24 (57.1%)	4 (57.1%)	0.747
TT	3 (7.1%)	0 (0.0%)	
MTHFR A1298C			
AA	25 (59.5%)	5 (71.4%)	
AC	15 (35.7%)	1 (14.3%)	0.392
CC	2 (4.8%)	1 (14.3%)	
MTR A2756G			
AA	28 (66.7%)	3 (42.9%)	
AG	10 (23.8%)	3 (42.9%)	0.474
GG	4 (9.5%)	1 (14.3%)	
MTRR A66G			
AA	14 (33.3%)	2 (28.6%)	
AG	18 (42.9%)	4 (57.1%)	0.757
GG	10 (23.8%)	1 (14.3%)	
MTHFD1 G1958A			
GG	17 (40.5%)	2 (28.6%)	
GA	19 (45.2%)	4 (57.1%)	0.819
AA	6 (14.3%)	1 (14.3%)	

Data represents number of women for each genotype and percentage (%) from the total study group

Appendix 10: Distribution of various maternal genotypes involved in one carbon metabolism genes in high risk pregnancies with normal and adverse outcomes

Maternal	Normal (n = 28)	Adverse (n = 61)	Chi-square P
MTHFR C677T			
CC	11 (39.3%)	33 (54.1%)	
CT	13 (46.4%)	22 (36.1%)	0.424
TT	4 (14.3%)	6 (9.8%)	
MTHFR A1298C			
AA	17 (60.7%)	32 (52.5%)	
AC	9 (32.1%)	25 (41.0%)	0.726
CC	2 (7.1%)	4 (6.6%)	
MTR A2756G			
AA	17 (60.7%)	36 (59.0%)	
AG	11 (39.3%)	21 (34.4%)	0.373
GG	0 (0.0%)	4 (6.6%)	
MTRR A66G			
AA	10 (35.7%)	16 (26.2%)	
AG	16 (57.1%)	32 (52.5%)	0.228
GG	2 (7.1%)	13 (21.3%)	
MTHFD1 G1958A			
GG	6 (21.4%)	14 (23.0%)	
GA	16 (57.1%)	29 (47.5%)	0.659
AA	6 (21.4%)	18 (29.5%)	

Data represents number of women for each genotype and percentage (%) from the total study group

Appendix 11: Distribution of various maternal genotypes involved in one carbon metabolism genes in low risk healthy pregnancies (controls) and uteroplacental insufficiency

Maternal	Control (n = 42)	UPI (n = 48)	Chi-square P
MTHFR C677T			
CC	15 (35.7%)	24 (50.0%)	
CT	24 (57.1%)	19 (39.6%)	0.415
TT	3 (7.1%)	5 (10.4%)	
MTHFR A1298C			
AA	25 (59.5%)	27 (56.3%)	
AC	15 (35.7%)	19 (39.6%)	0.609
CC	2 (4.8%)	2 (4.2%)	
MTR A2756G			
AA	28 (66.7%)	26 (54.2%)	
AG	10 (23.8%)	18 (42.8%)	0.336
GG	4 (9.5%)	4 (8.3%)	
MTRR A66G			
AA	14 (33.3%)	14 (29.2%)	
AG	18 (42.9%)	24 (50.0%)	0.604
GG	10 (23.8%)	10 (20.8%)	
MTHFD1 G1958A			
GG	17 (40.5%)	14 (29.2%)	
GA	19 (45.2%)	22 (45.8%)	0.201
AA	6 (14.3%)	12 (25.0%)	

Data represents number of women for each genotype and percentage (%) from the total study group

Appendix 12: Distribution of various maternal genotypes involved in one carbon metabolism genes in low risk healthy pregnancies (controls) and those who develop preeclampsia

Maternal	Control (n = 42)	PE (n = 15)	Chi-square P
MTHFR C677T			
CC	15 (35.7%)	7 (46.7%)	
CT	24 (57.1%)	7 (46.7%)	0.984
TT	3 (7.1%)	1 (6.7%)	
MTHFR A1298C			
AA	25 (59.5%)	6 (40.0%)	
AC	15 (35.7%)	8 (53.3%)	0.306
CC	2 (4.8%)	1 (6.7%)	
MTR A2756G			
AA	28 (66.7%)	10 (66.7%)	
AG	10 (23.8%)	5 (33.3%)	0.400
GG	4 (9.5%)	0 (0.0%)	
MTRR A66G			
AA	14 (33.3%)	4 (26.7%)	
AG	18 (42.9%)	7 (46.7%)	0.760
GG	10 (23.8%)	4 (26.7%)	
MTHFD1 G1958A			
GG	17 (40.5%)	5 (33.3%)	
GA	19 (45.2%)	8 (53.3%)	0.954
AA	6 (14.3%)	2 (13.3%)	

Data represents number of women for each genotype and percentage (%) from the total study group.

Appendix 13: Distribution of various fetal alleles involved in one carbon metabolism genes in low risk pregnancies with normal and adverse outcomes

Fetal	Normal (n = 41)	Adverse (n = 7)	Chi-square P
MTHFR C677T			
C	52 (63.4%)	11 (78.6%)	0.269
T	30 (36.6%)	3 (21.4%)	
MTHFR A1298C			
A	57 (69.5%)	11 (78.6%)	0.491
C	25 (30.5%)	3 (21.4%)	
MTR A2756G			
A	64 (78.0%)	9 (64.3%)	0.265
G	18 (22.0%)	5 (35.7%)	
MTRR A66G			
A	52 (63.4%)	10 (71.4%)	0.644
G	30 (36.6%)	4 (28.6%)	
MTHFD1 G1958A			
G	46 (56.1%)	10 (71.4%)	0.282
A	36 (43.9%)	4 (28.6%)	

Data represents number of alleles for each group and percentage (%) within each group.

Appendix 14: Distribution of various fetal alleles involved in one carbon metabolism genes in high risk pregnancies with normal and adverse outcomes

Fetal	Normal (n = 21)	Adverse (n = 55)	Chi-square P
MTHFR C677T			
C	27 (64.3%)	76 (69.1%)	0.321
T	15 (35.7%)	34 (30.9%)	
MTHFR A1298C			
A	30 (71.4%)	78 (70.9%)	0.402
C	12 (28.6%)	32 (29.1%)	
MTR A2756G			
A	29 (69.0%)	80 (72.7%)	0.652
G	13 (31.0%)	30 (27.3%)	
MTRR A66G			
A	26 (50.0%)	66 (60.0%)	0.230
G	26 (50.0%)	44 (40.0%)	
MTHFD1 G1958A			
G	21 (50.0%)	61 (55.5%)	0.546
A	21 (50.0%)	49 (44.5%)	

Data represents number of alleles for each group and percentage (%) within each group.

Appendix 15: Distribution of various fetal alleles involved in one carbon metabolism genes in low risk healthy pregnancies and UPI

Fetal	Control (n = 41)	UPI (n = 47)	Chi-square P
MTHFR C677T			
C	52 (63.4%)	68 (72.3%)	0.205
T	30 (36.6%)	26 (27.7%)	
MTHFR A1298C			
A	57 (69.5%)	68 (72.3%)	0.978
C	25 (30.5%)	26 (27.7%)	
MTR A2756G			
A	64 (78.0%)	66 (70.2%)	0.238
G	18 (22.0%)	28 (29.8%)	
MTRR A66G			
A	52 (63.4%)	55 (58.5%)	0.506
G	30 (36.6%)	39 (41.5%)	
MTHFD1 G1958A			
G	46 (56.1%)	50 (53.2%)	0.699
A	36 (43.9%)	44 (46.8%)	

Data represents number of alleles for each group and percentage (%) within each group.

Appendix 16: Distribution of various fetal alleles involved in one carbon metabolism genes in low risk healthy pregnancies and PE

Fetal	Control (n = 41)	PE (n = 15)	Chi-square P
MTHFR C677T			
C	52 (63.4%)	21 (70.0%)	0.517
T	30 (36.6%)	9 (30.0%)	
MTHFR A1298C			
A	57 (69.5%)	18 (60.0%)	0.434
C	25 (30.5%)	12 (40.0%)	
MTR A2756G			
A	64 (78.0%)	26 (86.7%)	0.309
G	18 (22.0%)	4 (13.3%)	
MTRR A66G			
A	52 (63.4%)	16 (53.3%)	0.121
G	30 (36.6%)	14 (46.7%)	
MTHFD1 G1958A			
G	46 (56.1%)	16 (53.3%)	0.794
A	36 (43.9%)	14 (46.7%)	

Data represents number of alleles for each group and percentage (%) within each group.

Appendix 17: Distribution of various fetal alleles involved in one carbon metabolism genes in low risk healthy pregnancies and IUGR

Fetal	Control (n = 41)	IUGR (n = 18)	Chi-square P
MTHFR C677T			
C	52 (63.4%)	29 (80.6%)	0.307
T	30 (36.6%)	7 (19.4%)	
MTHFR A1298C			
A	57 (69.5%)	25 (69.4%)	0.688
C	25 (30.5%)	11 (30.6%)	
MTR A2756G			
A	64 (78.0%)	27 (75.0%)	0.717
G	18 (22.0%)	9 (25.0%)	
MTRR A66G			
A	52 (63.4%)	26 (72.2%)	0.736
G	30 (36.6%)	10 (27.8%)	
MTHFD1 G1958A			
G	46 (56.1%)	16 (44.4%)	0.243
A	36 (43.9%)	20 (55.6%)	

Data represents number of alleles for each group and percentage (%) within each group.

Appendix 18: Distribution of various fetal genotypes involved in one carbon metabolism genes from high risk pregnancies with normal and adverse pregnancy outcomes

Fetal	Normal (n = 21)	Adverse (n = 55)	Chi-square P
MTHFR C677T			
CC	8 (38.1)	26 (47.3%)	
CT	11(52.4%)	24 (43.6%)	0.763
TT	2 (9.5%)	5 (9.1%)	
MTHFR A1298C			
AA	9 (42.9%)	29 (52.7%)	
AC	12 (57.1%)	20 (36.4%)	0.126
CC	0 (0.0%)	6 (10.9%)	
MTR A2756G			
AA	11 (52.4%)	32 (58.2%)	
AG	7 (33.3%)	16 (29.1%)	0.901
GG	3 (14.3%)	7 (12.7%)	
MTRR A66G			
AA	7 (33.3%)	18 (32.7%)	
AG	12 (57.1%)	30 (54.5%)	0.927
GG	2 (9.5%)	7 (12.7%)	
MTHFD1 G1958A			
GG	7 (33.3%)	16 (29.1%)	
GA	7 (33.3%)	29 (52.7%)	0.240
AA	7 (33.3%)	10 (18.2%)	

Data represents number of fetal genotypes and percentage (%) from the total study group.

Appendix 19: Distribution of various fetal genotypes involved in one carbon metabolism genes in low risk healthy (control) pregnancies and those who develop preeclampsia

Fetal	Control (n = 41)	PE (n = 15)	Chi-square P
MTHFR C677T			
CC	18 (43.9%)	6 (40.0%)	
CT	16 (39.0%)	9 (60.0%)	0.226
TT	7 (17.1%)	0 (0.0%)	
MTHFR A1298C			
AA	19 (46.3%)	5 (33.3%)	
AC	19 (46.3%)	8 (53.3%)	0.563
CC	3 (7.3%)	2 (13.3%)	
MTR A2756G			
AA	23 (56.1%)	12 (80.0%)	
AG	18 (43.9%)	2 (13.3%)	0.051
GG	0 (0.0%)	1 (6.7%)	
MTRR A66G			
AA	16 (39.0%)	4 (27.7%)	
AG	20 (48.8%)	8 (53.3%)	0.543
GG	5 (12.2%)	3 (20.0%)	
MTHFD1 G1958A			
GG	14 (34.1%)	4 (26.7%)	
GA	18 (43.9%)	8 (53.3%)	0.782
AA	9 (22.0%)	3 (20.0%)	

Data represents number of fetal genotypes and percentage (%) from the total study group.

Appendix 20: Distribution of various fetal genotypes involved in one carbon metabolism genes in low risk healthy normal (control) pregnancies and those who develop IUGR

Table 36. Distribution of fetal polymorphisms in control and IUGR pregnancies

Fetal	Control (n = 41)	IUGR (n = 18)	Chi-square P
MTHFR C677T			
CC	18 (43.9%)	11 (61.15)	
CT	16 (39.0%)	7 (38.9%)	0.144
TT	7 (17.1%)	0 (0.0%)	
MTHFR A1298C			
AA	19 (46.3%)	9 (50.0%)	
AC	19 (46.3%)	7 (38.9%)	0.817
CC	3 (7.3%)	2 (11.1%)	
MTR A2756G			
AA	23 (56.1%)	11 (61.1%)	
AG	18 (43.9%)	5 (27.8%)	0.074
GG	0 (0.0%)	2 (11.1%)	
MTRR A66G			
AA	16 (39.0%)	10 (55.6%)	
AG	20 (48.8%)	6 (33.3%)	0.530
GG	5 (12.2%)	2 (11.1%)	
MTHFD1 G1958A			
GG	14 (34.1%)	3 (16.7%)	
GA	18 (43.9%)	10 (55.6%)	0.382
AA	9 (22.0%)	5 (27.8%)	

Data represents number of fetal genotypes and percentage (%) from the total study group.

Appendix 21 Distribution of various maternal alleles involved in one carbon metabolism genes in low risk healthy normal (control) pregnancies and those who develop UPI in their index pregnancy

Maternal	Control (n = 41)	UPI (n = 24)	Chi-square P
MTHFR C677T			
C	53 (63.8%)	29 (61.7%)	0.806
T	30 (36.2%)	18 (38.3%)	
MTHFR A1298C			
A	64 (64.0%)	28 (70.0%)	0.499
C	36 (36.0%)	12 (30.0%)	
MTR A2756G			
A	64 (67.4%)	18 (51.4%)	0.094
G	31 (32.6%)	17 (48.6%)	
MTRR A66G			
A	45 (63.4%)	37(62.7%)	0.937
G	26 (36.6%)	22 (37.3%)	
MTHFD1 G1958A			
G	25(46.3%)	23 (30.3%)	0.061
A	29 (53.7%)	53 (69.7%)	

Appendix 22 *CpG*lobal™ Protocol in detail.

The AmberGen *CpG*lobal™ kit provides sufficient material to analyse 8 DNA samples in triplicate.

A) Digestion of DNA (HpaII, MspI, undigested control)

1) Placement of DNA samples in assay plate:

- Aliquot 10µl of a 10 ng/µl dilution of the DNA sample to 9 wells of the provided 96-well white plate. Place DNA sample 1 in Row A columns 2 to 4, 6 to 8, and 10 to12). DNA samples 2, 3, 4, 5, 6, 7 and 8 are placed in Rows B, C, D, E, F, G and H respectively using the same column configuration outlined above.

Plate setup for 8 samples

2) Preparation of enzyme mixes:

- Spin down enzyme tubes briefly to get contents to bottom.
- Hpa II Mix: Add entire contents of **Hpa II tube** (14 µl) to 546 µl of **Buffer 1** and mix well by gentle agitation and inversion.
- Msp I Mix: Add entire contents of **Msp I tube** (7 µl) to 546 µl of buffer 1 and 7 µl distilled water (provided) and mix well by gentle agitation and inversion.
- No Enzyme Mix: To 546 µl of **Buffer 1** add 14 µl distilled water (provided) and mix well by gentle agitation and inversion.
- Add 20 µl of the Mix solutions to the following wells
    - Hpa II Mix to columns 2 to 4,
    - Msp I Mix to columns 6 to 8,
    - No Enzyme Mix to columns 10 to 12.

3) Mix the content of the wells by pipetting up and down 4 times using a multi-channel pipette.

4) Seal the plate with a foil or plastic film seal, spin briefly and put in a 37°C air incubator for 2 hr.

5) After the 37°C incubation spin plate briefly.

B) Biotinylation of DNA

1) Prepare biotinylation mix.

- Add the entire contents of the **biotinylation enzyme** (80 µl) to the **biotinylation buffer tube** and mix well by gentle agitation and inversion.

2) Add 20 µl of biotinylation mix per well.

3) Mix with a multi channel pipette.

4) Seal the plate with a foil or plastic film seal, spin briefly and put in a 37°C air incubator for 30 minutes.

5) After incubation spin plate briefly.

C) Binding of DNA to Assay Plate

1) Aliquot 100 µl of **DNA Binding Solution** to the wells.

2) Mix the contents of the well (150 µl) using a multi-channel pipette with 200 µl tips. Mixing is accomplished by pipetting up and down 4 times.

3) Seal the plate with a plastic film seal, spin briefly and incubate overnight on an orbit shaker platform at room temperature at medium speed (150 rpm).

Preparation of **Wash Solution 1**:

- Add the entire contents of the **10X wash solution 1** (12 ml) to 108 ml of distilled water.

4) Remove the plate from the orbit shaker platform and aspirate the solution in the wells. An 8 or 12 channel vacuum aspirator works well.

5) Wash the wells once with 200 µl **Wash solution 1** and 3 times with 400 µl **Wash solution 1**.

D) Assay for Biotinylation

Pre-block

1) Preparation of **Blocking Solution**:

Add the entire contents of **Blocking Powder** to 24 mL water and stir on a magnetic stirrer until completely dissolved (about 10 to 15 minutes). Add all 6 ml of the **5X Blocking Liquid**.

2) Add 200 µl of **Blocking Solution** per well using a multi-channel pipette. Incubate the plate at room temperature without shaking for 30 minutes.

Add detection reagent

3) To prepare the **Biotin Detection Reagent** add the entire contents (6 µl) of the **Biotin Detection Reagent** to 12 ml of **Blocking Solution**.

4) The **Blocking Solution** is removed by aspiration.

5) 150 µl per well of **Blocking Solution** containing **Biotin Detection Reagent** is added.

6) Incubate the plates at room temperature without shaking for 30 minutes.

Detect biotinlyation

7) To prepare **Wash Solution 2** add the entire contents of **10X Wash Solution 2** (14 ml) to 136 ml water.

8) Prewarm **Luminescence Reagent A** and **Luminescence Reagent B** to 37°C.

9) Aspirate the **Blocking Solution** containing **Biotin Detection Reagent** from the wells.

10) Wash the wells once with 200 µl **Wash Solution 2** and 4 times with 400 µl Wash Solution 2.

11) To prepare **Luminescence Reagent Mix** add the entire contents of **Luminescence Reagent A** to the tube containing **Luminescence Reagent B**.

12) Add 150 µl **Luminescence Reagent Mix** per well.

13) After 1 to 2 minutes, read the luminescence of the plate on a luminometer.

15.1.1.1 *Calculate Methylated Cytosine Fraction*

14) Calculate average values for luminescence of triplicates for "no enzyme control" to determine background signal. Subtract average luminescence for no enzyme control from individual Hpa II and Msp I values to get net luminescence.

15) Calculate average net luminescence for MspI

16) Divide individual Hpa II net luminescence values by average Msp I net luminescence to get proportion of unmethylated cytosine (Unmet Cyt).

18) The fraction of methylated cytosine is equal to 1- Unmet Cyt.