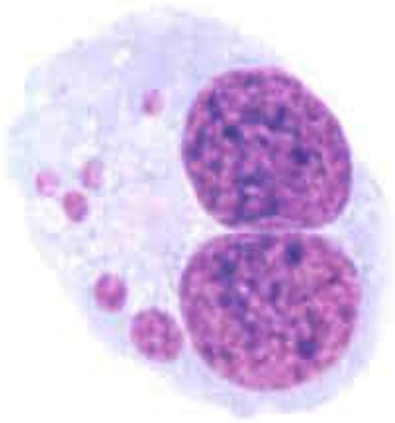

Diet and DNA damage in infants
The DADHI study

Mansi Dass Singh

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The DADHI study

Mansi Dass Singh

MSc (Nutrition & Dietetics)

A thesis submitted for the degree of Doctor of Philosophy

University of Adelaide, School of Health Sciences

Discipline of Obstetrics and Gynaecology

And

CSIRO Health & Biosecurity

Genome Health and Personalised Nutrition

November 2016

This thesis is dedicated to my guide and father Mr Harikishan Dass

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Abstract

Accumulation of DNA damage during infancy may increase risk of accelerated ageing and degenerative diseases such as cancers. Pregnancy is understood to be a state of high expression of inflammatory genes. It may be possible that infants, born to women at high risk of pre-eclampsia (PE): a condition associated with increased oxidative stress, inflammation and altered gene expression, may have increased DNA damage compared with infants born to women at low risk of developing PE. However, currently there are no baseline DNA damage data for infants born to mothers in relation to their low/high risk of developing PE in Australia.

This PhD project had four phases:

***A systematic literature search** was conducted with the aim to explore the literature and identify knowledge gaps in the role of folate in the etiology and prevention of PE. The review found (i) deficiency of folate and other B vitamins, with higher concentrations of oxidative stress biomarkers in maternal tissues and body fluids of women with PE when compared with women at low risk of PE, and (ii) some of this dysregulation may be balanced epigenetically with oral intake of methyl donors including folate and vitamins $B_{2.}$

***A prospective cohort study** was conducted; 'Diet and DNA damage in Infants' (The DADHI study), with the aim to study:

(i) DNA damage, cytostasis, and cytotoxicity utilizing a comprehensive Cytokinesis block micronucleus cytome (CBMN-Cyt) assay in lymphocyte of Australian born infants [at birth (cord blood, n=82), 3 (n=64) and 6 months (n=53) (heel prick blood)] of mothers at low risk of PE

(ii) association of maternal factors and infant birth outcomes with CBMN-Cyt biomarkers

(iii) whether mode of feeding influences CBMN-Cyt biomarkers in infants at 3 and 6 months after birth

This study found significant positive associations of infant birth outcomes (gestation age, birth weight, head circumference, birth length and APGAR score) and maternal anthropometric variables with CBMN-Cyt biomarkers, suggesting possible genotoxic effects on infant's DNA by metabolic processes that promote excessive growth and higher body mass index.

* The next aim was to determine

(i) association of **blood micronutrient status** with CBMN-Cyt biomarkers in cord blood at birth and infant's blood at 3 and 6 months

(ii) whether mode of feeding influences blood micronutrient status at 3 and 6 months after birth

The study observed significant associations of DNA damage biomarkers with infant birth outcomes and micronutrient status suggesting that both under and oversufficiency of some nutrients may be detrimental for cell growth and repair.

*A **pilot project** [in 'Investigations in the Folic acid clinical trial' (INFACT study)] with the aim to collect DNA damage data in the cord blood collected from infants of women at increased risk of developing PE. The study found that (i) maternal anthropometric variables may influence infant birth outcomes, mainly birth size, and (ii) INFACT cases (n=10) had higher frequency of CBMN-Cyt biomarkers compared with gender and birth weight matched DADHI controls (n=15).

These preliminary data could be used to form the design of larger studies required to confirm the association of maternal factors and PE with DNA damage in the infants at birth and later in life in the first 1000 days.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, 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. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Mansi Dass Singh (----------2017)

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And last but above all, my Father who has been the inspiration, initiator and motivating luminous for my dreams and aspirations and mom for her endearing and blessings.

Abbreviations

8-OHdG: 8-hydroxy-2′- deoxyguanosine
5-methyl THF: 5 methyl tetrahydro folate
5-LTR: 5-long terminal repeat

AOAC: Association of official analytical methods
ATP: Adenosine triphosphate
ADP: Adenosine diphosphate
ATM: Ataxia-telangiectasia mutated
ANOVA: Analysis of variance

BNC: Binucleated lymphocyte cells
BMI: Body mass index
BF: Breast fed
BP: Blood pressure

CBMN-Cyt: Cytokinesis block micronucleus-cytome assay
CO_2: Carbon dioxide
CH3: methyl group
Cob: Cobalamin
Cfu: Colony forming units
CVD: Cardiovascular disease
CI: Confidence interval
Cyto-B: Cytochalasin-B
CpG: cytosine-phosphate-guanine
CSIRO: Commonwealth Scientific and Industrial Research Organisation
CV: Coefficient of variation
CB: Calibration blank
CIROS: circular optical systems
COBRA: combined bisulfate restriction analysis
COMT: catechol-*O*-methyltransferase
CRH: corticotropin-releasing hormone
CT: cytotrophoblasts

DADHI: Diet and DNA damage in Infants
DHF: Di hydrofolate
DNA: Deoxyribonucleic acid
d-ROM: derivatives of reactive oxygen metabolites

dUMP: deoxy uridine monophosphate
dTMP: deoxy thymidine monophosphate
dTTP: deoxy thymidine triphosphate
dUMP: deoxy uridine monophosphate
DMSO: Dimethylsulphoxide
DS: Down syndrome

EDTA: Ethylene diamine tetra acetic acid
ELISA: Enzyme-linked immunosorbent assay
FA: Folic acid
FFQ: Food frequency questionnaire
FBS: Foetal Bovine serum
FAn: Fanconi Anemia
FACT: Folic Acid Clinical Trial
GA: Gestation age
HELLP: haemolysis, elevated liver enzymes, low platelet count
HIF-1α: hypoxia induced factor-1α
Hcy: Homocysteine
HBSS: Hanks Balanced Salt solution
HPLC: High Performance Liquid Chromatography
HT: Hypertension
IUGR: Intrauterine growth restriction
IGF: Insulin growth factor
IMVS: Institute of Medical and Veterinary Science
IRR: Incident rate ratio
IVF: In vitro fertilization
ICP: Inductively coupled plasma analysis
ICPAES: Inductively coupled plasma atomic emission spectrometry
IQ: Intelligence quotient
INFACT: Investigations in Folic Acid Clinical trial
ICAM-1: intercellular adhesion molecule-1
ICR: imprinting control region

L *casei: Lactobacillus casei*
LBW: Low birth weight
LGA: Large for gestational age
LOD: Limit of detection

MTHF: Methyl tetrahydro folate
MTHFD1: methylenetetrahydrofolate dehydrogenase
MTHFR: methylenetetrahydrofolate reductase
MTRR: methionine synthase reductase
MTR: methionine synthase
MN: Micronuclei
MNC: Mononucleated lymphocyte cells
MMA: Methylmalonic acid
MDA: malondialdehyde
MS: Microsoft

MA: Microbiological assay
MRL: method reporting limits
MMP: matrix metalloproteinase
MS-SNuPE: methylation-sensitive single-nucleotide primer extension

NHANES: National Health and Nutrition Examination Survey
NHMRC: National Health and Medical Research Council's levels of evidence
NPB: Nucleoplasmic bridges
NBUD: Nuclear buds
NDI: Nuclear division index
NTD: Neural tube defects
NSW: New South Wales

OR: Odd ratio
OCM: One carbon metabolism
OSI: oxidative stress index

PE: Pre-eclampsia
PCR: Polymerase chain reaction
p: significance value
PHA: Phytohemagglutinin
PABA: Para amino benzoic acid
PBL: Peripheral blood lymphocyte
PTPE: preterm pre-eclampsia

RCT: randomized controlled trial
RBC: Red blood cells
RCF: red cell folate
r: correlation coefficient
RR: relative risk
RNA: Ribonucleic acid
ref-1: redox factor
RT-PCR, reverse transcription polymerase chain reaction

SD: standard deviation
SEM: standard error of mean
SAM: S-adenosylmethionine
SAH: S-adenosyl homocysteine
SGA: Small for gestation age
SSE: sister chromatin exchange
THF: tetra hydro folate
TNF: Tumor necrosis factor
TLR-9: toll like receptor-9
TS: thymidylate synthase
TAS: total antioxidant status
TOS: and total oxidant status
WCH: Women's and Children Hospital

Publications arising from this thesis

1. Singh MD, Thomas P, Owens J, Hague W, Fenech M, 2005. 'Potential role of folate in Pre-eclampsia', Nutrition Reviews .Oct; 73 (10):694-722. Impact factor 6
2. Singh MD, Thomas P, Hor M, Almond T, Owens J, Hague W, Fenech M 2016. 'Infant birth outcomes are associated with DNA damage biomarkers as measured by CBMN-Cyt assay-The DADHI study'. Submitted with major revisions to Mutagenesis journal

Presentations arising from this thesis

1. 'Genome stability of infants as measured by CBMN-Cyt assay and influence of feeding during six months after birth' at Nutrition society of Australia-Adelaide Student presentation event, 19 November 2015
2. 8th Congress of the International Society of Nutrigenetics/Nutrigenomics 2-3 May 2014, Gold Coast, Australia
3. Florey postgraduate Research Conference, 24th September, 2015
4. Joint Annual Scientific Meeting of the Nutrition Society of NZ and the Nutrition Society of Australia, 1st - 4th December 2015
5. 'Genome stability in lymphocytes of South Australian babies as measured by Cytokinesis Block Micronucleus assay', Oral presentation as part of Annual review at joint HDR seminar programme for the Disciplines of Obstetrics and Gynaecology and Robinson Institute, 12th March 2015
6. Folate and Genome Integrity in Infants', Oral presentation as part of Annual review at joint HDR seminar programme for the Disciplines of Obstetrics and Gynaecology and Robinson Institute, 10th June 2014
7. Diet and DNA Health in Infant', Oral presentation at CSIRO Nutrigenomic Laboratory, June 2014

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Mansi Dass Singh (Candidate)
Wrote manuscript and contributed to planning of the article.

Certification
This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.

22.11.16.

Phil Thomas
Provided critical evaluation of the manuscript

Signed by Prof. Michael Fenech on behalf of Dr. Phil Thomas

Date 29/11/2016

Julie Owens
Provided critical evaluation of the manuscript

Date 24/11/16

William Hague
Contributed to planning of the article and provided critical evaluation of the manuscript

Date 23/11/16

Michael Fenech
Contributed to planning of the article, methodology and provided critical evaluation of the manuscript

Date 22/11/2016

1 Literature review: The potential role of folate in pre-eclampsia

1.1 Abstract

Dietary deficiencies of folate and other B vitamin cofactors involved in one carbon metabolism, together with genetic polymorphisms in key folate-methionine metabolic pathway enzymes, are associated with increases in circulating plasma homocysteine, reduction in DNA methylation patterns and genome instability events. All of these biomarkers have also been associated with pre-eclampsia. The aim of this review is to explore the literature and identify potential knowledge gaps in relation to folate's role at the genomic level in either the etiology or prevention of pre-eclampsia. A systematic search strategy was designed to identify citations in electronic databases for the following terms: Folic acid supplementation AND Pre-eclampsia, Folic acid supplementation AND genome stability, Folate AND genome stability AND Pre-eclampsia, Folic acid supplementation AND DNA methylation, Folate AND DNA methylation AND Pre-eclampsia. 43 articles were selected according to predefined selection criteria**.** The studies included in the present review were not homogeneous that made poled analysis of data very difficult. The present review highlights associations between folate deficiency and certain biomarkers observed in various tissues of women at risk of pre-eclampsia. Further investigation is required to understand role of folate in either etiology or prevention of pre-eclampsia.

1.1 Introduction

1.1.1 Pre-eclampsia

The Society of Obstetric Medicine of Australia and New Zealand defines Pre-eclampsia (PE) as a "multi-system disorder characterized by hypertension (HT) and the involvement of one or more other organ systems and/or the foetus"(1). *De novo* HT (≥140/90 mmHg after 20

weeks gestation) is commonly (but not always) the first manifestation of PE. Proteinuria or other evidence of multisystem dysfunction, such as abnormal liver and/or renal function tests and/or thrombocytopenia and/or evidence of placental insufficiency, may also be observed among women affected by PE (1). PE affects approximately 5-7% of pregnancies all over the world (2). Epidemiological data show that women who have experienced PE are more prone to develop HT (3,4), renal disease and cardiovascular disease (5-8) later in life. PE is also associated with intra-uterine growth restriction (IUGR) (9), small for gestational age (10) and preterm delivery of the foetus (11). PE may be classified as early-onset pre-eclampsia (diagnosis prior to 34 weeks) and late-onset pre-eclampsia (diagnosis after 34 weeks gestation) (12). Although the exact cause is still unknown, genetic and epigenetic features are being explored to explain the pathogenesis of PE (13), which may influence this two-stage disorder. The first stage is marked by defective trophoblast invasion during early implantation (14,15) that may contribute to release of vasoactive agents such as nitric oxide (16,17) and subsequent remodelling of the uterine spiral arteries (18). These reactions manifest into defective uteroplacental blood circulation and ensuing placental ischemia (19). This ultimately leads to a second stage of systemic inflammatory responses and maternal endothelial dysfunction leading to manifestation of clinical symptoms (15).

Numerous studies have reported increased plasma or serum homocysteine (Hcy) among women with PE, suggesting that Hcy may be an independent risk factor for this disorder (20-29). Hcy promotes the generation of hydrogen peroxide and oxygen-derived free radicals through the oxidation of its sulfhydryl component (30,31). This results in abnormal changes to the vascular endothelial cell cytoskeleton, acceleration of LDL oxidation and blood vessel thickening (32). Hcy may also induce apoptosis in human umbilical vein endothelial cells and smooth muscle cells by accumulation of unfolded proteins in the lumen of the endoplasmic

reticulum (33). It may also increase thromboxane formation, increase leucocytes adhesion to endothelial cells and increase the concentration of pro-inflammatory cytokines within blood vessels (34). Hcy down regulates intracellular glutathione peroxidase leading to a decrease in bioactive nitric oxide which is body's primary vasodilator as observed in aortic endothelial cell cultures (35). Thus Hcy may either cause maternal endothelial dysfunction through oxidative stress (36) or may interfere with nitric oxide function leading to placental vasoconstriction and ischemia in PE (37). However, whether Hcy is causative or is merely a bystander in the process remains unclear (38).

At present, diagnosis, and treatment and early prevention of PE are limited by the absence of reliable biomarkers to detect PE prior to manifestation of classic clinical symptoms. Current prevention strategies for PE include early screening for those with risk factors, such as obesity, chronic HT, renal disease, autoimmune disorders, diabetes, previous and family history of PE (39), and assessment of poor placentation with first trimester pregnancy- associated plasma protein A measurements (40) and second trimester uterine artery Doppler resistance indices (41,42). This is followed by careful monitoring for the associated clinical signs and symptoms of PE, such as the development of proteinuria (43). Furthermore, use of aspirin (50-150 mg/d) may have small to moderate benefits in reducing the risk of PE, mainly when treatment is commenced before 16 weeks of gestation (44-50). Women at high risk of PE may also benefit from calcium supplementation (0.6-1.0 g/d), especially if the usual dietary intake of calcium is low (51-56), Vitamin D (57-60) and L-arginine (61) supplementation. Other dietary components have also been explored to provide a protective therapy against the development of PE such as low salt intake (62,63), fish oil containing n-3 fatty acids (64), garlic (65), protein and energy restriction in obese women (48,50), high fiber, potassium (66) and antioxidants (vitamin C and E) (67-70), all with discouraging results. Some studies, however,

conducted over the last 2 decades have shown that folic acid (FA) supplementation may have protective effects on reducing PE risk (71-73).

1.1.2 **Folate**

Folate (Vitamin B_9) is an essential water soluble vitamin, required for DNA synthesis and repair, as well as for methionine regeneration (74). Folate acts as a methyl donor in single carbon reactions that are important in amino acid metabolism and various biosynthetic pathways (75), and in the establishment and maintenance of epigenetic patterns (76). The term 'folic acid' (pteroylmonoglutamic acid) refers to the synthetic monoglutamate non-reduced and non-methylated form of the vitamin, which is used in supplements and food fortification (77). The term 'folate' generally applies to all forms of the vitamin, both dietary and synthetic (78). Mammals cannot synthesize folate *de novo* and hence, it must be acquired from the dietary intake of foods rich in folate, such as green vegetables (asparagus, broccoli, and spinach), legumes, liver (79), aleurone flour (milled from wheat germ cell wall) (80) and foods fortified with FA such as wheat flour used for making bread (81) in order to avoid deficiency. Folate is transported across the cell membrane either by a membrane carrier or a folate-binding protein, such as the reduced folate carrier, a transmembrane protein that mediates the uptake of serum 5-methyl tetrahydro folate (THF) across most tissues in the body (82). The emerging importance of folate in epigenetic and genetic mechanisms (83) may be best understood through the participation of folate in one carbon metabolism (OCM) (84) (**Figure 1.1**) as a methyl donor along with vitamins B_2, B_{12} and B_6 as essential cofactors (85). Under normal dietary conditions, absorbed folate is metabolized to 5-methyl THF in the intestine/liver and subsequently to 5,10-methylene THF within all tissues where it is required for the synthesis of deoxythymidine triphosphate from deoxyuridine monophosphate (77). Both *in vitro* and *in*

vivo folate deficiency cause excessive incorporation of uracil into DNA, leading to genome instability events, such as single and double strand breaks, chromosome breakage and ultimately micronucleus formation, a robust and validated biomarker of whole chromosome loss and/or breakage (86-88).

Alternatively in OCM, 5-methyl THF participates in the synthesis of methionine through the remethylation of Hcy, utilizing B_{12} as a cofactor, and subsequently synthesis of S-adenosylmethionine (SAM) (89). SAM is the universal methyl donor in over 100 methylation reactions, including genomic methylation, and after donating its methyl group, is converted to S-adenosylhomocysteine (SAH) (90,91). As SAH is a competitive inhibitor of numerous methyl transferases, including DNA methyltransferase (91), the ratio of SAM to SAH determines the methylation capacity of a cell and subsequently gene expression (92).

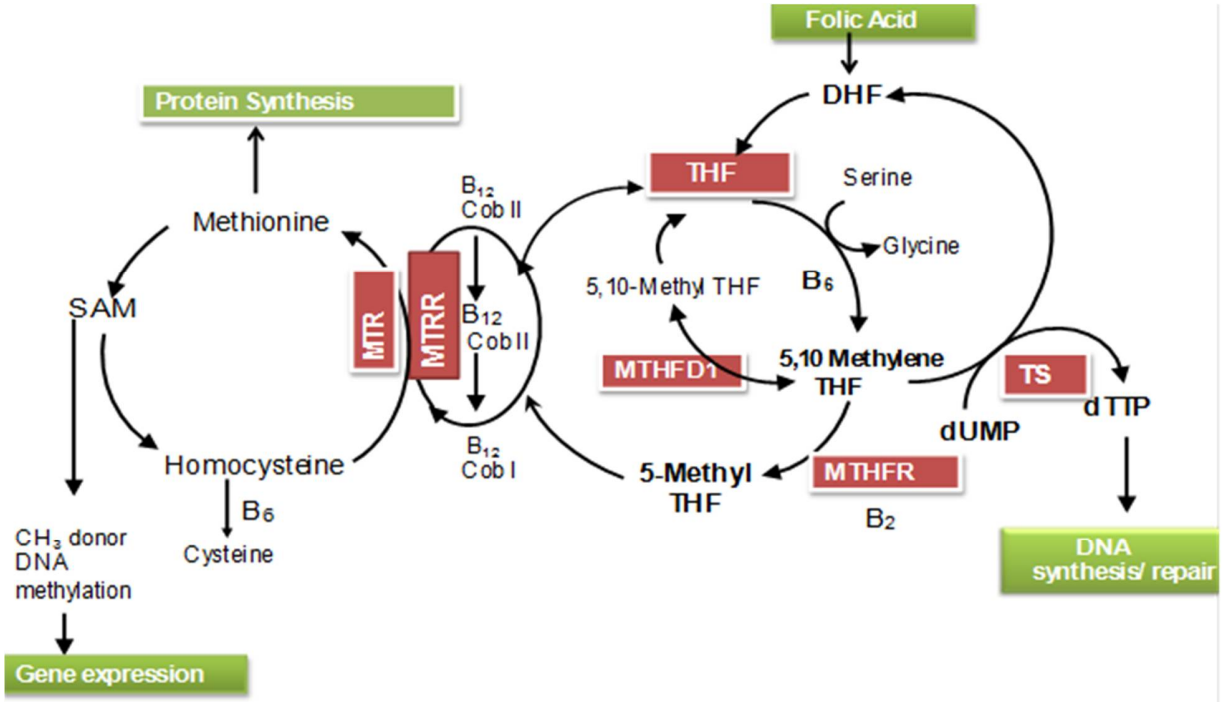

Figure 1.1: Scheme of one-carbon metabolism

Adapted from Hague 2003 and Furness *et al* 2008
Abbreviations: B_6, pyridoxine; B_2, riboflavin; CH_3, methyl group; Cob I, II, III, vitamin B_{12} in different oxidative stages; DHF, dihydrofolate; dTTP, deoxythymidine triphosphate; dUMP-deoxyuridine monophosphate, MTHFD1, methylenetetrahydrofolate dyhydrogenase; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; SAM, *S*-adenosyl methionine; THF, tetrahydrofolate; TS, thymidylate synthase.

1.1.3 **Current practice in assessing folate status**

Folate status may be assessed by measuring total folate in serum, plasma, or red blood cells (93). While recent changes in an individual's folate status may be indicated by serum folate, red blood cell folate reflects long term tissue folate stores (94). Commonly used laboratory methods include microbiological and protein binding assays (95,96). More recently, mass spectrometry methods

have been applied to measure individual folate one-carbon metabolites in human blood (97). Plasma Hcy can be considered a functional biomarker of folate status, as folate deficiency directly impairs conversion of Hcy to methionine in OCM, thus increasing plasma Hcy concentration (97).

1.1.4 **Assessing genome stability and oxidative stress**

Human genome is susceptible to damage by various exogenous (pollutants, UV radiation, smoking, etc.) and endogenous factors (free radicals) that manifest in oxidation, alkylation, hydrolysis, bulky adduct formation in DNA bases in human cells (98). When excessive oxidative damage exceeds the body's repair and antioxidant defence mechanisms it may lead to single and double strand breaks in cellular DNA, gene mutations and altered gene expression (99). These contributors to DNA damage may have particularly adverse consequences in early life when DNA synthesis is at its highest (100). There are a number of assays that can be used to measure oxidative stress, DNA damage and cellular response to DNA damage and oxidative stress during pregnancy including 8-hydroxy-2′- deoxyguanosine (8-OHdG): an oxidized form of guanine (101), 8-isoprostane (a marker of lipid peroxidation and excessive systemic oxidative stress) (102), activin A: a member of the transforming growth factor β family of cytokines (102), thioredoxin expression: a reductive enzyme involved in repair of oxidatively damaged proteins in various tissues including placenta (103), apurinic/redox factor-1: an essential enzyme in the DNA base excision repair possessing both DNA repair and redox regulatory activities (104), the terminal deoxynucleotidyl transferase-mediated or assay: direct method for the assessment of DNA fragmentation (105), the Comet assay (106) and phosphorylated H2AX (107): measure double strand breaks. The lymphocyte "cytokinesis block micronucleus cytome (CBMN-Cyt) assay is one of the most comprehensive and

best validated methods to measure chromosomal DNA damage in humans (108). In this assay, chromosomal damage is assessed by scoring micronuclei and other nuclear anomalies, such as nucleoplasmic bridges and nuclear buds (109). Micronuclei originate in dividing cells when either chromosome breaks, lacking centromeres (acentric fragments) and/or whole chromosomes (centromere positive) fail to move towards spindle poles during anaphase. The lagging acentric fragment or whole chromosomes are covered by a nuclear envelope during the subsequent telophase of the mitotic cycle. The displaced chromosomes or fragments then uncoil and slowly assume the morphology of an interphase nucleus which are smaller than the main cellular nucleus, hence named "micronucleus" (110) (**Figure 1.2**). Micronuclei frequency, therefore, provide a robust and reliable biomarker of both chromosome breakage and/or chromosome loss. An elevated micronuclei frequency in lymphocytes has been associated with anaemia (111), cancer (112,113), cardiovascular diseases (114), neurodegenerative diseases (115), reproductive and pregnancy complications including pregnancy loss (116), infertility (117) and PE (118). Moreover, micronuclei have been consistently shown to be sensitive to deficiency of micronutrient, such as of folate due to the induction of chromosome fragmentation or malsegregation (119).

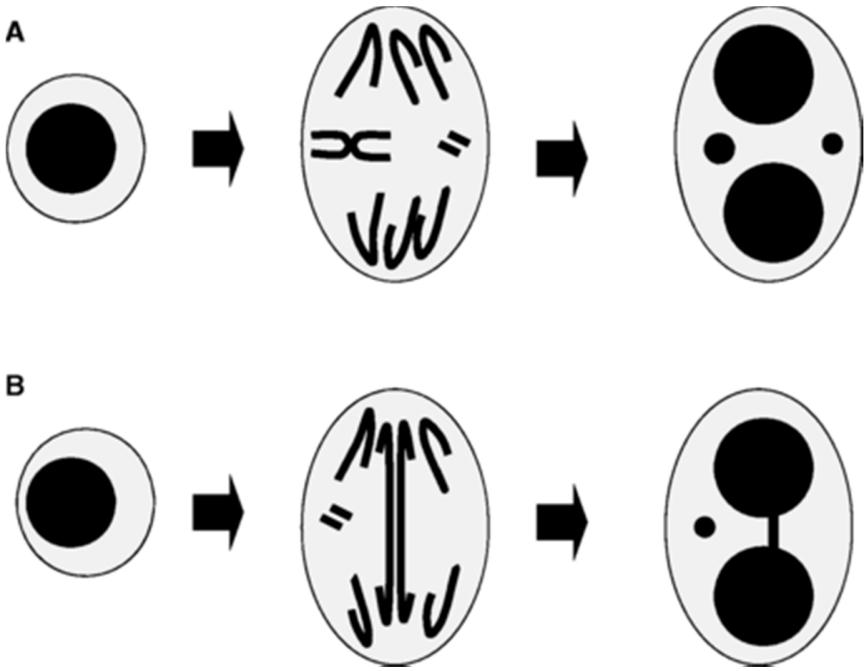

Figure 1.2: Diagrammatic representation of origin of micronuclei

(A) The origin of mononuclei from lagging whole chromosomes and acentric chromosome fragments at anaphase. (B) The formation of a nucleoplasmic bridge from a dicentric chromosome in which the centromeres are pulled to opposite poles of the cell, and the formation of a mononuclei from the accompanying acentric chromosome fragment. Fenech *et al* 2011

1.1.5 **Assessing DNA methylation and gene expression**

DNA methylation is one of the main epigenetic processes through which gene expression is modulated among humans (120). SAM donates methyl groups for the conversion of cytosine to methyl cytosine: a reaction catalysed by DNA methyltransferase (121). The cytosine nucleotide that precedes a guanosine nucleotide in the DNA sequence becomes covalently linked by phosphodiester bonds to form a CpG dinucleotide. These dinucleotide cluster in small stretches of DNA, termed CpG islands. 70% to 80% of the CpG sites in DNA contain methylated cytosine in

humans (122) which is associated with the silencing of genes (123). By contrast, most CpG islands in gene promoters of housekeeping genes are unmethylated and are associated with active expression of the gene (124).

Global DNA methylation may be quantified with bisulphite-based polymerase chain reaction (PCR) methods (125,126). However, global methylation does not give information on site specific DNA methylation in relation to specific gene expression, hence it is difficult to utilize such information in regard to potential roles in specific diseases (127).

DNA methylation analysis at specific gene loci principally includes sodium bisulphite modification of DNA, which converts unmethylated cytosine to uracil, without altering methylated cytosine (128). This is followed by the use of methylation-sensitive restriction enzymes to cleave DNA and by PCR with specific primers to distinguish between methylated and unmethylated DNA (129). *Gene-specific methylation* analysis applicable to candidate gene approaches include sensitive methods or quantitative methods such as Methylight and methylation sensitive PCR (130-132). Site specific *DNA methylation on a genome-wide scale* can also be assessed using microarrays or by pyro sequencing: sequencing-by-synthesis method (133-135).

Altered methylation status can then be further correlated with altered gene expression, using technologies available for analysing mRNA expression levels such as, northern blots, reverse transcription PCR microarrays, serial analysis of gene expression, comparative expressed sequence tag analysis, and massively parallel signature sequencing (136,137).

Hence, studies that have investigated genome stability events and global or gene specific methylation in various tissues of women with PE were assessed in this review, along with studies into the effect of FA supplementation among women at high risk of PE. The main objective of this

review was to explore the literature and identify potential knowledge gaps in relation to folate's role at the genomic level in either the aetiology or prevention of PE.

1.2 **Methods**

A systematic search strategy was designed (138) to identify citations from electronic databases for the following terms: Folic acid supplementation AND Pre-eclampsia, Folic acid supplementation AND genome stability, Folate AND genome stability AND Pre-eclampsia, Folic acid supplementation AND DNA methylation, Folate AND DNA methylation AND Pre-eclampsia. The search used the following databases: Medline, CINAHL, Web of Knowledge, Scopus, Academic Search Premier and Science Direct up till June 2014. The studies were selected in 2 stages (**Figure 1.3**). The abstracts were retrieved after the online search (n=1123), were reviewed and narrowed to 110 articles. The articles were further searched for relevant publications.

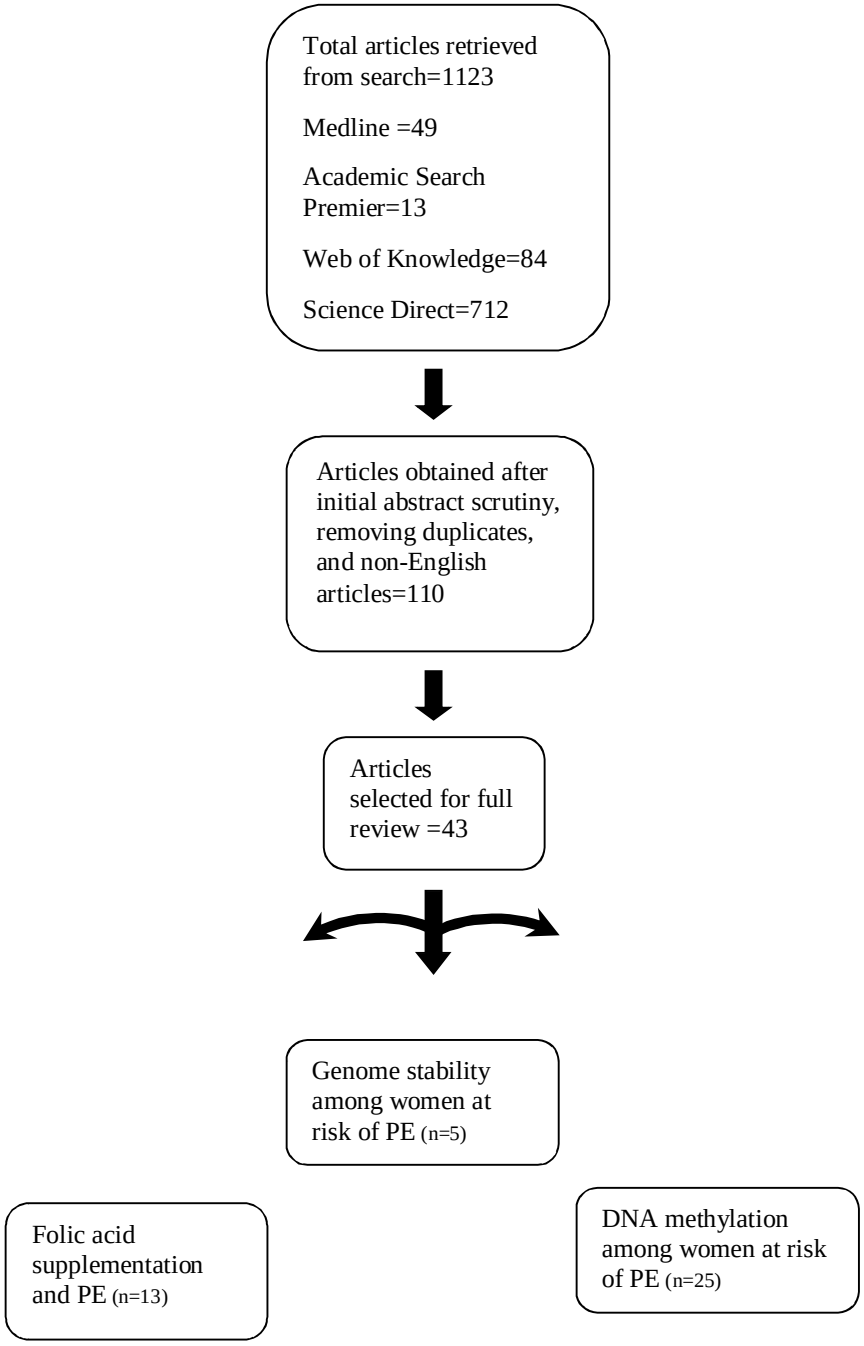

Figure 1.3: Flow chart of the search and selection process for research studies

The criteria for study inclusion were: Studies/ reviews that evaluated the effect of FA supplementation and/or folate status in women with PE; the primary or secondary outcome in the research studies was PE; PE diagnosed with at least one measurement of blood pressure (BP) (140/90mm Hg) and/or proteinuria; the role of folate status/supplementation studied in the context of differing genotype in pregnant women with PE; genome stability events studied in maternal blood for women at risk of PE; global and/or gene specific methylation patterns studied in tissues of women at risk of PE; only full-text English language articles and studies on animals were excluded. The articles were assigned a level of evidence, according to the Australian National Health and Medical Research Council criteria for level of evidence (**Table 1.1**) (139)

Table 1.1: Australian National Health and Medical Research Council's levels of evidence

Level of evidence	Type of studies
I	Evidence obtained from a systematic review of all relevant randomized controlled trials
II	Evidence obtained from at least 1 properly designed randomized controlled trial
III-1	Evidence obtained from well-designed pseudo randomized controlled trials (alternate allocation or some other method)
III-2	Evidence obtained from comparative studies (including systematic reviews of such studies) with concurrent controls: nonrandomized experimental trials, cohort studies, case–control studies, or interrupted time series with a control group
III-3	Evidence obtained from comparative studies with historical control, 2 or more single-arm studies, or interrupted time series without a parallel control group
IV	Evidence obtained from case series, with either post-test or pre-test/post-test outcomes

1.3 **Results and Discussion**

An online search for the terms 'Folic acid supplementation AND Pre-eclampsia, Folic acid supplementation AND genome stability, Folate AND genome stability AND Pre-eclampsia, Folic acid supplementation AND DNA methylation, Folate AND DNA methylation AND Pre-eclampsia' resulted in a total of 1123 articles. Two authors (MS and BH) independently assessed eligibility, using the predefined inclusion criteria. Any disagreements were resolved by discussion. A high number of duplicated results were obtained in the search of the different databases. The studies that were excluded either reported the effect of nutrients other than folate in women at risk of PE or their primary outcome was not PE. The studies selected on the basis of inclusion criteria (n=43) were then grouped into Genome stability in women at risk of PE (n=5) (**Table 1.2**), DNA methylation in women at risk of PE (n=25) (**Table 1.3**) and 'Folic acid supplementation in PE' (n=13) (**Table 1.4**) for a narrative synthesis. The diverse subject group and different type of variables studied across the articles selected prohibited statistical assessment of heterogeneity and meta-analysis.

1.3.1 **Genome integrity in women at risk of PE**

The extent of DNA damage can be measured by studying levels of oxidative stress markers in serum/plasma/lymphocytes/placenta of pregnant women (140), both at the DNA base sequence level and at the chromosomal and nuclear level (141). Increased oxidative damage in PE may be caused by elevated plasma Hcy (38), which has also been previously shown to be associated with increased micronuclei frequency in lymphocytes in young adults (87).

Studies that investigated DNA damage in relation to PE are outlined in **Table 1.2**. In all 5 studies were included consisting of one prospective study (118) and four case control studies (102,142-144). The first prospective cohort study to investigate the association between genome integrity and PE was conducted on women at both low risk (no previous history of adverse pregnancy outcomes such as PE) and high risk of adverse pregnancy outcomes (women with pre-existing condition of PE/HT/diabetes) in Australia (118). Increased micronuclei frequency, as measured by the CBMN-cyt assay, in maternal peripheral lymphocytes at 20 weeks gestation was associated prospectively with PE and IUGR. The odd ratios (OR) for PE and/or IUGR in the cohort of only high risk pregnancies (n=91) was 17.85 (p=0.007) if the micronuclei frequency was greater than 39 per 1000 cells (118). The study suggests that the frequency of micronuclei is increased in lymphocytes of women who later develop PE and/or IUGR compared with women with normal pregnancy outcomes. A case control study in Australia reported genome instability (micronuclei frequency and Nuclear buds) to be positively associated with Hcy concentrations in peripheral maternal blood of women at increased risk of PE (r=0.179, p=0.038 and r=0.171, p=0.047, respectively) (142). A recent case-control study in Japan, demonstrated that oxidative DNA damage, as measured by 8-OHdG was greater in the placenta of women with early onset of PE (143).

A further case control study in Australia reported a significant positive relation (r^2=0.72, p<0.001) between circulating levels of 8-isoprostane and activin A among women with PE (n=21) compared with normal pregnant women (n=20) (102). A case control study conducted in Japan observed significantly higher concentration of 8-OHdG among women with PE and IUGR (n=11) (p=0.0021), thioredoxin expression in PE (n=13) (p=0.045), and expression of redox factor-1 in

PE (p=0.017) as well as in PE and IUGR (p=0.0038) compared with normal pregnant women (n=23) (144). Interestingly, increased cellular 8-OHdG is correlated with formation of micronuclei in lymphocytes (109), while increased micronuclei have been consistently associated with low folate status (145,146). Further research in a cohort of women at risk of PE may help in explaining the significance of observed genome instability in relation to the folate deficiency and prognosis of PE. As a consequence, the CBMN-cyt assay, together with biomarkers of oxidative damage, may be useful as potential diagnostic markers for the early detection of PE.

Table 1.2: Studies of genome integrity in women at risk of pre-eclampsia

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
Furness *et al.* (2010)	South Australia	III-2	Prospective cohort	136 pregnant women: high-risk (n = 91) and low-risk (n = 41)	CBMN-cyt assay in lymphocytes collected at 20 weeks gestation	Increased DNA damage in maternal peripheral lymphocytes at 20 weeks gestation associated prospectively with PE and IUGR. When genome damage increased to a frequency of 36.7 micronuclei per 1000 binucleated cells, the OR of developing PE and/or IUGR was 15.97	First study to investigate the association between chromosomal DNA damage at midpregnancy and pregnancy outcomes in a cohort of women at high risk of PE
Kimura *et al* (2013)	Japan	III-2	Case–control	Women with uncomplicated pregnancies (n = 10), early-onset PE (n = 13), and late-onset PE (n = 12)	Immunohistochemical analysis conducted to measure the proportion of placental trophoblast cell nuclei staining positive for 8-OHdG and redox factor-1	The proportion of nuclei that stained positive for 8-OHdG was significantly higher in both PE groups compared with the control group, with a higher proportion in the early-onset PE group ($p < 0.001$) than in the late-onset PE group ($p < 0.05$)	

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
Mandang *et al.* (2007)	Australia	III-2	Case–control and *in vitro*	Women (26–40 weeks gestation) with established PE (n = 21) and gestationally matched healthy pregnant women (n = 20). Placental tissue (n = 11), umbilical cords (n = 6), and maternal peripheral blood (n = 6) from women with a healthy, singleton pregnancy undergoing an elective caesarean section at term (37–40 weeks gestation)	Serum isoprostane and activin A measured in the 2 groups of women. Trophoblast explants, human umbilical vein endothelial cells, and peripheral blood monocytes exposed to oxidative xanthine/xanthine oxidase in vitro	Maternal plasma levels of 8-isoprostane and activin A were significantly higher in women with PE than in controls (333.8 ± 70 vs 176.3 ± 26.2 pg/ml, $p = 0.04$, and 49.5 ± 7 vs 13.1 ± 1.2 ng/ml, $p < 0.001$, respectively). Serum 8-isoprostane and activin A significantly and positively correlated ($r^2 = 0.72$; $p < 0.001$) in women with PE vs women with normal pregnancy	Activin may be a useful marker of systemic oxidative damage, as observed in women with PE
Takagi *et al.* (2004)	Japan	III-2	Case–control	Placental tissues from 42 healthy women (6–40 weeks gestation) and women with PE (n = 24). For Western blotting,	Immunohistochemistry and Western blotting for 8-OHdG, 4-hydroxynonenal, thioredoxin, and redox factor-1 in the placentas of women with PE,	8-OHdG levels significantly higher in IUGR or PE+IUGR group compared with normal pregnancy; thioredoxin expression and redox factor-1 expression significantly higher in PE ($p = 0.017$),	Oxidative DNA damage as measured by 8-OHdG is increased in PE with IUGR but not in PE without IUGR. However, the redox

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
				placental tissue was collected from 8 women with a normal pregnancy (9–39 wk), 5 with PE (28–39 wk), 3 with IUGR (28–36 wk), and 1 with PE + IUGR (36 wk)	IUGR, PE+IUGR, or normal pregnancy	IUGR ($p = 0.016$), and PE + IUGR ($p = 0.0038$)	function is accelerated in both PE and IUGR
Furness *et al* (2013)	South Australia	III-2	Prospective case–control	Women (<20 weeks gestation) grouped as high (n = 91) or low risk (n = 46) of adverse pregnancy outcomes	Demographic, clinical, and dietary data along with fasting blood samples collected at 18–20 weeks gestation. Detailed information collected on type and dose of multimicronutrient supplement consumption	Maternal folate and plasma Hcy were not increased at 18–20 weeks gestation in those who developed PE. Micrononuclei frequency and nucleoplasmic buds in lymphocytes were positively correlated with Hcy ($r = 0.179$, $p = 0.038$, and $r = 0.171$, $p = 0.047$, respectively). Multivariate regression analysis showed that RBC folate was a strong predictor of IUGR ($p = 0.006$)	Despite high-dose supplementation with FA in women with high-risk pregnancies, RBC folate was similar to, and plasma Hcy was lower but not statistically different from, that in women with low-risk pregnancies ($p = 0.095$)

Abbreviations: CBMN-cyt, cytokinesis-block micronucleus cytome assay; FA, folic acid; Hcy, homocysteine; IUGR, intrauterine growth restriction; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; OR, odds ratio; PE, pre-eclampsia ;*r*, correlation coefficient for bivariate analysis; r^2, coefficient of determination for bivariate analysis; RBC, red blood cells

1.3.2 **DNA methylation in women at risk of PE**

A number of studies have investigated both gene specific and global methylation in diverse tissues of women with PE, identifying large numbers of genes whose expression is either up-regulated or down-regulated in various tissues collected from women with PE. These researchers have also been able to correlate specific gene expression with CpG methylation patterns in promoter regions of these genes and have thus paved the way towards identifying key biomarkers in the development of PE. There were 25 studies included in the present review that investigated methylation patterns in diverse tissues of women at risk of PE: 22 case control studies, two prospective studies, one review as outlined in **Table 1.3**. Hyper methylation and reduced expression of genes encoding various proteins involved in placental implantation involving trophoblast invasive functions have been discovered in placentae from women with PE. Examples of these include ASTN1 (cell adhesion), ABC 6, MOVI0 (ribonucleotide binding) (147), NR3C1 (glucocorticoid receptors), CRHBP (corticotrophin releasing hormone binding) (148), H-19 (trophoblast invasion) (149), syncytin-1 (cell fusion and trophoblast invasion) (150-152), and also genes involved in transcription, lipid metabolism, membrane transport and the immune system (153).

Conversely, significant over-expression of certain genes has been attributed to decreased methylation in the placental tissue of patients with PE, such as VEGF (154), EPAS1 and FLT1 (155) (angiogenic factors), TIMP3 (matrix metalloproteinase inhibitor) (156,157), LAIR-2 (gene encoding for a trophoblast protein), DNAJC5G (gene coding a neuroprotective protein), LAMA3 (gene encoding laminins that are important for endothelial repair) (158), LEP (encoding for protein

for regulatory function in reproductive maturity) (159,160), placental matrix metalloproteinase 9 (MMP9; a member of family of zinc-dependent proteases that may interfere extra villous trophoblast invasion) (161) and SERPIN3A (homeostasis in inflammation and coagulation pathway) (134,162). Some studies have also reported non-association of hypomethylation in certain genes (COMT promoter and H19/IGF2) in the placentae of women with PE (163,164).

In addition to these placental studies, maternal omental arteries, leucocytes, cell free and cell free foetal DNA in maternal plasma have also been investigated for both global and gene specific methylation status (165-169), with the aim of identifying a biomarker for pre-symptomatic diagnosis of PE. The primary outcome of these studies confirmed considerable differences in methylation patterns of some genes among women with PE compared with normal pregnant women. These mainly involved reduced methylation of inflammatory genes in omental arteries (166), foetal-derived hypermethylated RASSF1A (tumour suppressor gene) sequences in maternal plasma (165,169), placental-derived hypermethylated RASSF1A in maternal plasma (167) and hypermethylation of genes (involved in neuropeptide signalling pathway and seizures) observed in maternal leucocytes (168). Thus, it is speculated that altered expression of these genes may be contributing to inflammatory response and endothelial dysfunction during placental implantation in women who develop PE. Furthermore, a case control study conducted in India reported altered placental global DNA methylation patterns in a small group of women with both preterm and term PE (n=57). The study found that such women had increased plasma Hcy when compared with normotensive women in the control group (n=30), and also showed a positive correlation between global DNA methylation and systolic ($r=0.56$; $p<0.01$) and diastolic ($r=0.49$; $p<0.05$) BP in the

term PE group (170). Thus the study suggests a possible role of Hcy in affecting global DNA methylation and BP among women with PE.

In summary, this section of the review highlights that altered DNA methylation is consistently reported in various tissues of women with PE, highlighting possible defects in OCM or inadequate intake of dietary methyl donors. As folate (171) and Hcy concentrations have been inversely associated with altered global DNA methylation (172,173), it is inferred that modulation of DNA methylation of the CpG dinucleotide with methyl donors may influence the regulation of gene expression involved during early placentation. Further research may pave the way for identifying distinct DNA methylation patterns in women during early pregnancy that may predict PE prior to its clinical presentation.

Table 1.3: Studies of DNA methylation in women at risk of pre-eclampsia

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
Huang *et al.* (2014)	China and USA	II	Review	Described in various articles	Analysis of *syncytin-1*methylation and expression profiles in different tissues	Decreased*syncytin-1*expression associated with increased DNA methylation levels of the 5′-LTR region in placentas from women with IUGR and PE	Downregulation of syncytin-1 during hypoxic conditions, as observed in PE, may affect formation of syncytiotrophoblasts
Yan *et al.* (2013)	China	III-2	Case–control	Placentas collected from women with PE (n = 30) and healthy women who delivered by caesarean section (n = 30)	Samples from 5 cases of severe PE and 5 control cases were tested using DNA methylation array and gene expression microarray. Quantitative PCR was used to verify result of gene expression test in placental tissue	Significantly altered expression of more than 10 genes, along with changed methylation, reported in the placental tissue of patients with PE. Genes include *LAIR2* (gene encoding for a trophoblastic protein),*DNAJC5G* (gene encoding a neuroprotective protein), and*LAMA3* (gene encoding laminins that are important for endothelial repair). Among genes that were found to be downregulated in placentas of women with PE	Various genes that may influence trophoblast invasion and endothelial function during the early placentation stages of pregnancy reported in women with PE

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
						were *SSTR1*,*synaptotagmin VI*(involved in acrosomal exocytosis), and*TPSAB1*(involved in reproductive functions)	
Anderson *et al.* (2014)	Ohio, USA	IV	Prospective	Nulliparous, normotensive women (n = 55) during first trimester of pregnancy	Genome-wide DNA methylation quantified in white blood cells and placental chorionic tissue from women with PE (n = 6) and compared with findings in aged-matched normotensive women (n = 6)	Significant differences in DNA methylation identified in 207 individual linked CpG sites in maternal white blood cells collected in the first trimester Genes associated with cell-signal transduction involving lipid binding, protease enzyme inhibition, protein–protein interaction, cell cycle processes, and adhesion showed hypermethylation, while those with signaling pathways involving cellular metabolic processes had significant hypomethylation	Though conducted on a small sample, the study demonstrated that DNA methylation analysis may be pursued as a clinical biomarker for early screening of PE
Zhuang *et al.* (2013)	USA	III-2	Case–control	Placentas of pregnant women with uncomplicated	Methylation in the 5'-LTR of syncytin-1 promoter was quantified	Methylation levels were inversely correlated with*syncytin-1* mRNA	

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
				outcomes and of women with PE	by COBRA, methylation-specific PCR, and DNA sequencing	levels, suggesting that hypermethylation may lead to*syncytin-1*downregulation	
White *et al.* (2013)	USA	III-2	Case–control	Women with PE (n = 14) and normotensive controls (n = 14)	Genomic DNA extracted and Human Methylation Assay (Illumina, San Diego, CA) run on all samples	729 genes were hypermethylated in leukocyte DNA of women with PE compared with normotensive controls. 268 genes were hypomethylated in women with PE	
Blair *et al.* (2013)	USA	III-2	Case–control	Women with PE (n = 14) and normotensive controls (n = 14). Methylation at 27 578 CpG sites in 14 495 genes in maternal leukocyte DNA collected at delivery on the fetal side of the placenta from women with early onset of PE (n = 20) and	Illumina HT-12v4 Expression Bead Chip (Illumina, San Diego, CA) used to assess gene expression of >45 000 transcripts in a subset of cases and controls, performed using a subset of samples and controls (n = 8 each), and to assess gene expression of >45 000	Study identified 38 840 CpG sites with significantly altered DNA methylation among women diagnosed with early-onset PE, of which 282 had a 12.5% methylation difference compared with controls. Of the candidate CpGs, 74.5% were hypomethylated and 25.5% hypermethylated in women with early-onset PE compared with controls. Genome-wide expression in	PE associated with global hypermethylation in the leukocytes of venous blood in a small group of women

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
				compared with gestationally matched controls (n = 20)	transcripts in a subset of cases and controls	a subset of samples showed that expression of genes responsible for angiogenesis (such as *EPAS1* and *FLT1*) were negatively correlated with DNA methylation changes ($p < 0.05$)	
Hogg *et al.* (2013)	Canada	III-2	Case–control study with candidate gene approach	Placental samples from 3 chorionic villus sites collected at delivery from normotensive pregnant women (controls, n = 19) and women with early-onset PE (n = 19). DNA methylation quantified by bisulfite pyrosequencing in a cohort of controls (n = 111), in women with early-onset PE (n = 19), late-onset PE (n = 18), and	Selection of candidate genes by Infinium HumanMethylation450 Bead Chip array (Infinium, San Diego, CA), bisulfite pyrosequencing to assess CpG methylation, gene expression array for expression of mRNA	DNA methylation (percentage points) was increased at CpG sites within genes encoding the glucocorticoid receptor (*NR3C1* exon 1D promoter and CRH-binding protein intron 3) and decreased within CRH in placental tissue of women with early-onset PE as compared with controls. Significant hypomethylation of steroidogenic genes was observed in PE placentas	Study provides evidence for altered methylation and subsequent difference in expression of cortisol-signaling genes in early-onset PE

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
				normotensive IUGR (n = 13)			
Sundrani *et al.* (2013)	India	III-2	Case–control	Placentas from normotensive women with term delivery (≥37 wk, n = 46), women with PE delivering preterm (<37 wk, n = 45), and women with PE delivering at term (≥37 wk, n = 48)	Expression levels and promoter CpG methylation of *VEGF*, *FLT-1*, and *KDR* genes in placentas determined by Taqman-based quantitative real-time PCR (Life Technologies, Grand Island, NY) and by the Sequenom MassARRAY (Sequenom, San Diego, CA), respectively	Hypomethylation of CpGs in the promoter region and an increased expression of*VEGF* gene among term and preterm women with PE compared with controls. Higher expression of*FLT-1* and *KDR* in preterm women with PE compared with control group, although mean methylation in the*FLT 1* and *KDR*promoters was similar between the 3 groups	Altered expression of genes responsible for encoding proteins involved in angiogenesis reported in placentas of women with PE
Xiang *et al.* (2013)	China	III-2	Case–control	Placental tissues from women with PE (n = 23) and women with uncomplicated pregnancies (n = 22) with singleton pregnancies	PCR validation done on PE (n = 7) and normotensive (n = 6) pregnancies. DNA methylation analysis used for PE (n = 16) and control (n = 16) samples	Expression of the *LEP* gene encoding for leptin protein was significantly elevated in PE placentas compared with normal placentas and was inversely related to DNA methylation in promoter	Hypomethylation of*LEP* and hypermethylation of*SH3PXD2A* genes observed in placentas of women with PE but their role in pathophysiology

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
						regions, though at a nonsignificant level.	requires further investigation
Papantoniou *et al.* (2013)	Greece	III-2	Retrospective and case-control	Peripheral blood samples from Caucasian normotensive pregnant women, at low risk of PE (n = 48) and with PE (n = 24) at 11–13 weeks gestation	Cell-free DNA and cell-free fetal DNA found in apoptotic syncytiotrophoblast fragments determined by quantifying*RASSF1A* by qRT-PCR. A second qRT-PCR was performed following methylation-sensitive enzyme digestion by BstUI to quantitate hypermethylated*RASSF1A* sequences of fetal origin	Cell-free DNA and cell-free fetal DNA levels were significantly increased in women who developed PE compared with controls	
Ruebner *et al.* (2013)	Germany	III-2	Case–control	4 isolated villous cytotrophoblasts from placentas: control (n = 3), IUGR (n = 3), PE (n = 3), PE/IUGR (n = 3), and	Human cytotrophoblasts isolated using the trypsin-DNase-dispase collagenase-hyaluronidase/percoll method. The trophoblast-like cell	Hypermethylation by 49% in IUGR, 53% in PE, 47% in PE/IUGR, and 64% in HELLP/IUGR observed compared with 29% in control CTs. DNA demethylation of the	

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
				HELLP/IUGR (n = 2)	lines derived from choriocarcinomas were cultured. Absolute and semiquantitative real-time PCR with specific primers used to quantitate *syncytin-1*. Bisulfite treatment of genomic DNA performed with the EpiTect Bisulfite Kit (QIAGEN, Valencia, CA)	trophoblast-like cell lines showed an elevated syncytin-1 expression and fusion ability in all cell lines	
Hogg *et al.* (2013)	Canada	III-2	Case–control	Chorionic villous samples for DNA methylation (normal pregnant women, n = 111) at 28–41 wk .*LEP* methylation compared between controls and women with early-onset PE (n = 19), late-onset PE (n = 18), or IUGR (n = 13)	DNA extracted from pooled placenta samples; plasma leptin measured using a Leptin ELISA Kit (Life Technologies, Grand Island, NY); genotype analysed for an SNP within *LEP*exon 1	Maternal leptin concentrations significantly increased in both early- and late-onset PE cases compared with controls but were not altered in IUGR pregnancies and were not related to DNA methylation	

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
Kim *et al.* (2013)	South Korea	III-2	Prospective case–control	Maternal plasma at 7–41 gestational weeks from women with normal pregnancies (n = 161), IUGR (n = 43), PE (n = 22), or placental previa (n = 14) and plasma from nonpregnant women (n = 20)	Real-time quantitative PCR performed to quantify *RASSF1A* concentrations before and after methylation-sensitive restriction digestion in maternal plasma	Concentration of hypermethylated *RASSF1A* was relatively high at 7–14 gestational weeks in all patient groups. Hypermethylated *RASSF1A* concentration at 15–28 wk was significantly higher in women who subsequently developed IUGR ($P = 0.002$), PE ($P < 0.001$), or PP ($P < 0.001$) compared with women in control group	Measuring *RASSF1A* methylation patterns in maternal plasma during first trimester may be further pursued for investigation as a biomarker for PE
Xiang et al. (2013)[157]	China	III-2	Case–control	Placentas from women with PE (n = 41) and from normotensive women as controls (n = 22); maternal peripheral blood from cases (n = 3) and controls (n = 6); and cord blood from cases (n = 7) and controls (n = 8)	Genomic DNA isolated from placentas and blood samples using the QIAamp DNA Mini Kit (QIAGEN, Valencia, CA). qRT-PCR performed to determine the mRNA expression of *TIMP3*. Total RNAs were extracted from placentas	The 2 analyzed CpG sites (2699 and 2880 bp, upstream of the transcription start site) in the promoter region were significantly hypomethylated in PE placentas compared with normal placentas. Expression of the *TIMP3* gene was increased nearly 2-fold in placentas of PE women with	*TIMP3* is likely to be involved in the etiology of PE

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
						a low level of CpG methylation compared with that in normal placental samples ($P = 0.007$)	
Mousa *et al.* (2012)	USA	III-2	Case–control	Omental fat biopsies of ≈ 2 cm × 2 cm × 0. 5 cm in size collected from normal pregnant women (n = 5) and women with severe PE (n = 7) (26–40 weeks gestation)	DNA extracted from omental arteries using QuickGene DNA tissue kit (Wako, Mountain View, CA). Infinium HumanMethylation27 BeadChip assay (Illumina, San Diego, CA) used for analysis of global DNA methylation	65 hypomethylated genes (false discovery rate of <5% and difference in methylation of >0.10) were identified, among which thromboxane synthase gene was the most hypomethylated gene in women with PE	Small sample size of different gestational ages could not clearly identify the expression of genes in early- or late-onset PE. Moreover, the entire genome methylation could not be ascertained
Jia *et al.* (2012)	China	III-2	Case–control	Placental tissue from women with PE delivering after 33 wk (n = 9) and women with normal-term pregnancies as controls (n = 9)	DNA extracted from frozen placental tissue and a genome-wide analysis of the DNA methylation profile done using methylated DNA immunoprecipitation and the NimbleGen HG18 Microarray (Roche NimbleGen,	296 genes showed significant aberrant DNA methylation in placental tissues of women with PE. In addition, the methylation profile of 6 of these genes (*CAPN2*, *EPHX2*,*ADORA2 B*,*SOX7*, *CXCL1*, and *CDX1*) in 9 patients with PE was validated by	Genome-wide hypermethylation was obvious in CpG sites in multiple genes; however, gene-specific methylation analysis will augment understanding of pathways of

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
					Branford, CT). Methylation status of identified candidate genes was validated by bisulfite sequencing PCR	bisulfite sequencing PCR. The promoter CpG regions in most of the genes were hypermethylated by 60% in placentas of women with PE compared with controls	epigenetic control of placental implantation in women with PE
Gao *et al.* (2011)	China	III-2	Case–control	Placental tissue collected from cases (24 women with PE: 10 with early-onset PE, 14 with late-onset PE) and controls (women with normal pregnancies, n = 24)	Immunohistochemistry analysis performed. Total RNAs from cells and placental tissue isolated with TRIzol reagent (Life Technologies, Grand Island, NY). DNA methylation level quantified using bisulfite PCR and pyrosequencing	Global DNA methylation and DNA (cytosine-5) methyltransferase 1 mRNA were significantly higher in placentas of women with early-onset PE compared with normal controls. Hypermethylation of the promoter region of the *H19*gene and reduced expression of the*H19* gene were both observed in early-onset PE placentas compared with normal controls	Role of H19 gene in trophoblast invasion during early placentation needs further investigation
Zhao *et al.* (2011)	China	III-2	Case–control study with	Genomic DNA extracted from center of placenta (toward mother side) from	Two isoforms of *COMT*gene (soluble cytoplasmic and membrane-bound)	Significant hypomethylation of the soluble cytoplasmic*COMT* promote	Differential methylation of*COMT* gene does

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
			candidate gene approach	women with PE (n = 16) and women with normal pregnancies (controls, n = 21), along with maternal peripheral blood (n = 4 cases, n = 6 controls) and umbilical cord blood (n = 8 cases; n = 8 controls)	studied. Genomic DNA isolated using QIAamp DNA Mini Kit (QIAGEN, Valencia, CA) and bisulfite treatment of genomic DNA performed using the EpiTect Bisulfite Kit (QIAGEN). Quantitative methylation measured using the mass array compact system	r in placental tissue observed (mean, 28.6%) compared with blood samples (mean, 74.5%, $p < 0.001$). No significant difference between the methylation patterns of women with PE and controls (28.7% and 28.6% methylation, respectively; $p = 0.818$) in placental tissue and peripheral blood	not correlate with development of PE
Kulkarni *et al.* (2011)	India	III-2	Case–control	Fresh placental tissue and venous blood samples from 87 women with singleton pregnancies: 30 with PE, 27 with PTPE, and 30 normotensive women with term pregnancies (controls)	Folate and vitamin B_{12}measured by fluorescence polarization immunoassay and Hcy by microparticle enzyme immunoassay. Genomic DNA extracted from placental tissues with the QIAGEN Blood and Tissue Kit (QIAGEN, Valencia, CA). Global DNA methylation	Positive association found between global DNA methylation and systolic ($p < 0.01$) and diastolic ($p < 0.05$) BP in the term PE group, along with high Hcy concentrations. No difference in folate concentrations, though vitamin B_{12} levels were significantly higher ($p < 0.05$) in PTPE when compared with term PE and normotensive groups. Mean	First study to report association of BP and global DNA methylation in women with PE

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
					measured using the Methylamp Quantification Kit (Epigentek, Farmington, NY)	global DNA methylation levels were significantly higher among term PE ($0.68\% \pm 0.26\%$, $p < 0.05$) and PTPE ($0.72\% \pm 0.37\%$, $p < 0.05$) groups compared with the normotensive ($0.53\% \pm 0.24\%$) group	
Yuen *et al.* (2010)	Canada	III-2	Case–control	Placental tissue from women with PE [early onset (n = 4), late onset (n = 4)], IUGR (n = 4), and early (n = 4) and late controls (n = 5)	DNA extracted and RNA expression from placental tissue studied using the Illumina microarray and human gene expression array (Illumina, San Diego, CA). DNA samples extracted from blood of 5 normal females and from fetal tissues (brain, kidney, and lung) of 3 abortuses to assess tissue specificity of methylation in the candidate loci. Bisulfite pyrosequencing done to	1505 CpG sites associated with 807 genes in 26 placentas from all groups were analyzed for methylation patterns. Thirty-four loci were hypomethylated (false discovery rate < 10% and methylation difference >10%) in early-onset PE placentas compared with 0 and 5 in late-onset PE and IUGR placentas, respectively. The promoter of*TIMP3* was confirmed to be significantly hypomethylated in early-	Further studies required to investigate reasons for hypomethylation and subsequent altered expression of *TIMP3* gene in placentas of women with PE; findings may help define a possible biomarker of PE

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
					validate methylation loci	onset PE placentas ($p = 0.00001$)	
Bellido *et al*. (2010)	Switzerland	III-2	Case–control study using candidate gene approach	Venous blood samples from nonpregnant (n = 30) and pregnant women (n = 20). Placental samples from women with normal pregnancies (n = 25) and PE (n = 8)	Placental tissue used for DNA extraction and plasma used for extracting cell-free DNA with the High Pure PCR Template Preparation Kit (Roche Life Sciences, Branford, CT). Methylation quantified using high-throughput mass spectrometry on matrix-assisted laser desorption/ionization time-of-flight mass array	Methylation at CpG sites for tumor suppressor gene *RASSF1* gene was significantly different (43% hypomethylated and 32% hypermethylated) between placental (normal and PE) and plasma samples of pregnant women. The high-throughput profiling of methylation of the *RASSF1* gene revealed hypermethylated patterns in placental DNA (normal and PE) but hypomethylated patterns in cell-free DNA from plasma of pregnant women. Although the *SERPINB5* gene was more hypomethylated in placental DNA than in plasma DNA, there was no significant difference between the 2 groups	

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
Bourque *et al.* (2010)	Canada	III-2	Case–control study using candidate gene approach	Two placental tissue samples collected (1 near the cord insertion and 1 near the placental periphery) and used for extraction of genomic DNA from women with normal pregnancies (n = 22), IUGR (n = 13), PE (n = 17), and PE+IUGR (n = 21)	Methylation assessed using the Illumina Golden Gate Methylation Cancer Panel I array (Illumina, San Diego, CA), with pyrosequencing and MS-SNuPE assays used in imprinting control regions (ICR1 and ICR2) known to influence fetal and placental growth	Mean methylation at ICR1 site was significantly decreased in normotensive IUGR placentas ($P < 0.001$), but not in any other group, while methylation at ICR2 remained unaffected. Gene expression also seemed unaffected at the sites studied	
Wang *et al.* (2010)	China	III-2	Case–control	Placenta and fetal membrane collected from women with normal pregnancies (n = 18) and women with PE+IUGR (n = 20)	DNA extracted and methylation status of the promoter regions of MMP9 analyzed with methylation-sensitive restriction enzymes, followed by PCR amplification	Decreased methylation of promoter sites and higher expression of MMP9 reported in placentas of women with PE compared with normal women	

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
Tsui *et al.* (2007)	Hong Kong	III-2	Case–control	Placental tissues from women with PE (n = 5), women with normal pregnancies (n = 10). Maternal blood samples from women with PE (n = 10) (median GA: 39 wk) and women with normal pregnancies (n = 20)	DNA extracted from plasma with the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA). DNA extracted from placental tissues with the QIAamp DNA Mini Kit (QIAGEN). Bisulphite sequencing used to quantify methylation status	Median concentrations of hypermethylated*RASSF1A* were 4.3-fold higher in maternal plasma of women with PE than in controls. No significant difference between the extent of*RASSF1A*hypermethylation in placental tissues obtained from PE and control pregnancies	Though the reason for hypermethylation of the *RASSF1A*gene in maternal plasma from women with PE is unclear, further research may clarify its role as a noninvasive biomarker of PE
Chelbi *et al.* (2007)	France	III-2	Case–control	Placentas collected from women with normal pregnancies (controls, n = 9), PE (n = 7), PE + IUGR, and IUGR (n = 8)	DNA extracted by mechanical grinding using electric Ultra-Turax homogenizer (IKA, Wilmington, NC), followed by study of gene expression (qRT-PCR) and analysis of CpG methylation status by sequencing	Of the 18 *SERPIN*genes studied in placental tissues,*SERPIN A* was underexpressed as compared with*SERPIN B* ($P = 0.036$) in both PE and PE+IUGR samples. Ten promoter regions of *SERPIN*showed altered methylation.	

Abbreviations: BP, blood pressure; COBRA, combined bisulfate restriction analysis; COMT, catechol-*O*-methyltransferase CRH, corticotropin-releasing hormone; CT, cytotrophoblasts; HELLP, hemolysis, elevated liver enzymes, low platelet count; ICR, imprinting control region; IUGR, intrauterine growth restriction; 5-LTR, 5-long terminal repeat; MMP, matrix metalloproteinase 9; MS-SNuPE, methylation-sensitive single-nucleotide primer extension; PCR, polymerase chain reaction; PE, pre-eclampsia; PP, placental previa; PTPE, preterm pre-eclampsia; qRT-PCR, quantitative RT-PCR; RT-PCR, reverse transcription polymerase chain reaction; SNP,single-nucleotide polymorphism

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1.3.3 **Genetic polymorphisms in the folate/methionine pathway and PE**

When reviewing folate metabolism in a disease such as PE, the role of genetic polymorphisms within the enzymes of the folate/methionine pathway need to be considered, given that they influence folate bioavailability and influence human folate requirements (83). One of the most common autosomal recessive polymorphisms is the Methylene tetrahydrofolate reductase (MTHFR) C→T (cytosine to thymine nucleotide) substitution at the 677 nucleotide (174), resulting in a thermolabile enzyme with diminished enzyme activity (175). This results in a reduced capacity to convert 5-10, methylene THF to 5-methyl THF. The MTHFR C667T polymorphism affects about 10% of people worldwide, and more frequently in certain ethnic groups (26% in Italian and 32% in Mexican populations) (176). It has been demonstrated that both heterozygous (CT) and homozygous (TT) variants of MTHFR C677T have elevated thermolability and reduced enzyme activity, and are associated with increased circulating Hcy concentrations in plasma (177), especially under conditions of suboptimal folate status (178). The TT homozygous genotype is also susceptible to increased risk of PE (179-181) among Asian (182) and white population (177), possibly as a result of hyperhomocysteinemia (183), but not among Mexican pregnant women (184). TT homozygous individuals are also prone to have elevated BP among Chinese, Indian, Australian and Japanese populations (185-189) The TT genotype individuals are reported to have lower erythrocyte folate concentrations compared with those without this genetic variant, implying that folate requirements may be increased in these individuals (36); nevertheless FA supplementation among women with TT genotype (4mg/d for 6 months) was proven ineffective in

reducing plasma Hcy (190). Further investigations are therefore required to understand the associations of folate status and MTHFR polymorphisms observed among women at risk of PE (191-193). Carriage of the MTHFR C667T polymorphism has also been associated with DNA hypomethylation particularly when folate status is low (173). The possible reason may be that altered MTHFR activity causes an increase in 5-10, methylene THF concentration, with a resultant promotion of deoxy thymidine triphosphate synthesis over CpG island methylation (88,194-197), resulting in a subsequent increase in DNA hypomethylation. Investigations of other gene variants of key enzymes in OCM have been inconclusive in identifying their role in the pathology of PE (198-200). Interestingly, polymorphisms in the reduced folate carrier gene encoding the reduced folate carrier protein (A80G) that has been reported to be of significance in neural tube defects risk in an Italian population (201) were also found to be associated with increased micronuclei frequency in the lymphocytes of a South Australian cohort (202).

1.3.4 Is FA supplementation the answer to preventing aberrant metabolic defects of OCM among women at risk of PE?

FA supplementation has proven to be a cost effective and successful public health approach in reducing the incidence of neural tube defects (203) as well as that of worldwide megaloblastic anemia (204,205). The role of FA supplementation in reducing PE risk, and associated adverse pregnancy outcomes such as small for gestation age has been explored for more than a decade (206-209).

Thirteen studies were selected for the present review including two randomized controlled trials (RCTs) (73,210), three prospective studies (71,206,211), four retrospective studies (72,212-214),

one cross sectional study (215), non-randomized clinical study (216), one systematic review (217) and one retrospective case control study (218) and are outlined in **Table 1.4**.

In a double blind RCT, the effect of multivitamin (20 mg thiamine, 20 mg riboflavin, 25 mg B6, 50 µg B_{12}, 500 mg vitamin C, 30 mg vitamin E, and 0.8 mg FA) and vitamin A supplements (30 µg beta-carotene plus 5000 IU preformed vitamin A) was assessed in relation to HT in pregnancy among 955 HIV-positive pregnant Tanzanian women for 2 years (210). Vitamin A failed to show any effect, as did other antioxidants such as vitamin C and E, as shown in separate randomized trials (68,219). The multivitamin containing FA reduced the risk of HT during pregnancy by 38% [relative risk (RR) =0.62, 95% confidence interval (CI) 0.40-0.94] (210). The study included all forms of HT in pregnancy as measured by a single reading only at any time during pregnancy, data on proteinuria were not collected and there was a baseline supplementation of 5 mg FA in both trial and placebo arms, which could have confounded the observed effect.

Charles *et al* re-analyzed the data from a large RCT (Aberdeen Folate Supplementation Trial), which was performed between 1966 and 1967 (73). A total of 2928 women were randomized: 1977 were allocated to placebo, 466 to FA 200 mg/day and 485 to FA 5 mg/day. The primary objective was to study the effect on pregnancy outcomes such as birth weight, placental weight, gestational weight and PE. The study reported low adjusted OR for risk of PE for daily FA supplementation of either 0.2 mg (OR=0.46 [CI: 0.20, 1.05]) or 5 mg (OR=0.59 [CI: 0.26, 1.32]) (p for trend =0.1) (73). However, the birth outcome data was a post hoc analysis. Also, the number of PE cases was small and confidence intervals wide; the observed effect may therefore be attributable to chance. Moreover, 91.8% of women did not start supplementation until after 12 weeks gestation, by when the early placentation stage in human pregnancy is known to be complete.

The Ottawa and Kingston prospective study of a cohort of 2951 Canadian pregnant women between 12-20 weeks gestation, reported that supplementation with ≥1.0 mg FA, or a multivitamin containing ≥1.0 mg FA, in the early second trimester was associated with increased serum folate, lower plasma Hcy and a 63% reduction in risk of PE (OR, 0.37; 95% CI, 0.18-0.75) (71). Another prospective cohort study reported that the use of a periconceptional multivitamin containing FA was associated with a 45% reduced risk of PE compared with non-supplementation (OR= 0.55, 95% CI: 0.32, 0.95) (206).

A retrospective study collected information on FA antagonist users (n=14 982) and nonusers (n=59 825) among Canadian pregnant women with a singleton birth. A multi variate analysis demonstrated that maternal exposure to FA antagonists during one year before pregnancy increased the risk of PE (OR 1.52, 95% CI 1.39, 1.66) (72). Another retrospective case-control study collected information on multivitamin consumption and BP from 2100 mothers of non-malformed infants in the US and Canada. 81% of women reported FA use before 12 weeks of gestation (212). The multivariate-adjusted RR of developing gestational HT following one month of supplementation with multivitamin containing FA (0.4-1 mg), compared with not using FA during that same month, was 0.55 (95% CI, 0.39, 0.79) (212). The study also demonstrated significant association between the MTHFR T677T genotype and the risk of gestational hypertension. A recent retrospective study also reported decreased plasma Hcy and a reduced risk of PE among Korean women taking prenatal FA supplementation of 0.4-1.0 mg/d (OR 0.27; 95% CI 0.09–0.76; p=0.014) (214). A retrospective population based longitudinal study conducted in Canada examined the trend in frequencies of PE and HT during pregnancy before and after implementation of mandatory FA fortification. A substantial decrease in associated risk of PE following

fortification was reported (unadjusted prevalence rate 0.96; 95% CI 0.94-0.98), though monthly rates of PE and HT during pregnancy remained unaffected (213).

A cross-sectional study investigated the role of iron and FA supplementation in a cohort of healthy Tanzanian women with singleton pregnancy (n=21 889). The study reported that the OR of FA supplement use with PE/eclampsia risk was 0.48. However, the self-reported data gave insufficient information on dose, timings and frequency of FA (215). Another case study assessed the effect of FA (5 mg/d) and B_6 (250 mg/d) supplementation in 37 pregnant women with hyperhomocysteinaemia and previous history of PE (216). Women taking FA supplements showed a decrease in plasma Hcy concentration. However, there was no control group in the study and women were also administered aspirin, hence no clear inference could be drawn on the effect of FA supplementation on the risk of PE (216).

A systematic review of 18 published articles selected 5 case control studies to examine the role of FA, Hcy, MTHFR and B_{12} in PE and reported no effect of FA in reducing the risk of PE (217). Also, a recent prospective population based study did not find any effect of 400 µg FA intake among a Chinese cohort at risk of PE (211). A retrospective case control study in South Australia did not find any association between red blood cell folate status and PE (218); however, as the study was conducted on a small number of women with PE (n=22) who had highly varied folate status at the beginning of the study, the results cannot be generalized.

Although the above data is accumulated from diverse types of studies with varied subject group numbers and variables, the review provided evidence for a possible effect of FA supplementation in reducing the risk of PE. There is some evidence from that FA supplements (mean dose 5.6 mg/d) may have protective effect on adverse birth outcomes associated with PE, including low birth

weight, and rate of preterm birth (OR 0.41 95% CI: 0.18, 0.94) in pregnant women with early onset of PE (207). However whether similar benefits can be achieved for reducing the risk developing PE still needs to be investigated.

Table 1.4: Studies of folic acid supplementation in women at risk of pre-eclampsia

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
Charles *et al*. (2005)	Multiple countries	II	Double-blind randomized controlled trial (1966–1967) combined with a Cochrane review	2928 pregnant women at 30 weeks gestation: placebo (n = 1977), FA supplementation of 200 µg/d (n = 466), and FA supplementation of 5 mg/d (n = 485)	Data on demographics and serum folate levels collected. Main pregnancy outcomes were birth weight, placental weight, and gestational age at delivery; preterm delivery; antepartum haemorrhage; PE; foetal abnormality; and stillbirths	No evidence of an effect of supplements (0.2 mg or 5 mg FA/d) on mean birth weight, placental weight, or gestational age at delivery. Slight nonsignificant reduction in risk of LBW and PE after FA supplementation at all doses	FA supplementation started after 12 wk, when placenta is considered to have been formed

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
Merchant *et al.* (2005)	Tanzania	II	Double-blind, placebo-controlled, randomized clinical trial	1078 HIV-positive pregnant Tanzanian women	Effect of multivitamin (20 mg thiamin, 20 mg riboflavin, 25 mg vitamin B_6, 50 µg vitamin B_{12}, 500 mg vitamin C, 30 mg vitamin E, and 0.8 mg FA) and vitamin A supplements (30 µg beta-carotene plus 5000 IU preformed vitamin A) on BP assessed for 2 y	Women who received multivitamin containing FA were 38% less likely to develop HT during pregnancy than those who did not (RR = 0.62; 95%CI, 0.40–0.94; $p = 0.03$). There was no overall effect of vitamin A on HT during pregnancy (RR = 1.00; 95%CI, 0.66–1.51; $p = 0.98$)	Data on proteinuria not collected; BP reading taken only once, any time during pregnancy
Wen *et al.* (2008)	Canada	III-2	Prospective cohort	2951 pregnant women recruited from the Ottawa and Kingston Birth Cohort between 12 and 20 weeks gestation during 2002 and 2005	Demographic and clinical data collected. Blood analyzed for serum folate, plasma Hcy, and the presence of the*MTHFR* thermolabile variant gene	Supplementation with multivitamin containing FA associated with increased serum folate (average, 10.51 µmol/L), decreased plasma Hcy (average, 0.39 µmol/L), and reduced risk of PE (adjusted OR 0.37; 95%CI, 0.18–0.75)	

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
Li *et al.* (2013)	China	III-2	Prospective, population-based cohort	193 554 pregnant women (during the year 1993) not affected by diabetes mellitus or HT, before 20 weeks gestation	Public health medical records examined for detailed information on BP and FA intake. PE diagnosed on the basis of BP and proteinuria	The incidence of gestational HT and PE in women who received FA was 9.7% and 2.5%, respectively, compared with 9.4% and 2.4% in women who did not. The adjusted OR associated with FA use was 1.08 (95%CI, 1.04–1.11) for gestational HT and 1.11 (95%CI, 1.04–1.18) for PE. The study did not find a decrease in the risk of gestational HT or PE among women who took FA supplements, as compared with those who did not	The study with 99.9% power to detect change in HT did not assess dietary folate. Nonsignificant difference was observed in the distribution of early- or late-onset gestational HT and PE among women with and without FA use
Bodnar *et al.* (2006)	Pittsburgh, PA, USA	III-2	Prospective cohort	1835 women aged 14–44 y, carrying singleton infants, at	Interview conducted to collect data on FA use and sociodemographic	Multiple logistic regression model showed regular use of a multivitamin associated	Data on dose or brand of supplement were not collected. Information about

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
				16 weeks gestation (1997–2001)	and behavioral variables. Primary outcome was FA supplement use and PE diagnosed by BP (average of 5 BP readings ≥150 /90 mmHg) and proteinuria	with a 45% reduction in PE risk compared with no use (OR = 0.55; 95%CI, 0.32–0.95). PE was 0.29 times as likely in lean women who used a periconceptional multivitamin compared with lean nonusers, whereas there was no relation between multivitamin use and PE risk in overweight women	multivitamin use based on self-reported data
Hernández-Díaz *et al.* (2002)	USA and Canada	III-2	Retrospective case–control	2100 mothers of nonmalformed infants	Interview conducted to collect information on multivitamin consumption and high BP	The multivariate-adjusted RR of developing gestational HT after 1 mo of supplementation with a multivitamin containing FA (0.4–1 mg), compared with not using FA during that same month, was 0.55 (95%CI, 0.39–0.79)	Presence of high BP depended on self-report by participants. Study also limited by small sample size and the potential cross-classification of PE and gestational HT of PE

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
Wen *et al.* (2008)	Canada	III-2	Retrospective population-based cohort	Pregnant women with a singleton birth (both live births and stillbirths) (January 1, 1980, to December 2000, n = 31); 14 982 were exposed to FA antagonists and 59 825 were not exposed to FA antagonists	Information collected from provincial outpatient prescription drug database on exposure to FA antagonists during the 1-y period before delivery	Risks of PE (adjusted OR = 1.52; 95%CI, 1.39–1.66), severe PE (OR = 1.77; 95%CI, 1.38–2.28), placental abruption (OR = 1.32; 95%CI, 1.12–1.57), and fetal growth restriction defined as less than the 10th percentile (OR = 1.07; 95%CI, 1.01–1.13)	Information on smoking status of women not available, and most information was collected retrospectively
Kim *et al.* (2014)	Korea	III-2	Retrospective	Pregnant women with singleton pregnancies (n = 227)	Maternal blood and cord blood collected. Plasma total Hcy concentration measured using an automated enzymatic assay; Hcy methyltransferase, D-amino acid oxidase, and folate measured by an iodine-125-based radioimmunoassay	Maternal blood had significantly higher FA concentrations following FA supplementation (24.6 ng/mL vs 11.8 ng/mL), while plasma Hcy level was lower (5.5 mmol/mL vs 6.8 mmol/mL). Rates of PE (OR = 0.27; 95%CI, 0.09–0.76) were	

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
						reduced after FA supplementation	
Ray and Mamdani (2002)	Canada	III-2	Retrospective population-based longitudinal	1 001 141 women with live births and stillbirths, grouped into before (n = 792 213) and after (n = 209 228)food fortification	Details about HT or PE obtained from discharge summaries	No significant decline in HT ($p = 0.6$) or PE ($p = 0.9$) observed in either group. Study showed a small but significant decrease in associated risk of PE after mandatory fortification with FA (unadjusted prevalence ratio of 0.96; 95%CI, 0.94–0.98)	
Ogundipe *et al* (2012)	Tanzania	III-2	Cross-sectional observational cohort	21 889 women with normal singleton deliveries (1999–2008)	Interview and antenatal care records examined. Logistic regression models used to describe patterns of reported intake of prenatal FA and iron supplements	OR for FA supplement use with PE/eclampsia was 0.48	Timing and frequency of FA supplementation not available for all subjects. Information on medical conditions was based on self-reported data
Leeda *et al* (1998)	Netherlands	IV	Clinical trial	207 women at 10 wk postpartum	Methionine loading test repeated on 37	Vitamin B_6 and FA improved the	Study limited by its small size and the

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
				(181 with history of PE and 26 with history of IUGR). 171 were primiparous and 36 were multiparous	patients with abnormal results 10 wk after supplementing with 5 mg FA/d and 250 mg vitamin B_6/d	methionine loading test in patients with hyperhomocysteinemia, as reported postload Hcy value decreased from 68.5 mmol/L (95%CI, 60.8–76.2) to 29.3 mmol/L (95%CI, 25.6–33.0) ($p < 0.0001$)	absence of a control group, hence very high CI
Furness *et al* (2012)	Australia	III-2	Retrospective case–control	137 potential low-risk and high-risk pregnant women (6 and 20 weeks gestation, mean age 33 y) with viable singleton pregnancies	Fasting blood samples obtained, questionnaires administered, and RBC folate measured at 10–12 weeks gestation. Pregnancy outcome data obtained from patient case notes	Women with low folate status were likely to have SGA infants (OR = 6.9; 95%CI, 2–24.3) Those who were folate insufficient were also at increased risk of SGA (OR = 3.0; 95%CI, 1.3–7.7). No association found between folate status and PE	

Reference	Location	Level of evidence	Type of study	Participants or type of tissue samples	Methods/intervention	Results	Comments
Ray and Laskin (1999)	Multiple countries	II	Systematic review	Search in Ovid MEDLINE between 1966 and February 1999 for studies with measurement of vitamin B_{12}, FA, *MTHFR*, or Hcy and studies in subjects with PE/placental abruption/infarction or spontaneous and habitual abortion. Only human studies published in English selected	18 studies were included	Five case–control studies were examined for a relationship between PE and vitamin B_{12}, folate, Hcy, or *MTHFR*polymorphism. Only 1 study showed no association between folate deficiency and PE; but increased Hcy and homozygosity for*MTHFR* variant were both associated with a moderate risk of PE	Only 1 study reviewed for effect of FA on PE

Abbreviations: BP, blood pressure; CI, confidence interval; FA, folic acid; Hcy, homocysteine; HT, hypertension; IU, international units; LBW, low birth weight; MTHFR, methylene tetrahydrofolate reductase; OR, odds ratio; PE, pre-eclampsia; RBC, red blood cells; RR, relative risk; SGA, small for gestational age.

1.3.5 **Proposed mechanisms of a protective effects of FA in PE**

There has been recent evidence from both RCT's and longitudinal studies to suggest that high dose FA supplementation (1-15mg/d) may be effective in reducing systolic and diastolic BP among normal adults and post-menopausal women (220-224) as well as in reducing plasma Hcy (225,226). However, whether a high dose of FA may influence BP and other biomarkers in PE needs to be investigated in a cohort of women at risk of PE.

There are numerous mechanisms through which folate may influence the abundance of biomarkers of various hypothesized casual pathways, which are reported to be altered in PE (**Table 1.5**).

Table 1.5: Potential pharmacological effects of folate in relation to biomarkers associated with risk of pre-eclampsia

Pharmacological effect	**Influence on biomarkers/metabolic pathway**	
	Homocysteine dependent	**Independent of homocysteine**
Lowers Hcy concentrations	Increases methylation of Hcy to methionine	
Reduces blood pressure	- Reduces Hcy-induced extracellular matrix elastolysis, thereby reducing arterial stiffness - Increases nitric oxide availability	Folate may influence BP by modulating the availability of nitric oxide, which is a vasorelaxant, via the following mechanisms: - The structure of 5-MTHF is similar to that of tetrahydrobiopterin, an essential cofactor of endothelial nitric oxide synthase. Thus, folate may bind the pterin site in nitric oxide synthase and may directly interact with nitric oxide synthase. - 5-MTHF may increase the effectiveness of tetrahydrobiopterin on nitric oxide synthase uncoupling, enhancing one-electron oxidation of tetrahydrobiopterin. - Folate can enhance the regeneration of tetrahydrobiopterin from the inactive form
Improves endothelial dysfunction	Reduces Hcy-mediated oxidative stress, generation of hydrogen peroxide, and oxygen-derived free radicals in the endothelium	- Folate may influence tetrahydrobiopterin-mediated regulation of nitric oxide synthase and increase availability of nitric oxide for vasorelaxation. - FA may directly cause reduction of intracellular endothelial superoxide and influence endothelial dysfunction - Folate may also increase endothelium-derived hyperpolarizing factor, which may improve vessel relaxation and endothelial function
Prevents DNA damage and influences DNA methylation	Controls Hcy-induced oxidative stress and DNA damage	Folate is indispensable for genome stability, owing to its function as a methyl donor in one-carbon metabolism
Decreases thrombotic effect	Lowers Hcy and reduces generation of hydrogen peroxide and oxygen-derived free radicals	Folate may cause significant reduction in plasma fibrinogen and D-dimer levels, both markers of a prothrombotic state
Affects antioxidant activities directly and indirectly		- Folate may reduce generation of xanthine oxide–induced superoxide - Improves tissue concentrations of the antioxidant vitamins such as ascorbic acid and alpha- and gamma-tocopherol - Prevents lipid peroxidation and restores the circulating and cellular fatty acid composition, thereby influencing the balance of eicosanoid synthesis of platelets

- *Abbreviations*: BP, blood pressure; FA, folic acid; Hcy, homocysteine; 5-MTHF, 5-methyltetrahydrofolate

Lower plasma Hcy: A high dose FA supplementation is known to reduce plasma Hcy (30,225) which may manifest into multiple protective actions such as a fall in BP, increase nitric oxide availability (227,228) decreased extracellular matrix elastolysis, reduced arterial stiffness (30,32,229), reduced oxidative stress (182), decreased thrombosis in the endothelium (32,177) and subsequent prevention of endothelial dysfunction (230,231).

Reduce BP: FA may reduce BP through direct interaction with endothelial nitric oxide synthase (228,232-236).

Improve endothelial dysfunction: FA may directly cause reduction of intracellular endothelial superoxide (237-239) and increase endothelium derived hyperpolarizing factor (240) and thereby improve vessel relaxation and endothelial function.

Decrease thrombotic effect: Folate may control thrombosis by lowering plasma fibrinogen and D-dimer levels (241).

*Prevent DNA damage and influence DNA methylation***:** There have been consistent reports from experiments on humans that genome instability as measured by the appearance of micronuclei in lymphocytes is sensitive to folate status in peripheral blood (242) and folate depletion and repletion influences DNA hypomethylation and micronuclei frequency in humans (87,171,226,243). Post FA fortification, higher RBC folate status among postmenopausal women is reported to be associated with attenuation in leukocyte global DNA methylation but the reverse was true pre-fortification suggesting a complex relationship with FA supplementation (172). Primarily, folate may stabilize genome integrity during the early placentation stages, owing to the major role of folate in *de novo* nucleotide synthesis. Low cellular folate results in enhanced incorporation of uracil instead of thymidine in DNA.

Persistent accumulation of uracil in DNA results in DNA strand breaks, due to the action of uracil glycosylases during DNA excision repair causing high rates of transient DNA breaks (242). Folate deficiency thus induces DNA replication stress and the resultant DNA damage reduces the cellular viability and proliferation capacity (109). It may be inferred that increased intake of folate may influence placental OCM during early implantation (244,245) and thus the prognosis of both stages of PE; by either modulating epigenetic or genetic processes (246). More research on the role of FA in preventing uracil incorporation into DNA and chromosome fragmentation is hence required.

Direct and indirect antioxidant effects: Folate may reduce xanthine oxide-induced superoxide generation (247), improve tissue concentrations of the antioxidant vitamins such as ascorbic acid and alpha-and gamma-tocopherol (248), and possibly inhibit lipid peroxidation (249).

1.3.6 **Possible role of other methyl donors**

In addition to folate, other methyl donors have not been exclusively studied in relation to PE despite some evidence that vitamins B_2, B_6, B_{12} and choline may influence genome stability (145,242), oxidative stress (250) and endothelial vascular function (251) among healthy adults, patients with acute ischemic stroke and normal pregnant women respectively. Choline, a methyl-rich amine, may be oxidized to betaine in the mammalian liver or kidney cells, further promoting the remethylation of Hcy to methionine (252). Choline supplementation has been reported to decrease fms-like tyrosine kinase-1, an anti-angiogenic PE risk marker in the placental tissues and blood samples collected from normal women (251). Vitamin B_6 supplementation is known to correct the methionine load test among women at risk of PE (216), to reduce urinary 8-OHdG concentration in normal Japanese men (250), to reduce multiple plasma inflammatory biomarkers among US men and women (253-255), to reduce Hcy (255)

and to decrease systolic BP and increase serum nitric oxide among diabetic patients (256). Furthermore, vitamin B_6 may reduce oxidative damage through facilitating the synthesis of glutathione, a natural antioxidant (257). Deficiency of vitamin B_{12} may also cause hyperhomocysteinaemia (258,259). Additionally, the relationship between one-carbon biomarkers, mainly choline, betaine, B_6 and B_{12}, and global DNA methylation is reported to be dependent on folate availability among American postmenopausal women during pre/post FA fortification period (172). Dietary supplementation with vitamin B_{12} and FA in young Australian adults has also been shown to be significantly inversely correlated with micronuclei frequency (87). Interestingly, riboflavin status may also influence both total plasma Hcy concentrations and BP in individuals carrying the MTHFR T677T genotype (260-264).

Thus, it may be suggested that the interrelation and interdependence of choline, folate and other methyl donors needs further investigation among a cohort of women at risk of PE to assist in formulating a preventive regime with the aim to alleviate risk of PE.

1.3.7 **Potential hazards of High doses of FA supplementation in Pregnancy**

Supplementation of FA is regarded as safe and generally non-toxic in humans (265). The absorption and biotransformation process of folate is readily saturated at doses less than 400 µg/day (266,267). As human liver has a low capacity to reduce FA, a high oral intake of synthetic FA may eventually lead to saturation and subsequent entering of unmetabolized FA into the systemic circulation (268). However, in a population-based, prospective, epidemiologic study of 559 Hungarian pregnant women who consumed a variety of drugs, including FA (n=4), to attempt suicide, no acute or long term adverse effects of high doses of FA (120-150 mg) were detected at the birth of their newborn infants (269). A follow up study investigating the health status of both mothers and children showed no adverse effects (265). Furthermore,

supplementing with a high dose of FA (6mg/kg body weight), has no effect on chromosome damage in mice erythrocyte progenitor cells (270), suggesting that high intakes of FA are not genotoxic *in vivo*. Owing to the role of FA in DNA synthesis, it has been hypothesized that unmetabolized FA may promote growth of tumors and cancers, such as colorectal cancer (271). Conversely, some studies support a protective role of FA in colorectal (272), pancreatic, esophageal (273-275), gastric (274,276), oral(277) and ovarian cancers (278), while data on its effect on breast cancer is inconsistent (273,279). Although report from a prostate, lung, colorectal and ovarian cancer screening trial has shown an increased risk of breast cancer among postmenopausal women with a FA supplement use of 400 µg/d or more (280), a meta-analysis based on 8 prospective studies showed that dietary or total FA intake (200 µg/d) was not associated with risk of breast cancer (281).

A few studies have suggested that FA supplementation during mid to late gestation may increase asthma (282), allergic airways disease (283), adiposity and insulin resistance in young children (284). However, an increased risk of severe atopic sensitization was reported in male offspring born to women with PE (285) thus warranting further investigation into the origin of atopy and related symptoms among infants born to women with/at risk of PE.

1.4 **Limitations and Strengths**

The studies included in the present review were not homogeneous; hence they were sub-grouped in the categories (Table 2, 3 and 4) to allow assimilation and analysis of data. However, the studies in the sub-group measured diverse outcomes in relation to different genotypes or used dissimilar methods to measure genome integrity or were diverse in their design that made pooled analysis of data very difficult. Further, in order to have a wide understanding of the potential role of folate in PE, even studies with small sample size were included for the review

that may have been a source of bias. However, the strength of the review is the predefined selection criteria and the diversity of the participant studies that has helped in identifying the specific gaps in the literature. These can now form the basis of future investigations among a cohort of women at risk of PE to assist in understanding significance of folate intake by women at risk of PE and also in developing a preventive strategy to reduce risks associated with PE for pregnant women.

1.5 **Knowledge gaps**

- *Hyperhomocysteinaemia* may be observed during pregnancy in relation to folate deficiency, demonstrating involvement of the folate/methionine pathway. Increased plasma Hcy also occurs in women with PE, although the relationship of folate deficiency to the development of PE has not been established. It is also not clear in this circumstance whether Hcy is causal or an effect of some underlying metabolic defect in women with PE, or associated with decreased clearance of Hcy.
- FA supplementation *may reduce BP* among healthy individuals. Whether such an effect is possible among women at risk of PE needs to be investigated.
- Folate supplementation in the diet *may reduce plasma Hcy* concentrations in humans with an efficacy that may be dependent on genotype (e.g. MTHFR) and dose. Whether the same can be achieved in women with PE needs further investigation under placebo-controlled randomized conditions.
- *The amount of FA* required, the time of initiating supplementation and the duration for such an effect to become evident needs further investigation.
- Folate deficiency causes the *increased appearance of micronuclei* in human lymphocytes, which has also been observed in women at 20 week gestation to predict subsequent development of PE and/or IUGR. Intervention studies on a large cohort of women at risk of

PE are required to answer whether the micronuclei observed are a cause or a consequence of PE and also whether there is any change in the micronuclei frequency, alongside changes in plasma Hcy, in women at risk of PE following prophylactic treatment with high dose FA.

- It is also not known whether the *appearance of micronuclei in lymphocytes* correlates with DNA damage either in the uterine spiral arteries or in the placental cells.
- Whether the appearance of micronuclei in lymphocytes is due to insertion of *uracil* instead of thymidine in the DNA during placental cell proliferation among women at risk of PE is yet to be determined.
- Further investigations are required to clarify if any observed effect following folate prophylaxis is influenced by *common polymorphisms in the genes* coding for the key folate pathway enzymes.
- Folate deficiency has been reported to *alter lymphocyte DNA methylation* in humans. Altered global DNA methylation has also been reported in the placentae of women with PE. Nevertheless, intervention studies in a cohort of women at risk of PE are needed to determine if high FA therapy alters DNA methylation patterns in placental tissue consistently and in a beneficial manner. It also needs to be determined whether DNA methylation in lymphocytes correlates with that of placental tissue.
- As there is a complex interplay among all *methyl donors* including $B_{2,}$ B_6, B_{12}, choline and folate, in maintaining various metabolic functions, further research is warranted to unravel their possible utility in improving the prognosis and the prevention of PE.

1.6 **Conclusions**

Folate seems to be involved in the peri-implantation stages of human fetal and placental development, with its crucial function in both genetic and epigenetic processes. PE is well recognized as a disorder, which may originate from altered gene expression during the early

placental implantation stages. The present review highlights associations between folate deficiency and certain biomarkers observed in various tissues of women at risk of PE. It may be speculated that the biomarkers of PE risk observed in pregnant women are susceptible to change under FA supplementation. Accordingly, folate supplementation may overcome an underlying metabolic defect in folate metabolism among women at risk of PE. A large and adequately powered cohort study of women at risk of PE together with investigations on folate status and Hcy status in cord blood, along with genome wide gene specific DNA methylation of placenta and genotype data in relevant tissues (including endothelial cells in spiral arteries) may help in increasing the understanding about the underlying mechanisms. The Folic Acid Clinical Trial is currently being conducted as a worldwide study, initially investigating the impact of FA supplementation on clinical outcomes of pregnant women at increased risk of PE (286). This trial will allow the investigation of the impact of high dose FA on genome integrity biomarkers among women at risk of PE and their offspring and will test whether such effects are modifiable by genetic factors affecting folate metabolism. Consequently the possible role of the CBMN-cyt assay and/or DNA/gene specific methylation status in various tissues as biomarkers for early detection of PE and its prevention will also become clear.

2 General Introduction

2.1 Cellular DNA damage during infancy

The human genome is susceptible to damage at the molecular and chromosomal level caused by exposure to various exogenous factors, such as genotoxic pollutants (e.g.:bisphenol A) (287-291), ultraviolet radiation, smoking, etc., as well as endogenous factors (free radicals) that result in oxidation, alkylation, hydrolysis and adduct formation on DNA bases within human cells (98,289,292-294). Damage to the genome is recognised as an important pathological event that could lead to developmental defects, increases in inflammatory cytokines (295-300), immune system dysfunction and an increase in the risk for early onset of degenerative diseases, including cancer (301,302). DNA damage sustained during both the perinatal period and infancy (303-305) may also reflect the epigenomic impact of maternal diet, life-style and genotoxin exposures (304,306-312). Insults to the genome in the perinatal period are likely to be very important relative to other life-stages because of the higher probability that mutated and genomically unstable cells could populate the rapidly growing tissues of an infant (313-316). Pregnancy is observed to have increased angiogenesis and increased immune responses, especially at the site of implantation (317). Further, the hypoxic state during birthing may modulate expression of placental endothelial growth factors that control cellular growth, differentiation, proliferation and apoptosis (143,318-320). Numerous markers of oxidative DNA damage, repair functions, and hypoxia status] (reactive oxygen metabolites (d-ROMs), redox factor-1 (ref-1), and hypoxia-induced factor-1α (HIF-1α) respectively] were reported to increase in a small number of maternal and umbilical plasma collected from women with pre-eclampsia (PE) (n =12) when compared to normal, uncomplicated pregnancy (n =10) (143). Increased micronuclei frequency (MN): a measure of chromosomal loss and/or breakage in maternal peripheral lymphocytes at 20 weeks gestation was associated prospectively with PE and IUGR. The odd ratios (OR) for PE and/or IUGR in the cohort of only high risk pregnancies (n=91) was 17.85 (p=0.007) if the MN frequency was greater than 39 per 1000 cells (118). The study suggests that the MN frequency is increased in lymphocytes of women who later develop

PE and/or IUGR compared with women with normal pregnancy outcomes. It may therefore be speculated that infants born to women with complications during pregnancy, such as PE may be susceptible to more cellular DNA damage. Further, the Human MicroNucleus project compiled data on MN frequency assessed in lymphocytes of 6718 individuals (who were free of cancer at the time of testing) from 10 countries and found a significant increase of all cancers incidence in medium [relative risk (RR) 5 1.84; 95% CI: 1.28–2.66] and high MN frequency groups (RR 5 1.53; 95% CI: 1.04–2.25) (113,321,322) thereby showing that MN is a biomarker for early genetic effect and is predictive of cancer. Therefore, it is important that DNA damage in human tissues is detected and monitored at the earliest possible phase of life for infants. However, there are no baseline DNA damage data for infants born to mothers at low risk of complications in Australia. Hence, it is important to determine the normal range of DNA damage for infants born to women at low risk of complications in pregnancy. These data can then be used to compare with the degree of DNA damage for infants born to women at high risk of complications during pregnancy. Timely intervention may prevent the accumulation of DNA lesions and the potential manifestation of subsequent chronic diseases, such as cancer, at a later stage of life (113,322).

2.2 Measuring DNA damage in infants

There are a number of assays that can be used to measure oxidative stress, DNA damage and cellular responses to DNA damage and oxidative stress, including 8-hydroxy-2′-deoxyguanosine (8-OHdG), an oxidized form of guanine (101); apurinic/redox factor-1, an essential enzyme in DNA base excision repair that possesses both DNA repair and redox regulatory activities (104); the terminal deoxynucleotidyl transferase-mediated assay, a direct method for the assessment of DNA fragmentation (323); the comet assay that it is a single cell gel electrophoresis assay measuring single or double DNA strand breaks (324); and phosphorylated H2AX (314), which measures double-strand DNA breaks. During the past 30 years, the cytokinesis block micronucleus-cytome (CBMN-Cyt) assay has evolved into a robust

and reproducible assay for measuring genome damage and cell death at the cytological level and cell division rate. The CBMN-Cyt assay of peripheral blood lymphocytes is one of the most comprehensive and best validated methods to measure chromosomal DNA damage, cytostasis and cytoxicity (108). The ‘‘cytome’’ concept in the CBMN assay implies that every cell in the system studied is scored cytologically for its DNA damage, proliferation and viability status (108). In this assay, genome damage is measured by scoring:

(i) Micronuclei (MN): a biomarker of both chromosome breakage and/or loss;

(ii) Nucleoplasmic bridges (NPB): a biomarker of DNA mis-repair and/or telomere end-fusions

(iii) Nuclear buds (NBUD): a biomarker of gene amplification and /or the removal of amplified DNA and/or unresolved DNA repair complexes (109,110).

DNA damage biomarkers (MN, NPB and NBUD) are measured *ex vivo* in binucleated lymphocyte cells (BNC), because only cells that complete nuclear division can express molecular lesions in both DNA and the mitotic machinery as chromosome breakage or chromosome loss events respectively that lead to MN, NPB and NBUD formation. Genome damage already expressed *in vivo* as MN and NBUD is measured in mononucleated lymphocyte cells (MNC) that fail to divide *in vitro* in the CBMN-Cytassay (325,326).

Numerous studies have shown significant correlations between the frequency of DNA damage in mothers/fathers and their offspring, suggesting common environmental, nutritional or lifestyle insults (304,326-330). The available data for CBMN-Cyt biomarkers, primarily MN frequency measured in BNC in cord blood among various populations have been summarized in **Figure 2.1**. Despite this accumulating data of DNA damage, measured with the CBMN-Cyt assay, in lymphocytes collected from umbilical cord blood and from older infants (306,315,326,328-334), there have been no published data on baseline DNA damage biomarkers in infants born in Australia. Application of the CBMN-Cyt assay that has a diagnostic potential to assess DNA damage in cord blood and in infants could provide important

baseline data to design research studies to determine the causes of such pathology and plan interventions to mitigate loss of genome integrity in early life.

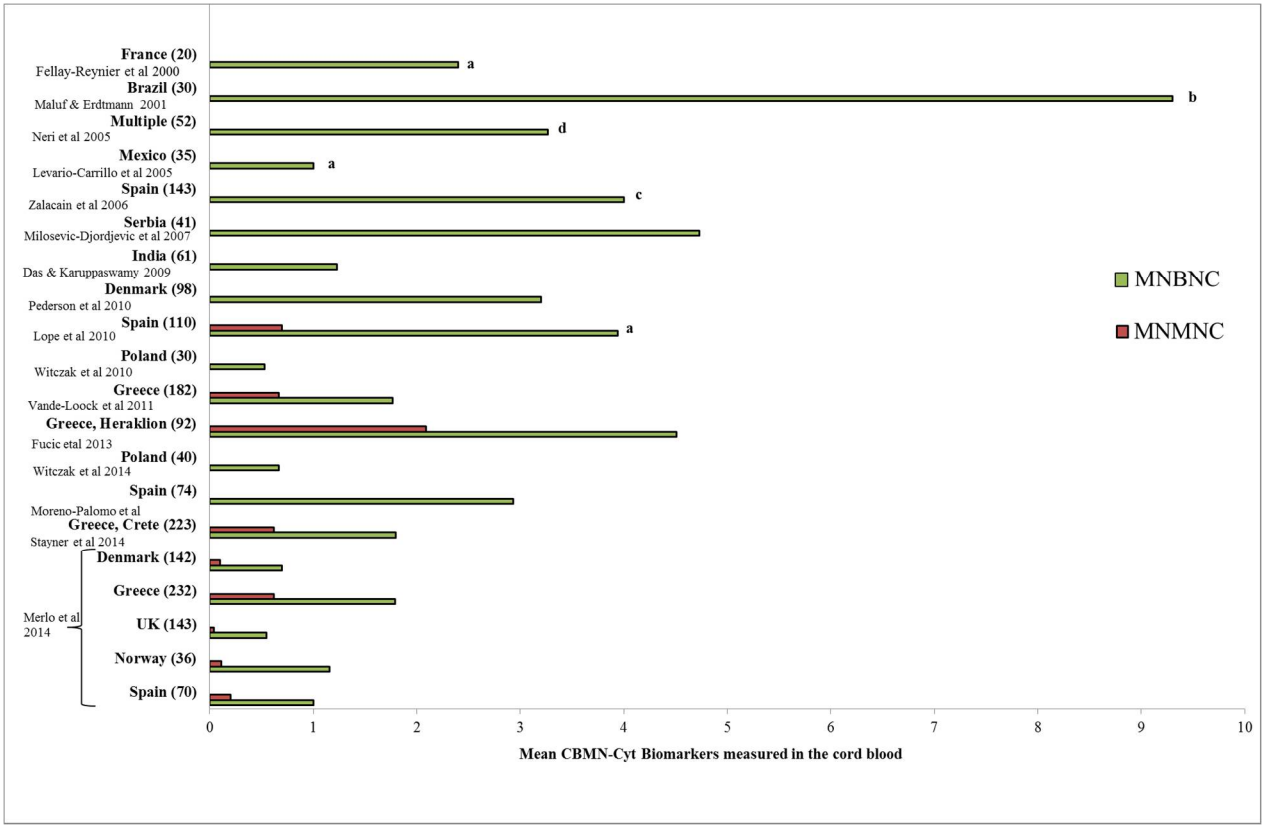


Figure 2. 1: Summary of mean MN frequency in BNC and MNC measured by CBMN-Cyt assay in cord blood of healthy infants
(number of subjects is shown in parenthesis and the names of authors are presented with the year of publication under the country's name)
Abbreviations: MN: micronuclei, BNC: binucleated lymphocyte cells, MNC: mononucleated lymphocyte cells,
a: represents data as micronucleated lymphocyte cells per 1000 BNC,
b: mean age of study participants =3.54 yrs and values per 2000 lymphocyte cells,
c: represents median value,
d: mean age of subjects ≤ 1 year, data represents pooled estimates

2.3 Neonatal outcomes, maternal factors and DNA damage markers

At birth, anthropometric measurements are the first indicators of an infant's general health (335). Growth assessment is an integral component of evidence-based care for newborns and infants and requires a comprehensive set of anthropometric standards that measure skeletal growth (head circumference and birth length) and fat and muscle mass (birth weight) (138,336,337). The APGAR score is a routine measure of comprehensive health at birth with respect to breathing effort, heart rate, muscle tone, reflexes and skin colour (338). The score is

usually assessed twice, at 1 and 5 minutes after birth, to determine both the neonate's tolerance of the birthing process and his/her adaptation to the extra-uterine environment (339). A low APGAR score at 5 minutes has been associated with increased infant mortality (339), but the tool is not proven to provide any predictive association with an infant's subsequent neurological or cognitive development (340). Head circumference is a measurement of a child's head around its largest diameters. A measure above the normal percentile may be a sign of hydrocephalus. A very small head circumference (microcephaly), on the other hand, often indicates a previous very slow growth rate and impaired brain development (341) and has been associated with nuclear replication stress caused by anaphase bridges, nucleoplasmic bridges and micronucleus formation in mice (342). Poor birth outcomes, such as low birth weight (LBW; <2500 g), due to prematurity or intra-uterine growth restriction, and small for gestational age (SGA, measured by low birthweight centiles), have been associated with adverse health outcomes during adulthood (337,343,344), both in underdeveloped and developing nations (345). At the other end of the continuum, in the developed countries overweight newborn infants may be considered "normal" (as their early obesity is not diagnosed) (346). Macrosomia (birth weight > 4000g) or large birth size may predict subsequent cardiometabolic imbalances in adult life (347-349) such as cardiovascular disease (350), type 2 diabetes (351), obesity (352) and some cancers (353). Further, a recent longitudinal cohort study observed that obese infants [with body mass index (BMI) $\geq 95^{th}$) at birth and at 6 months of age had shorter telomere length compared to non obese infants ($p=0.004$ and $p = 0.048$ respectively) during childhood (at 6 years of age) (354)

The maternal metabolic profile, including weight, age and BMI may be associated with adverse infant birth outcomes, such as birth defects (355,356) and preterm delivery (357). Maternal overweight may also be a causal factor for increased birth weight (358), as well as increasing the risk for cardiometabolic diseases of the offspring during his/her childhood and adult years (337,355,359,360). A meta-analysis has shown that maternal obesity increases the risk of

infants being born large for gestational age (LGA), having birth weight greater than 4000g (macrosomia) (360). Additionally, studies have consistently shown an association of increased maternal BMI with the infant's metabolic profile shift towards obesity (350,355,358,359,361,362), increased blood pressure (362,363), metabolic syndrome (364) and type 2 diabetes (365) during young adulthood. The experimental data indicate oxidative stress and inflammation to be the underlying mechanisms for prognosis of these metabolic disorders that leads to impaired DNA damage repair and cell cycle regulation (366-369). The inflammation-induced DNA damage, if it remains unchecked, may accumulate and may subsequently translate into an increased incidence of cancers (295,297-299,370,371).

Maternal factors including pre pregnancy BMI, weight, lifestyle variables such as smoking, diet and environmental exposures to pollutants are being investigated to study *in utero* genetic and epigenetic effects on infants' birth outcomes (306,308-312). A study conducted in Taiwan to measure DNA damage in the cord blood of neonates (n = 198), using the comet assay, reported higher DNA damage, reduced birth weight ($p = 0.005$), shorter birth length ($p = 0.021$) and smaller head circumference ($p = 0.013$) in neonates exposed to tobacco smoke *in utero* (n = 104) compared with those who were not so exposed (n = 94) (372). The study was conducted on a small group and DNA damage scores could not give comprehensive DNA damage data on DNA strand breaks or aneuploidy or cell death. Few studies have investigated association of maternal anthropometric variables and infant birth outcomes with DNA damage measures, utilizing CBMN-Cyt biomarkers. The NewGeneris study reported a significant inverse association between gestational age (GA) and MN frequency in MNC in the newborns (n =251), with significantly lower MN in MNC in preterm newborns (GA < 37 weeks) compared with those from term births at ≥ 37 weeks GA (333). Mother's age (>30 years) and infant birth weight was shown to modulate MN BNC in cord blood T lymphocytes in a small Mexican cohort (330). However, the Rhea mother-child cohort study found no association of gestational age with CBMN-Cyt biomarkers, measured in cord blood (326). The BioMadrid utilizing

automated image analysis system to measure MN frequency, also reported no association of MN frequency with both parental characteristics (including age and BMI) and infant birth outcomes (APGAR score at 1 minute, birth weight, GA) (328). In a prospective Boston-Birth cohort study, childhood z scores for BMI was observed to be positively associated with maternal pre-pregnancy BMI. The risk of childhood overweight or obesity (measured at 6 years of age) was significantly increased in overweight (RR=1.3[95% CI: 1.2, 1.6]) and obese (RR=1.6 [95% CI: 1.3, 1.8]) mothers' children compared to the risk of childhood overweight and obesity in children of normal-weight mothers (based on maternal pre-pregnancy body mass index). Additionally, the risk of childhood overweight increased significantly by 30% with each unit increase in maternal pre pregnancy BMI (RR=1.3[95% CI: 1.1, 1.4] (312). And in the NewGeneris cohort, maternal serum vitamin D (<50 nmol/L recorded at 14-18 weeks of gestation) was associated with increased MN BNC frequency in cord blood measured with automated image analysis system [incidence rate ratio (IRR= 1.32 (95%CI: 1.00, 1.72)]. This increase was higher for newborns with birth weight above the third quartile [≥ 3.5 kg; IRR = 2.21 (1.26, 3.89)] (310) indicating epigenetic influence of maternal factors on infants' metabolic profile.

As birth outcomes are predictors of the metabolic profile in adult life (373-375), it is important that baseline DNA damage profiles are assessed for an Australian population, to assist in designing chronic disease preventative strategies for the community.

2.4 Feeding methods and DNA damage during infancy

The public health significance of the relationship between infant feeding choice and chronic disease has been recognized in several major international reports (376-378). The type of feeding method adopted for infants may significantly influence the nutritional status of infants during the first few months of life. Children, who are breastfed for longer periods, have lower infectious morbidity and mortality than do those who are either breastfed for shorter periods or not breastfed (379,380). Recent literature also suggests that breastfeeding may protect against

the offspring being overweight later in life (381,382). The epidemiological evidence that pre and early postnatal nutrition influences the metabolic profile of adults, (Barker's hypothesis of 'early-life origin of health and disease') (344,383), has now been experimentally tested, the data demonstrating the epigenetic modulation of the key metabolic factor, the imprinted insulin-like Growth Factor (IGF)-2 gene, (384) during the fetal programming stage (383). The exact mechanisms underlying this methylation-driven shift (385) in programming, which might impact the risk of cardiometabolic diseases during adulthood, are still unknown. Animal experiments show that gene expression of metabolic markers may be switched 'on' or 'off' (386) by environmental factors, including nutrition *in utero* and during early postnatal development (386-390). Furthermore, trials in rats have demonstrated that an imbalance in metabolites in the one carbon pathway (homocysteine and folate), which increases oxidative stress and DNA damage (391-393), may be reversed by supplementation of methyl donors, such as folate and choline (394). An infant is dependent on optimal supplies of micronutrients from the mother's breast milk, complementary feeds or other dietary sources. The mechanisms of benefits of breast milk on infant DNA and gene expression is still not clear (395). Exclusive breastfeeding at 4–6 weeks of age may have long-term effects on child health, as evidenced by longer telomere length at 4 and 5 years of age in a recent longitudinal study on Latino children (354). Few studies have investigated the possible genome-protective effect of breast milk over formula feeds in infants. Shoji *et al* studied DNA damage in very low birth weight breast-fed [n=15, mean (±SD) GA 29.2 (±2.3) weeks and birth weight 1231 (±298) g] who received more than 90% of their intake as breast milk) and formula-fed (n=14) infants [mean (±SD)] GA 28.7 (±2.0) weeks and birth weig1182 (±281) g] who received more than 90% of their intake as commercial formula) at 2, 7, 14, and 28 days of age. They measured urinary OHdG as a biomarker of oxidative DNA damage, and observed that formula-fed babies had higher urinary OHdG concentrations than breast fed infants (396). The same investigators examined oxidative stress markers in one month old healthy infants (n = 41, 23M, 18F): the infants were divided

into four groups according to the type of feeding [Group 1 received 90% of their intake as breast milk; Group 2 received 50% to 90% of their intake as breast milk; Group 3 received 50% to 90% of their intake as formula; Group 4 received over 90% of their intake as formula]. The study found lower OHdG urinary concentrations in the mainly breast-fed group compared with the other groups (397). The sample size of both these studies, conducted in Japan, was small and it is possible that urinary 8OHdG may reflect more efficient excision of 8OHdG by DNA repair processes (110) but these preliminary data demonstrate a possible effect of mode of feeding on the DNA health of infants rather than the actual DNA damage. Hence, there is a need to utilize comprehensive and more robust DNA damage biomarkers when investigating genome health during vulnerable stages of human life, such as infancy. Another study, which utilized the comet assay to measure DNA damage in infants (n =70, aged 9-12 months), reported an increase both in limited DNA-damaged ($p < 0.001$) and in extensively DNA-damaged ($p < 0.001$) cells from infants fed cow's milk compared with cells from breast-fed infants (398). The aforementioned studies, however, neither collected the micronutrient status in blood samples or breast milk, nor considered the potential confounding effects of lifestyle factors, such as smoking, which are proven genotoxic agents (399-405).

Data from the Longitudinal Study of Australian Children (406) show that the proportion of infants who are exclusively breast fed (BF) declines rapidly after birth (**Figure 1.3**). In those babies who are not exclusively BF, breast milk may be replaced, to varying degrees, with formula milk, cow’s milk, soy milk and other drinks that differ in micronutrient and macronutrient composition relative to human breast milk (**Figure 1.4**) (406). However, it is not known whether such a shift in mode of feeding may modulate DNA damage biomarkers during first six months after birth.

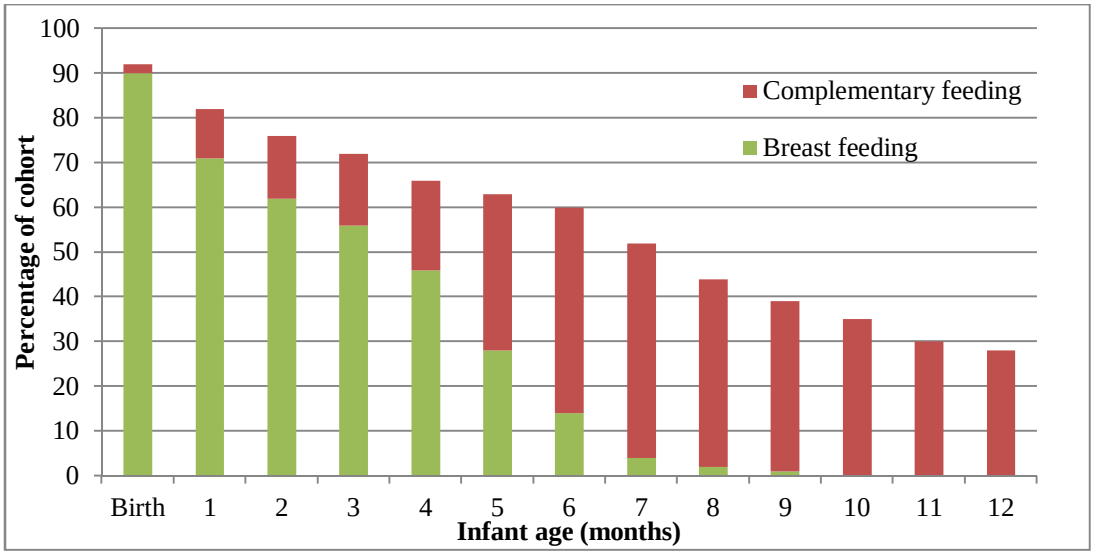


Figure 2. 2: Growing up in Australia: The Longitudinal Study of Australian Children (Annual report, Australian Institute of Family Studies 2006 2007, (Growing Up in Australia, Waves 1 and 2)

Figure 2. 3: Growing up in Australia: The Longitudinal Study of Australian Children (complementary feeds)
(Annual report, Australian Institute of Family Studies 2006 2007, (Growing Up in Australia, Waves 1 and 2)

2.5 Blood micronutrients and Infant DNA health

An optimal balance of dietary micronutrients is essential for maintenance of human cellular genome integrity (407). Dietary micronutrients, such as folate, vitamins B_{12}, B_6 and B_2 (254,408,409), magnesium (410), carotenoids (411,412), zinc (413-415), niacin (416), manganese (417,418), iron (419), selenium (420,421), copper (422), vitamin C, vitamin E (423-427) and vitamin D (428), are variably required as substrates or enzymatic cofactors involved in metabolic reactions (416,424,429-433). The roles of some of the micronutrients in human biological functions, including DNA replication and repair, are summarized in **Table 1.**

Table 2.1: Role of micronutrients in DNA health Role of micronutrients in DNA health

Micronutrient	Role in biological functions	Deficiency/ Excess
Iron (434-439)	❖ DNA synthesis: Enzyme ribonucleotide reductase is iron dependent ❖ DNA repair: iron is cofactor for DNA glycosylase and Alkyltransferases (enzymes for base excision repair and mismatch repair) ❖ Oxidative metabolism: iron is a cofactor of numerous enzymes that catalyse redox reactions, such as cytochromes responsible for oxidative phosphorylation in mitochondria ❖ Synthesis of organic and inorganic cofactors, such as haem and iron-sulphur clusters, is iron dependent ❖ Synthesis of oxygen transport proteins, in particular haemoglobin and myoglobin, is iron dependent ❖ Drug metabolism: iron is incorporated in cytochrome P450 ❖ Redox sensitive action: iron is involved in upregulation of nitric oxide synthase	❖ *Deficiency* may lead to anaemia and DNA damage ❖ *Excess* may cause increased production of free radicals and risk of, e.g., gastric cancer
Copper (440-444)	❖ Required for erythropoiesis ❖ Cofactor for numerous metallo-enzymes (such as superoxide dismutase) ❖ Development of central nervous system ❖ Functions as an electron acceptor/donor in key redox reactions, such as in mitochondrial respiration, synthesis of melanin and cross-linking of collagen ❖ Required in antioxidant pathway of superoxide dismutase, caeruloplasmin, catalase and glutathione ❖ Required for myocardial contractility	❖ *Deficiency* results in • Impaired energy production, abnormal glucose and cholesterol metabolism • Increased oxidative damage, increased tissue iron accrual • Altered structure and function of circulating blood and immune cells • Increased reactive oxygen species and oxidative damage to lipids, DNA and proteins

Micronutrient	Role in biological functions	Excess/Deficiency
Calcium (445-452)	❖ Provides rigidity to bone structure ❖ Enables Intracellular signalling pathways, such as the phosphoinositide and cyclic adenosine monophosphate systems ❖ Influences structural conformation of DNA Calcium is known to affect protein–DNA interactions by regulating secondary modifications, such as phosphorylation of various transcription factors, with consequences for gene transcription or DNA replication	❖ Dysregulation of mitochondrial Ca^{2+} homeostasis may generate reactive oxygen species ❖ *Deficiency* • Deficiency can cause paraesthesia, tetany, seizures, encephalopathy and heart failure ❖ *Excess* • Excessive Ca^{2+} concentrations may boost mitochondrial aspartate/glutamate carrier activity, mitochondrial metabolism and oxidative stress • Mitochondrial overload of Ca^{2+} may cause neuro-excitotoxicity, necrosis and apoptosis
Magnesium (111,410,453-457)	❖ Maintains genome stability: Mg is a cofactor of enzymes involved in DNA replication, gene expression and protein synthesis ❖ Changes in free Mg^{2+} concentrations serve as signals for cell	❖ *Deficiency* may manifest as • electrolyte imbalance, • Altered glucose homeostasis • Symptoms of depression

	cycle regulation and apoptosis ❖ Is a structural component of polyribosomes and nucleic acids ❖ Direct enzyme activation by complexion with ATP, ADP and GTP, (e.g., phosphofructokinase and pyruvate kinase) ❖ Maintains membrane function: cell adhesion ❖ Maintains low intracellular calcium concentrations ❖ Enables muscle contraction/relaxation ❖ Modulates neurotransmitter release ❖ Modulates action potential conduction in nodal tissue	• Unbalanced magnesium homeostasis is frequently observed in tumour cells • Inflammation and increased susceptibility to oxidative stress ❖ *Excess* • may cause neuromuscular symptoms by blockage of neuromuscular transmission and reduced serum calcium concentration
Micronutrient	**Role in biological functions**	**Excess/Deficiency**
Zinc (413-415,458-470)	❖ Structural component of proteins involved in DNA damage signalling and repair replicative enzymes, such as DNA and RNA polymerases, transcription factor tumour protein p53 ❖ Maintains the physiological values of metallothionein ❖ Cell cycle progression and apoptosis: allowing the cell to induce adequate repair of DNA before cellular division ❖ DNA damage response: Base excision repair; recognition and removal of 8-hydroxy-2-deoxyguanosine by hoGG1 glycosylase is Zn dependent ❖ Antioxidant: zinc is a free radical scavenger as an important cofactor for superoxide dismutase enzyme activity ❖ Important role in action of cobalamine independent methionine synthase enzyme that catalyses S alkylation reaction.	❖ *Deficiency* • causes oxidative stress • induces an increase in binding activity of transcription factors involved in regulating cell proliferation and apoptosis • results in a loss of DNA integrity and potential for increased cancer risk • impairs cognitive function ❖ *Excess* • inhibits the activity of some DNA repair proteins, including N-methylpurine-DNA glycosylase and DNA ligase 1 • causes chromosomal instability and DNA double strand breaks • induces Cu deficiency • may also induce cellular apoptosis • causes oxidative damage

Sodium and Potassium (471-482)	❖ Sodium (Na) and potassium (K) are the major extracellular and intracellular ions respectively in the human body. Together with chloride and bicarbonate ions, sodium is the major determinant of osmolarity of plasma and blood volume ❖ Maintain membrane potential in nerves and muscles ❖ Na/K gradient is the major active transport mechanism for nutrients, such as monosaccharides (sodium-glucose transporter 1), amino acids, pantothenic and lipoic acid (sodium dependent multivitamin transporter-SMVT), and is important in calcium homeostasis ❖ Interact with macro-ions to modulate solubility of proteins ❖ Activate major cell membrane enzyme sodium potassium ATPase ❖ Na+/K+ exchange can induce conformational switching of telomeric G-quadruplex (G4: G-rich portion of telomere)	❖ Dysregulation of homeostasis of sodium and potassium ions may lead to cell shrinkage ❖ Cytotoxicity of immune cells during carcinogenesis is dependent on sodium-potassium ion modulated calcium signalling
Micronutrient	**Role in biological functions**	**Excess/Deficiency**
Phosphorus (479,480,483)	❖ Structural component of deoxyribonucleic acid (DNA), ribonucleic acid (RNA), adenosine diphosphate (ADP), phospholipids and sugar phosphates ❖ Component of phosphate: a major intracellular buffer and helps to protect blood systemic acid/base balance, ❖ Acts as a temporary store and transport mechanism for energy ❖ Structural component of cell membrane (phospholipid) ❖ Aids in activating catalytic proteins through phosphorylation and dephosphorylation ❖ Indirectly involved in oxygen transfer (in red blood cells, synthesis of 2,3-diphosphoglycerate, which influences oxygen release from haemoglobin and requires phosphorus).	❖ Phosphorus *deficiency* is rare and may lead to leucocyte dysfunction, reduced cardiac output and neurological problems (such as encephalopathy, ataxia, seizure, neuropathy stimulating Guillain-Barré syndrome). ❖ Phosphorus *toxicity* may cause tetany and Hypocalcaemia
Sulphur (480,484-493)	❖ Sulphur is a constituent of various organo-sulphur compounds in the human body, such as thiols, amino acids (cysteine, methionine, taurine), biotin, Co A, Hcy, SAM, and contributes towards • Cellular energy production / metabolism	❖ *Deficiency* may: • impair growth and immune function • reduce gene expression (as component of methionine) • reduce cell growth and proliferation ❖ *Excess* may:

	• Protection of neural tissue – synthesis of neurotransmitters, improvement of neural memory and dampening of excessive firing of neurons • Antioxidant protection as thiols (e.g. glutathione, metallothionein) • Blood flow – produces both blood clotting factors as well as anticoagulants (fibrinogen, heparin) • Production of glycosaminoglycans, chondroitin sulphate and hyaluronic acid • Detoxification – by means of conjugation and chelation (required for metabolism of drug, steroids and xenobiotics) • Regulation of DNA replication and transcription- DNA processing enzymes contain Fe–S clusters • Methylation and gene expression (SAM) • As component of Hydrogen sulfide, it is known to protect endothelial cells against oxidative stress by enhancing activator protein 1 binding activity with the sirtuin3 (SIRT3) promoter	• increase oxidative stress (as component of homocysteine, inorganic sulphur derived through additives, pollutants) • Some forms are toxic, such as sulphite and sulphur dioxide
Micronutrient	**Role in biological functions**	**Excess/Deficiency**
Vitamin B_{12} (85,242,494-504)	Cobalamin plays a crucial role in ❖ DNA synthesis and regulation ❖ Synthesis of fatty acids ❖ DNA methylation ❖ Energy production ❖ One carbon metabolism along with folate ❖ Erythropoiesis ❖ Essential for normal neurodevelopment ❖ Coenzyme in reactions for conversion of methionine from homocysteine in the cytosol. and conversion of methylmalonyl-CoA to succinyl-CoA in the mitochondria	❖ *Deficiency* • Increases Hcy and MMA concentrations • Increased DNA damage • Pernicious anaemia • increased risk of PE, growth restriction and NTD • Hypomethylation of DNA ❖ *Excess* • MMA may cause increases in ROS in inflammatory cytokines (TNFα) • neurological abnormalities.
Folate (78,145,242,408,409,429,499,505-510)	❖ Maintenance methylation of DNA ❖ Synthesis of dTMP from dUMP ❖ Synthesis of methionine and SAM, the common methyl donor that determines gene expression and chromosome conformation	❖ *Deficiency* may lead to anaemia • Increased risk of neural tube defects • Hyperhomocysteinaemia • Reduced methylation of DNA • Genome damage owing to excessive

	❖ One carbon metabolism	incorporation of uracil instead of thymine • Inefficient DNA repair • Appearance of MN in lymphocytes • Increased cell apoptosis • Increased frequency of NPB and NBUD that may represent telomere-telomere end fusions or DNA misrepair and gene amplification respectively • Demethylation of heterochromatin causing structural centromere defects • Reduced or increased telomere length leading to telomere dydfunction • Mitochondrial DNA deletion

*Abbreviations :*DNA: deoxyribonucleic acid; RNA: ribonucleic acid; ADP: adenosine diphosphate;8-OHdG: 8 hydroxy deoxy guanosine; Na: sodium; K: potassium; Fe-S: iron-sulphur; CoA: coenzyme A; MMA: methylmalonic acid; Hcy: homocysteine; SAM: S-adenosyl methionine; dUMP: deoxyuridine monophosphate; dTMP: deoxythymidine monophosphate; TNF: tumor necrosis factor; ROS; reactive oxygen species, PE; pre-eclampsia, NTD: neural tube defects, MN: micronuclei, NPB: nucleoplasmic bridges, NBUD: nuclear buds

,

The dietary deficiency of these micronutrients, including trace minerals, at any stage of human development may induce DNA damage and epigenetic changes (98,511) and accelerated telomere shortening (99,409,512). Human cells are sensitive to both endogenous and exogenous insults during early phases of life. This is particularly evident *in utero* and during the early stages of infancy, where cells are more sensitive to the damaging effects of micronutrient deficiency (513). The pregnant woman's body undergoes preparation for labour, parturition and lactation at the same time while providing nutrients for foetal growth (514). During pregnancy an increase in inflammatory cytokines is required at the feto-placental interface for successful implantation and completion of pregnancy (515,516). This demands maximal output from endogenous antioxidant systems (glutathione peroxidase and superoxide dismutase) to counter the potential genome damaging effects of oxidative damage from inflammation (517). The deficiency of trace minerals required for efficient free radical quenching (mainly selenium, copper, zinc, iron, magnesium), along with cofactors necessary for strengthening immune and energy pathways (vitamin B_3, B_2, B_6, magnesium, copper, zinc, iron), may increase oxidative stress (517). Further, imbalances in the folate/methionine pathway, due to either genetic polymorphism (e.g. MTHFR) or deficiency of folate, B_2, B_6, folate and B_{12}, may increase Hcy (192,217,254,255,494,518-524). These imbalances are also associated with increased DNA damage (525,526). Status of some of these nutrients has been studied for their association with CBMN-Cyt biomarkers. Folate deficiency causes increased appearance of MN in human lymphocytes (145,499). There is also evidence to suggest that folate deficiency increases risk of PE (71,72,206-209,212,217,218,246,527,528). MN has also been observed in women at 20 weeks gestation to predict subsequent development of pre-eclampsia and/or intra-uterine growth restriction (IUGR) (118). Further, supplementation of folate, along with other B vitamins (B_2, B_6 and B_{12}), during pre- and peri-conceptional stages may potentially provide protective effects from complications arising from PE among women and their infants (71,523). Micronutrient status of iron in young subjects (434-436); calcium in children (529); zinc

(413,470,530) in *in vitro* human cells; nicotinic acid, vitamin E, retinol, beta-carotene, pantothenic acid, biotin and riboflavin *in vivo* in adults, have also been observed to influence CBMN-Cyt biomarkers (145).

There are few studies that have investigated plasma concentrations of trace minerals and their association with DNA damage biomarkers in infants and young children. A cohort study of young children (n=30, mean age 11.5 yrs) of poor economic status in Brazil found a negative association between the presence of both MN and NPB with red cell iron status (r= - 0.9, p = 0.002; r= 0.9, p= 0.01 respectively) (434). A cross sectional study in Western Australia of healthy children (3, 6 and 9 years, n=462) reported positive associations of plasma calcium with both MN (p = 0.01) and necrosis (p = 0.05), α tocopherol was negatively associated with NPB but lutein was positively associated with NPB (529). In the same cohort negative association of zinc concentration with telomere length was observed. And damage of the A allele of the reduced folate carrier A80G polymorphism was associated with shorter telomere and higher MN frequency (529). A biochemical and cytogenetic epidemiological study found a negative association of B_{12} with MN index in young subjects (aged 20-40 years, n =29, r = 0.20, p = 0.29) in South Australia (171,531).

Infant body composition and micronutrient status varies rapidly while adapting to internal (physiological) and external (mainly mode of feeding and environment) changes (532). Thus, in order to understand DNA damage in infants born to mothers with normal pregnancy or with pregnancy complications, it is important that the micronutrient status is assessed both in cord blood and in infant blood after birth.

2.6 Knowledge gaps

- There are no data on DNA damage, cell proliferation and cytotoxicity biomarkers in Australian infants, born from women at low risk of complications during pregnancy, both at birth and to six months after birth.

- It is not established whether there are any differences in the frequency of these biomarkers in infants with respect to gender and birth outcomes, such as weight, height, head circumference and APGAR score.

- No data are yet available whether DNA damage biomarkers increase or decrease in infants during the first six months after birth.

- It is not known whether mode of feeding (breast milk vs formula feed) influences DNA damage biomarkers in infants during first six months after birth

- It is not known whether blood micronutrient status of infants is associated with DNA damage biomarkers during first six months after birth.

- It is not known whether infants born to women with high risk of inflammatory conditions during pregnancy, such as pre-eclampsia, have increased DNA damage biomarkers compared with infants born to women with low risks of inflammatory conditions during pregnancy.

A prospective cohort study titled; 'Diet and DNA damage in Infants'- the DADHI study was therefore designed with the primary aim of collecting comprehensive data on DNA damage biomarkers in South Australian infants (0-6 months), utilizing the CBMN-Cyt assay. A pilot project on woman at high risk of complications, enrolled in the 'Investigations in Folic acid clinical trial' (INFACT) study was also planned.

The hypotheses and aims of the study were:

2.7 Hypotheses

1. The CBMN-Cyt biomarkers measured in cord blood are associated with infant birth outcomes

2. The CBMN-Cyt biomarkers measured in cord blood are associated with maternal demographic and lifestyle characteristics
3. Genome damage increases from birth to 6 months after birth
4. Genome damage is reduced in infants who are breast fed compared with those who are fed with complementary foods or formula milk
5. Plasma micronutrients are correlated with CBMN-Cyt biomarkers measured in lymphocytes collected from infants at birth, and at three and six months of age
6. Infants born to women at risk of pre-eclampsia have increased genome instability, as determined by the CBMN-Cyt assay, compared with infants born to mothers at low risk of pregnancy complications

2.8 Aims

1. To study association of infant birth outcomes with CBMN-Cyt biomarkers in cord blood
2. To study association of mother's demographic and lifestyle characteristics with CBMN-Cyt biomarkers
3. To measure the change in frequency of CBMN-Cyt biomarkers at birth, three and six months after birth
4. To determine whether mode of feeding influences CBMN-Cyt biomarkers in infants at 3 and 6 months after birth
5. To determine whether concentrations of plasma minerals considered necessary for genome stability are associated with CBMN-Cyt biomarkers in cord blood and at 6 months following birth

6. To determine whether infants born to mothers at risk of pre-eclampsia have increased frequency of CBMN-Cyt biomarkers compared with infants born to mothers with a lower risk of complications during pregnancy

3 Study design and general methodology

This chapter outlines the methods and protocol performed within the study. It also highlights the inclusion and exclusion criteria used for recruiting participants for the study.

3.1 Study Design

A longitudinal prospective cohort study – 'Diet and DNA damage in Infants'-The DADHI study was conducted on infants born to mothers at low risk of complications during pregnancy at the CSIRO Food and Nutrition and the Women's and Children's Hospital (WCH), Adelaide. The study was approved by the Human Experimentation Ethics committee of the CSIRO and the Human Research Ethics Committee of the WCH. All the participants were informed about the study aims and requirements through a detailed information sheet before giving their informed consent. A schematic representation of the study design is given in **Figure 3.1.**

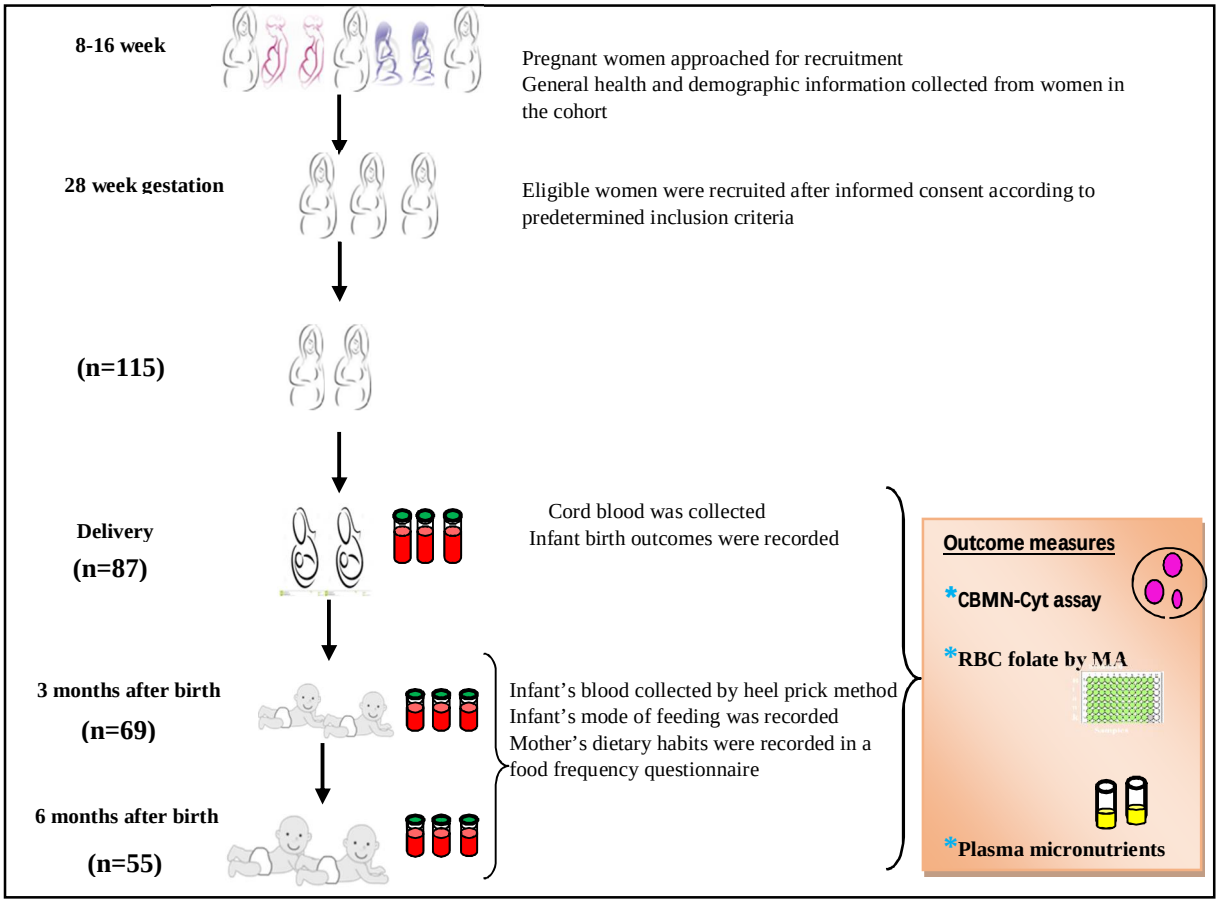


Figure 3.1: Schematic representation of the DADHI study design and recruitment
Abbreviations: **(**CBMN-Cyt: cytokinesis block micronucleus assay, RBC: red blood cell, MA: microbiological assay for folate)

3.2 Participants

Pregnant women at low risk of any complication during pregnancy were given information about the study at 8-16 weeks gestation during a regular check-up visit at WCH, Adelaide. Eligible women were enrolled at 16-28 weeks gestation and a signed informed consent was obtained from them.

3.2.1 Inclusion criteria

- Preferably second viable pregnancy (naturally conceived)
- Gestation age (GA) between $8^{0/7}$ and $16^{6/7}$ weeks of pregnancy (GA is based on the first day of the last menstrual period or ultrasound performed before $12^{6/7}$)
- No more than 2 previous first trimester losses

3.2.2 Exclusion criteria

- Multiple and/or IVF pregnancy
- Any disease or complication of pregnancy, including: hypertension, Type I or II diabetes mellitus, epilepsy, asthma, anaemia, inflammatory bowel syndrome, renal, liver or thyroid problems
- Body mass index (BMI) < 35 kg/m^2
- Infants born premature

3.2.3 Recruitment

A total of 1671 women were approached, attending the antenatal clinic at WCH to participate voluntarily for the study at 8-16 weeks gestation. A detailed Information sheet and consent form approved by the Human ethics committee of CSIRO and WCH was given to each interested woman to read and discuss with family members or friends prior to agreeing to participate in the study. The signed consent form was copied and attached to the medical record of each participant to ensure and facilitate proper collection of cord blood at the time of delivery.

Out of 1671 women, who were approached, 877 were assessed to be ineligible and 679 declined to participate in the study. The consort diagram for detailed information on recruitment of participants is presented in **Figure 3.2**.

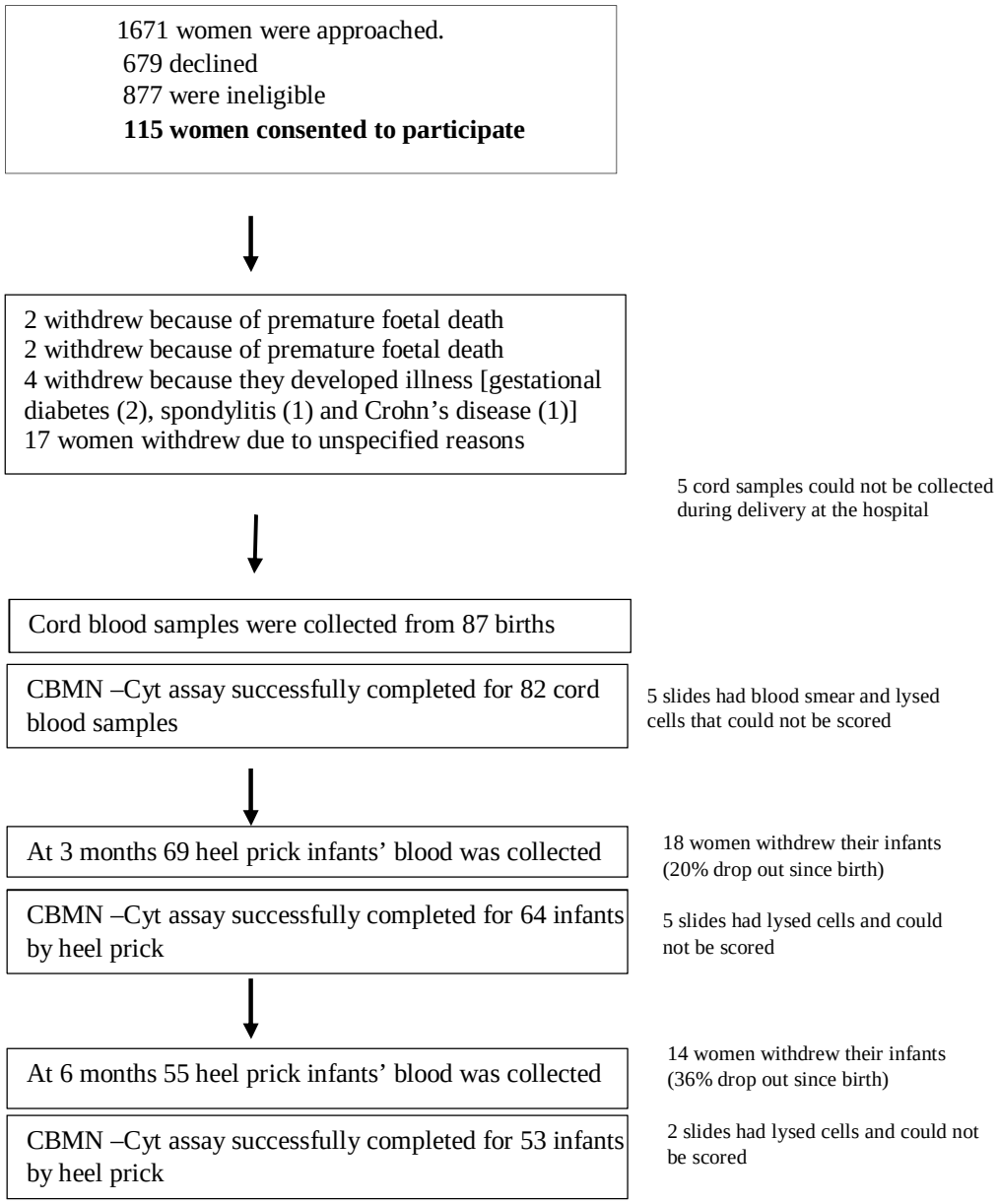


Figure 3.2: Consort diagram for DADHI study recruitment, blood collection and CBMN-Cyt assay completion
Abbreviation: CBMN-Cyt: Cytokinesis block micronucleus Cytome assay

3.3 **Power calculation**

Based on previously published data on 408 newborns (328,333,334,533) the expected mean (± SD) of micronucleus frequency measured in lymphocytes using the CBMN cytome assay is 1.20 (± 1.02). Using the SD value of 1.02 the study was powered to detect differences in micronucleus frequency between two groups ranging between 0.41 and 0.58 at 80% power and $p < 0.05$ (two-tailed) depending on the number of subjects per group (50-100) as indicated in **Table 3.1** below. The Table also lists detectable differences at higher power levels.

Table 3.1: Sample size to detect significant differences at different power levels

N per group	99%	95%	90%	80%
50	0.88	0.74	0.67	0.58
60	0.81	0.68	0.61	0.53
70	0.74	0.63	0.56	0.49
80	0.70	0.59	0.53	0.45
90	0.66	0.55	0.50	0.43
100	0.62	0.52	0.47	0.41

Note: Power calculations were made using GraphPad Statmate ver 2.0

3.4 **A pilot study**

A small group of women at high risk of complications during pregnancy was recruited from the Investigations in the Folic acid clinical trial (INFACT study) as a pilot study. The **Folic Acid Clinical Trial (FACT)** is a randomised, double-blind, placebo-controlled, Phase III, international multi-centre intervention of daily supplementation of 4.0 mg of folic acid (FA) from randomization until delivery of the infant for the prevention of pre-eclampsia (PE), funded through the Canadian Institutes of Health Research. Women were recruited for the FACT study on the basis of an increased risk of PE (previous PE, twin pregnancy, chronic hypertension, pre-existing diabetes, obesity), and those in the Adelaide cohort were approached for participation in the INFACT study. The INFACT study was designed to evaluate the effect of high dose folic

acid on maternal and infant folate status, on DNA damage markers in mother, neonate and the infant, on neonatal and infant adiposity, and on the development of an allergic cytokine profile in the offspring. The study was approved by the Human Research Ethics Committee of WCH, Adelaide. All the women were informed about the study aim and requirements through a detailed Information sheet before giving their informed consent. The schematic representation of the study design is given in **Figure 3.3.**

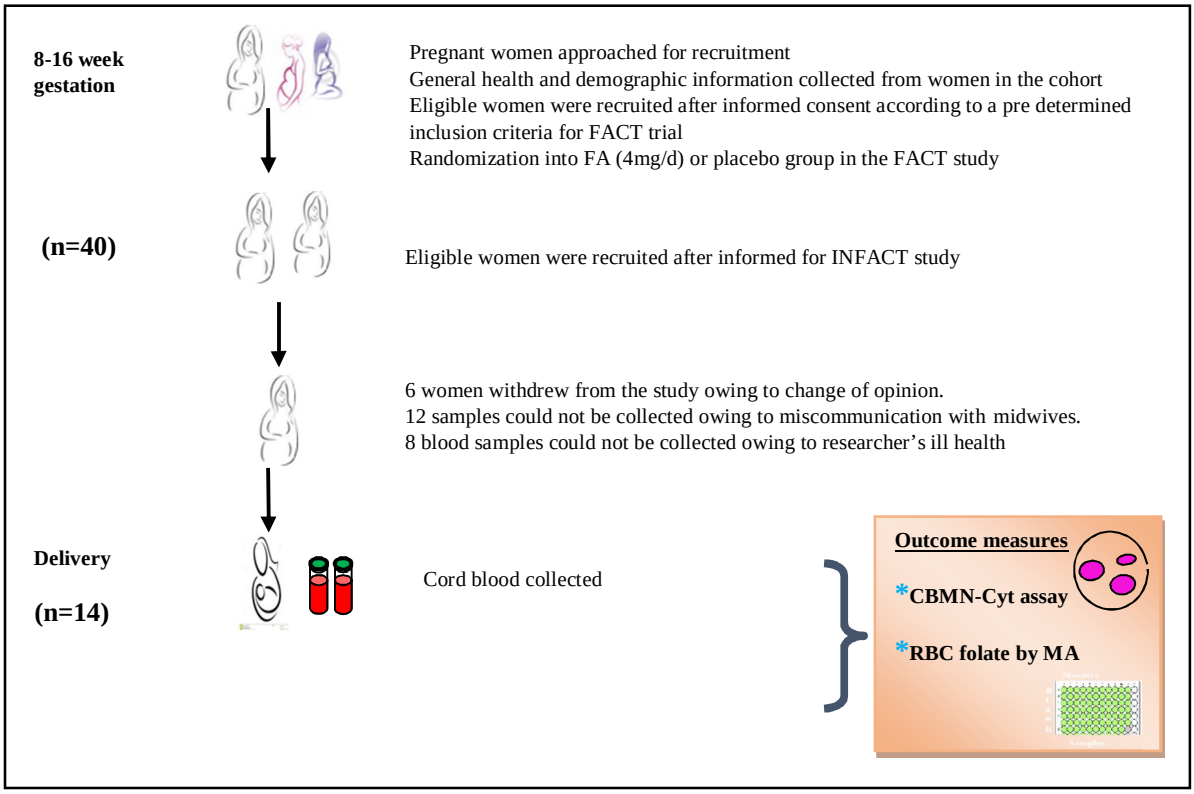


Figure 3.3: Schematic representation of the pilot project in the INFACT study
Abbreviations: **(**CBMN-Cyt: cytokinesis block micronucleus assay, RBC: red blood cell, MA: microbiological assay for folate, FACT: folic acid clinical trial)

3.4.1 **Inclusion criteria**

- ≥18 years of age at the time of consent
- Taking ≤1.1 mg of folic acid supplementation daily at the time of randomization.
- Live foetus

- GA between $8^{0/7}$ and $16^{6/7}$ weeks of pregnancy (GA is based on the first day of the last menstrual period or ultrasound performed before $12^{6/7}$).
- At least one of the identified risk factors for PE:

- Pre existing hypertension (documented evidence of diastolic blood pressure ≥90 mm Hg or use of hypertensive medication during this pregnancy specifically for the treatment of hypertension prior to randomisation)
- Pre pregnancy diabetes (documented evidence of Type I or Type II diabetes mellitus)
- Twin pregnancy
- Documented evidence of history of PE in a previous pregnancy
- BMI ≥35kg/m^2

3.4.2 **Exclusion criteria**

- Known history or presence of any clinically significant disease which would be a contraindication to FA supplementation
- Known foetal anomaly/demise
- History of medical complications including renal disease, epilepsy, cancer or use of FA antagonists
- Current enrolment in other clinical trials or who have received an investigational drug within 3 months of randomisation
- Higher order (>2) multiple pregnancy
- Known hypersensitivity to FA
- Known current alcohol abuse (≥2 drinks per day)

3.4.3 **Sample size**

In total, 124 women enrolled in the FACT study were approached to participate in the INFACT study up to March 2015. 40 women consented to be part of the sub study of INFACT project.

6 women withdrew from the study owing to change of opinion. 12 samples could not be collected owing to miscommunication with midwives. 8 blood samples could not be collected owing to the researcher's ill health. Thus, at delivery, cord blood was collected from 14 women enrolled in INFACT to be part of this pilot study. The control group comprised infants (n=19) born to women with low risk of pregnancy complications (subset from the DADHI study) that has been discussed in detail in chapter 6 and 7, and were matched for gender and birth weight (± 150g) at birth (indicated as DADHI control in this chapter 8).

3.5 General health and Food frequency questionnaire

A general health questionnaire was administered to participating women (in both DADHI and INFACT study) at between 8 and 16 weeks gestation to collect detailed information about the mother's demographics, medical and family history, lifestyle habits such as smoking, dose and duration of FA supplementation and other supplements and any medicines consumed during the pregnancy period. Mother's weight at recruitment was recorded using a digital balance accurate to within 100 g, and height was determined using a stadiometer accurate to within 1 cm of overall height. BMI was then calculated using the formula weight (kg)/ height $(m)^2$. Type of labour and delivery (Caesarean/induced, normal/spontaneous) and any complications during labour was also recorded. A Food Frequency questionnaire (FFQ) (The Cancer Council, Victoria) was administered at 3 and 6 months postpartum to collect information about the mother's intake of macro and micro-nutrients (534)**.** Details regarding infant's birth weight, height, head circumference, APGAR score at 1 and 5 minutes post birth, gender and gestation age were also recorded.

3.6 Infant's feeding record

During the first six months after birth, infants may vary significantly in their feeding history in terms of (i) the period that they were exclusively breast fed, (ii) the total cumulative duration of breastfeeding and (iii) the substitute or "complementary" foods used when the baby was not exclusively breast fed (406). The information regarding mode of feeding for the infants in the

cohort was collected during months 1-3 and 4-6 months for the DADHI cohort (**Appendix 1**). Based on the data collected each infant was given a score of 1 to 4 (**Table 3.2**). The scores were then averaged for the first 3 months and for the period between 3- 6 months. (**Appendix 1a**)

Table 3.2: Scoring criteria for infant mode of feeding

Mode of feeding	**Score**
Exclusive breast fed	4
Partially breast fed	3
Exclusive formula fed or other milk (soy or cow)	2
Partially formula fed or other milk	1

3.7 Blood collection

For INFACT participants: Approximately 20 ml of cord blood was collected immediately after birth into two 9 ml sterile Lithium Heparin coated collection containers (green top; Greiner Vacuette 2 mL Cat.No. 454089). The tubes were kept at 4^{o}C before being transported to the CSIRO Nutrigenomics laboratory in a lab top cooler within 4-6 hours of collection. The cord blood was kept at room temperature (18-22^{o}C) and was prepared for the CBMN-Cyt assay (The assay is explained in separate chapter 4). After removing the blood required for CBMN-Cyt assay (2*100µl) from cord blood samples, the whole blood tubes were centrifuged at 3000 rpm for 20 minutes to separate the plasma. The red blood cells cells (1*100 µl) were stored in cryovial at - 80°C at the CSIRO Nutritgenomic laboratory for microbiological assay of folate (The assay is explained in chapter 5).

For DADHI participants: The cord blood collection was same as approximately 20 ml of cord blood was collected immediately after birth into two 9 ml sterile Lithium Heparin coated

collection containers (green top; Greiner Vacuette 2 mL Cat.No. 454089). The tubes were kept at 4ºC before being transported to the CSIRO Nutrigenomics laboratory in a lab top cooler within 4-6 hours of collection. The cord blood was kept at room temperature (18-22ºC) and was prepared for the CBMN-Cyt assay.

After removing the blood required for CBMN-Cyt assay (2*100µl) from cord blood samples, the whole blood tubes were centrifuged at 3000 rpm for 20 minutes to separate the plasma. The red blood cells cells (1*100 µl) were stored in cryovial at - 80°C at the CSIRO Nutritgenomic laboratory for microbiological assay of folate.

2mL of plasma was isolated and stored for mineral/micronutrient analysis at -20°C, till transported to Institute of Medical and Veterinary Science (IMVS, Adelaide). Two tubes with 300 µl plasma were stored at -80°C till transported IMVS for serum folate and vitamin B_{12} by immunoassay method utilizing ADVIA Centaur XP Immunoassay System.

For DADHI infant cohort at three and 6 month time points after birth, 1 ml of infant blood was collected in a Vacuette® Lith/Hep coated tube by an experienced nurse at CSIRO clinic into a 1 ml mini vial from a heel prick using the tenderfoot method (535) and was stored in a labtop cooler (Nalgene 0ºC labtop cooler 3x4 tubes 17mm, Lot: 7111573010) at 18-22ºC and the CBMN-Cyt assay was performed. After removing the blood required for CBMN-Cyt assay (2*100µl) from infant samples, the whole blood tubes were centrifuged at 3000 rpm for 20 minutes to separate the plasma. The red blood cells cells (1*100 µl) were stored in cryovial at - 80°C at the CSIRO Nutritgenomic laboratory for microbiological assay of folate.

The remaining plasma was isolated and stored for mineral/micronutrient analysis at -20°C, till transported to Institute of Medical and Veterinary Science (IMVS, Adelaide). The process of blood collection for DADHI study is explained in **Figure 3.5.**

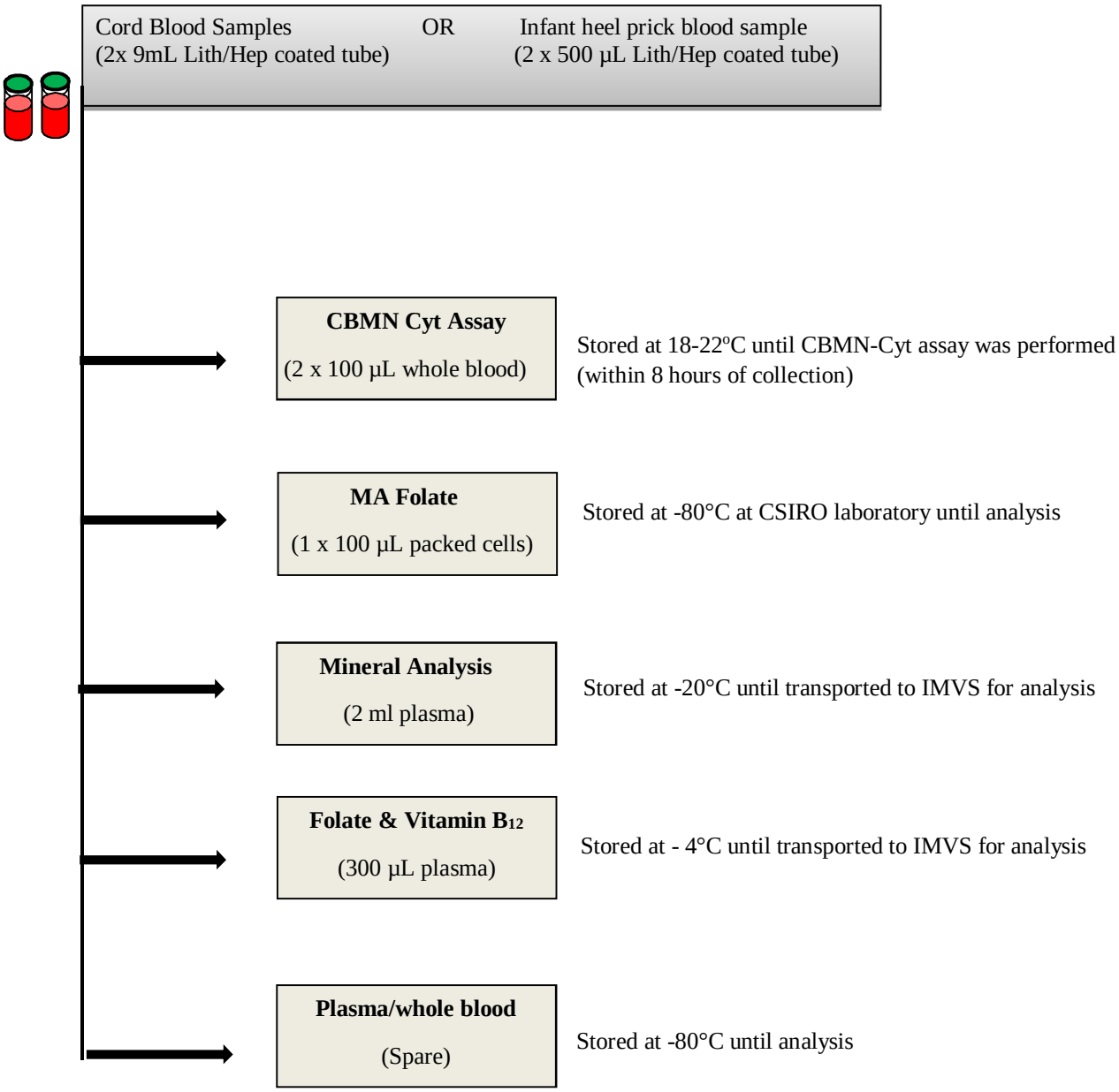


Figure 3.4: DADHI processing protocol for cord bloods and infant heel prick bloods

[Adapted from protocol designed by Maryam Hor (research assistant at CSIRO nutrigenomic laboratory)]
Abbreviations: MA Folate: Microbiological assay for Folate; IMVS: Institute of Medical and Veterinary Science

4 Cytokinesis block micronucleus- Cytome assay

4.1 Principle

The cytokinesis block micronucleus-cytome (CBMN-Cyt) has evolved into a comprehensive and robust method for measuring DNA damage in peripheral blood lymphocytes over the past

25 years (108,536). The assay is a broad system of analysing and measuring DNA damage, cytostasis, and cytotoxicity (**Table 4.1**) (108).

Table 4.1: Biomarkers assessed in CBMN-Cyt assay

Genome integrity measure	Biomarker
Genome damage	MN, NPB, NBUD in BNC and MN, NBUD in MNC
Cytostasis	MNC, BNC, Multinucleated cells, Nuclear division index
Cytotoxicity	Apoptosis, Necrosis

Abbreviations: MN: micronuclei; NPB: nucleoplasmic bridges; NBUD: nuclear buds, BNC: binucleated lymphocyte cells, MNC: mononucleated lymphocyte cells

The ‘‘cytome’’ concept in the CBMN assay implies that every cell in the system studied is scored cytologically for its DNA damage, proliferation and viability status (108). In this assay, genome damage is measured by scoring: Micronuclei (MN): biomarker of both chromosome breakage and/or loss; Nucleoplasmic bridges (NPB): a biomarker of DNA mis-repair and/or telomere end-fusions and Nuclear buds (NBUD): a biomarker of gene amplification and /or the removal of unresolved DNA repair complexes (109,110).

DNA damage biomarkers expressed *ex vivo* (MN, NPB, NBUD) are measured in once divided binucleated lymphocyte cells (BNC, that are accumulated by blocking cytokinesis with cytochalasin B) because only cells that complete nuclear division can express molecular lesions in DNA and in the mitotic machinery that lead to MN, NPB and NBUD formation. Genome damage already expressed *in vivo* as MN and NBUD is measured in mononucleated lymphocyte cells (MNC) that fail to divide *in vitro* in the CBMN-Cyt assay (110,325,326,537) (**Figure 4.1**). Expression of MN may also be a surrogate marker of DNA hypomethylation because

hypomeythylation of pericetromeric DNA leads to chromosome malsegregation and lagging chromosomes which form into micronuclei (108,109,538).

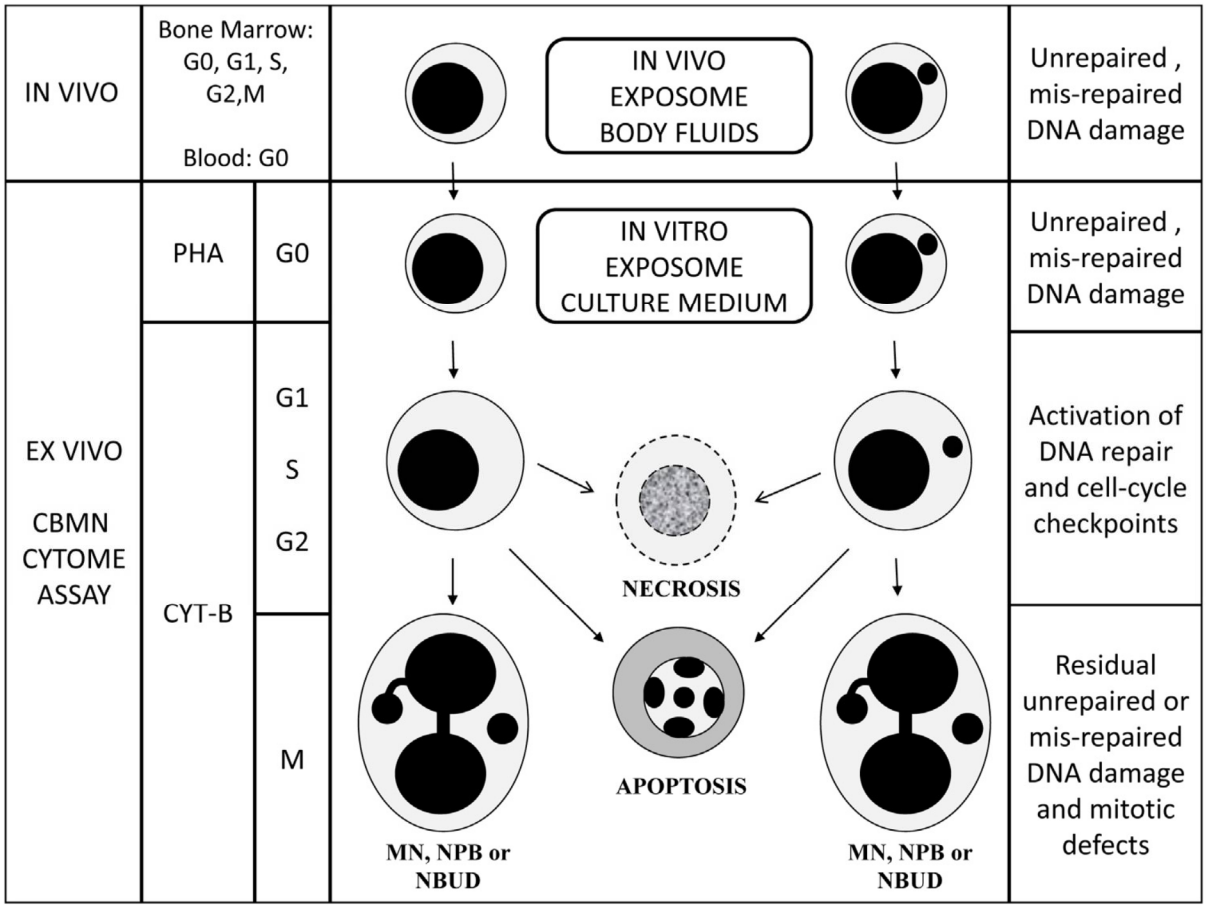


Figure 4.1: Cytokinesis-block micronucleus Cytome assay (109)
Abbreviations: MN: micronuclei; NPB: nucleoplasmic bridges; NBUD: nuclear buds, BNC: binucleated lymphocyte cells, MNC: mononucleated lymphocyte cells; DNA: deoxyribonucleic acid; CBMN: Cytokinesis-block micronucleus Cytome; Cyt-B: cytochalasin B; PHA: Phytohaemagglutinin; G0, G1, S, G2 and M: different phases during the interphase stage of cell division (G: gap, M: mitosis, S: synthesis)

4.2 **Lymphocyte CBMN-Cyt method**

Initially cells lymphocytes are stimulated to divide *in-vitro* using a plant lectin [Phytohaemagglutinin (PHA)] followed by exposure to cytochalasin-B (Cyto-B) solution to block the cells that have completed mitosis at the binucleated cell stage by inhibiting cytokinesis. Thus a nuclear division is completed and various chromosomal DNA damage biomarkers may be observed as nuclear anomalies in once divided binucleated cells. In the

present study, the whole blood CBMN-Cyt assay was conducted as previously described by Fenech M 2007 (108). The outline for the assay is depicted in **Figure 4.2**

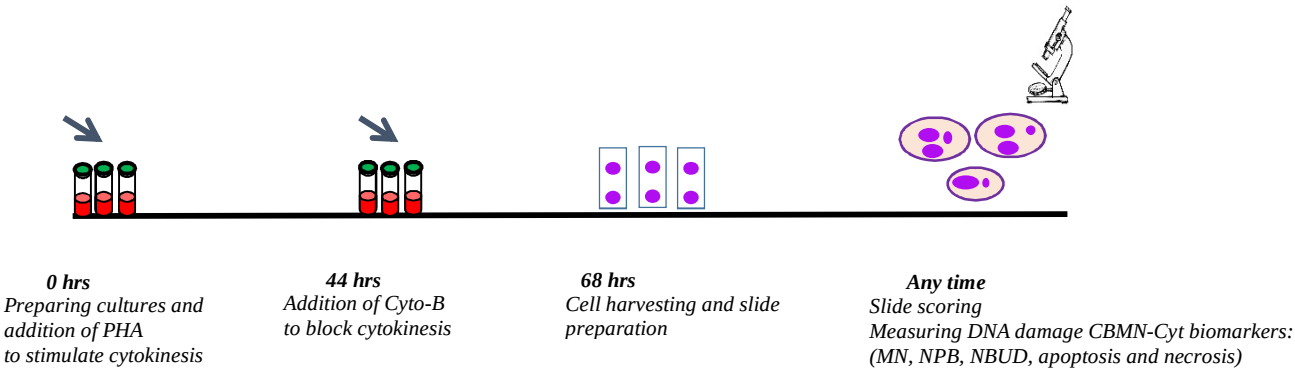


Figure 4.2: Outline of CBMN-Cyt assay
Abbreviations: PHA: Phytohaemagglutinin; Cyto-B: cytochalasin-B, MN: micronuclei; NPB: nucleoplasmic bridges; NBUD: nuclear buds

4.2.1 **Preparation of reagents**

Ficoll-Paque: 100 ml sterile liquid (Amersham Pharmacia Biotech, Sweden, cat no. 17144002). This product is stable if bottle remains unopened, however is susceptible to deterioration on exposure to air for prolonged duration. Hence, the bottle is dated on opening; the solution is extracted with the use of sterile needle and syringe without opening the seal and minimum amounts (100 ml) of solution is extracted for a single use.

Hanks Balanced Salt solution (HBSS): sterile with calcium and magnesium without phenol red (Trace Scientific, Melbourne, Australia, Cat no. 111010500-V); stored at 4 ºC but use at room temperature.

RPMI 1640 without L Glutamine: 100 ml sterile liquid (Sigma, R0883, Australia). Store at 4 ºC. Use at 37 ºC when preparing cultures.

Fetal Bovine serum (FBS): 100 ml, sterile FBS (Trace Scientific, Melbourne, Australia, cat no 15010-0100V) is stored at -20 ºC. Thaw in a 37 ºC water bath before adding to the culture medium. The thawed solution is stable for 4 weeks. Repeated thawing and refreezing were avoided.

L-Glutamine: 200mM sterile solution (Sigma, Sydney, Australia, cat no. G7513); stored in 1ml aliquots at -20 ºC for up to 2 years. The solution was thawed at room temperature before adding to the culture medium.

Sodium Pyruvate: 100 mM sterile solution (Sigma, Sydney, Australia, cat no. S8636), stored at -20 ºC in 1ml aliquots for up to 2 years. The solution was thawed at room temperature before adding to the culture medium.

Culture medium [RPMI, 10% FBS, 1% sodium pyruvate (100 mM) and 1% L-glutamine (200 mM)]. 20 ml of culture medium was prepared using 17.6 ml RPMI, 2 ml FBS, 200 µl Glutamine and 200 µl Sodium Pyruvate. The culture medium was prepared in a sterile tissue culture grade plastic bottles. It may be stored at 4 ºC for up to 1 week. Before use, the media was pre-warmed at 37ºC in a humidified incubator with a 5% CO_2 atmosphere.

DMSO: sterile filtered soulution of DMSO (Sigma, D2650, Australia) was stored at room temperature (20 ºC).

Cytochalasin-B (Cyto-B, Sigma, C6762, Australia): Five milligrams of solid Cyto-B was dissolved in 8.33 ml DMSO to give a Cyt-B solution 600 µg/ml. This stock Cyto B was stored at -20 ºC in a vial for 12 months. On the day of assay, 100 µl of the stock Cyto B was thawed, and 900 µl of culture medium was aseptically added room temperature to the vial to obtain a 1,000 µl solution of 60 µg/ml. This cytotoxic agent is a possible teratogen and hence was prepared in a Cyto guard cabinet and for precaution personal protective clothing including Tyvek gown, double nitrile gloves and safety glasses were used.

Phytohemagglutinin (PHA, Murex Biotech, Dartford, UK, Cat no 8e27-01): 45 mg freeze dried extract of PHA was dissolved in 20 ml sterile isotonic saline to give a concentration of 22.5 mg/ml. This stock PHA could be stored for 4 weeks only. On the day of assay, 100 µl of stock PHA was diluted with 900 µl of culture media to get a working solution of 2.25 mg/ml.

Diff Quick fixative set (Lab Aids, Narrabeen, Australia).

DePeX mounting medium (BDH laboratory, Poole, UK).

4.2.2 **CBMN-Cyt assay protocol**

On day 0, 100 µl of heparinised whole blood was cultured in 810 µl medium. The mitogenic activity in lymphocytes was initiated by adding 90 µl PHA solution to give a final concentration of 202.5 µg/ml. The time of PHA addition was recorded. The cells were incubated at 37 ºC with loosened lids in a humidified atmosphere containing 5% carbon dioxide for 44 h.

At 44 hrs, the cell cultures were carefully removed from the incubator and 100 µl of cytochalasin-B solution was gently mixed into the cultures. The cells were returned to the incubator for a further 24 hrs.

At 68 hrs, cultures were removed from the incubator, and the cells were mixed gently. The cell suspension was underlaid with 400 µl of Ficoll-Paque in a TV10 tube (Techno Plas, S9716VSU, Australia) using a ratio of 1 (Ficoll):3 (cell suspension) without disturbing the interface. The tube containing cell suspension overlaid on Ficoll was then centrifuged once at 400g for 30 min at 18 to 20ºC to separate the lymphocytes. Using a pipette with a 200 µl clear plugged tip, the 'buffy' lymphocyte layer at the interface of the Ficoll Paque and culture medium was removed carefully avoiding uptake of Ficoll. The lymphocyte suspension was washed in three times its volume of Hanks HBSS by gently pipetting in 1320 µl HBSS solution and then centrifuging at 180g for 10 min at room temperature to remove any residual Ficoll and cell debris.

The supernatant was gently removed, leaving approximately 200 µl cell suspension. Subsequently, 15 µl dimethyl sulfoxide (DMSO 7.5% v/v of cell suspension Sigma, Sydney, Australia) was added to prevent cell clumping and to optimize visualization of cytoplasmic boundaries.

This was followed by harvesting of cells: microscope slides were prepared by washing in absolute ethanol. The slides were allowed to dry. The slides were then labelled along with a filter card that was then together assembled with cytocentrifuge cup utilizing a slide holder. The

combined slide, filter card, and cytocentrifuge cup were as per manufacturer's instruction and spun in a cytocentrifuge (Model Cytospin 3, Shandon Southern Products, Cheshire, UK).

One hundred microliters of cell suspension was added to the cytospin cup corresponding to the numbered slide in the rotor and spun at 600 rpm for 5 min. A spot was obtained at the end of centrifugation. The card and the slide were inverted and the above process repeated in order to obtain a second spot. The slides were air dried in a biohazard hood for 10 minutes followed by fixing in Diff Quick fixative (Lab Aids, Narrabeen, Australia) for 10 min. Then the slides were transferred directly into Diff Quick stain: 10 dips in the orange stain followed by 5 dips in the blue stain. The extra stain was washed off with tap water and slides were left to air-dry for 10 minutes. The slides were finally cover slipped using DePeX mounting medium (BDH laboratory, Poole, UK) in a fume-hood.

Scoring of slides

The slides were scored for the various CBMN-Cyt biomarkers using standard criteria (108,539) and photomicrographs and criteria of endpoints that were measured are shown in **Table 4.2.** The scoring sheet for recording all the CBMN-Cyt biomarkers is included in **Appendix 1 and 2**. A conventional light microscope (Model Leica DMLB2: Leica Microsystem, Wetzlar, Germany) was used to examine the cells at 1000 x magnification. Two scorers (MH and TA) individually counted 500 cells for cytostasis markers [mononucleated, binucleated and multinucleated lymphocyte cells (>2 nuclei)] and cytotoxicity biomarkers (necrotic and apoptotic). The frequencies of MNC, BNC and multinucleated cell are used to measure the nuclear division index (NDI). The NDI provides a measure of the proliferative status of the viable cell fraction and thus indicates mitogenic response in lymphocytes (108).

The formula for calculating NDI is as follows (540).

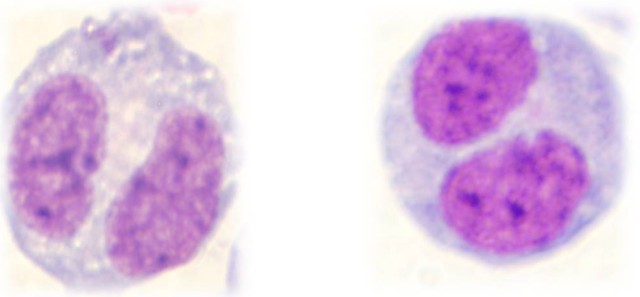
$$NDI = \frac{(M1 + 2M2 + 3M3 + 4M4)}{N}$$

*where M1–M4 represent the number of cells with 1–4 nuclei

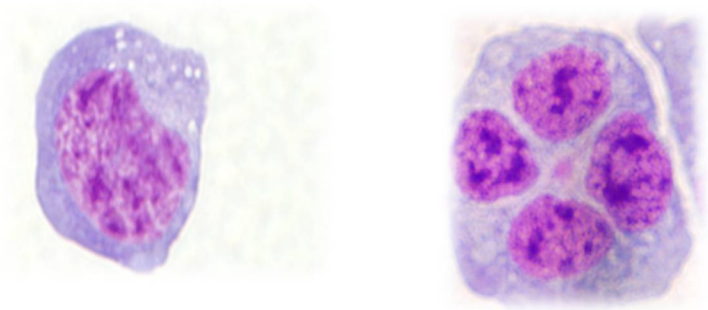
*N is the total number of viable cells scored (excluding necrotic and apoptotic lymphocytes).

A NDI score of 1 represents that all viable cells have failed to divide during the cytokinesis-block period and so all are mononucleated (540). A score of 2 indicates that all viable cells have completed one division and hence are binucleated. A score greater than 2 implies that some viable cells have completed more than one nuclear division during the cytokinesis-block phase and that a significant proportion of cells with two or more nuclei have been observed (108). Both the scorers independently counted the CBMN-Cyt assay biomarkers (MN, NPB, NBUD) in 1000 BNCs from each duplicate culture to give an overall total for each biomarker per 4000 BNC per sample. The results were then averaged and presented for every 1000 BNCs. A third scorer (MD) independently counted all MNC per slide spot in a slide, and DNA damage biomarkers were measured in MNC (MN and NBUDs), using criteria previously described (539). An average of 500 MNCs were scored for MN and NPB in each duplicate culture. The results in MNC were expressed as MN and NBUD per 100 MNC per subject. The HUMN scoring criteria recommends that the MN frequency be determined in a minimum of 1000 cells (539) but in 40% of our slides, there were insufficient MNC to score 1000 lymphocyte cells.

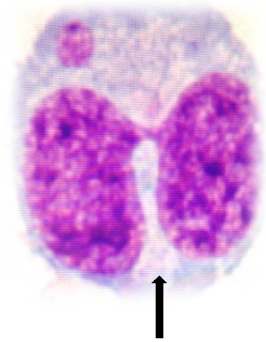
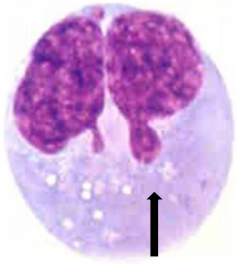
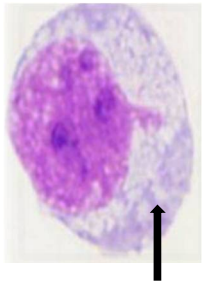
Table 4.2: Scoring criteria with photomicrographs of CBMN-Cyt biomarkers

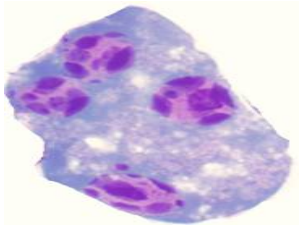
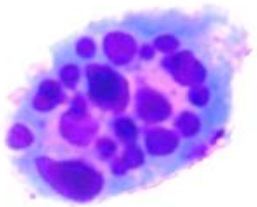
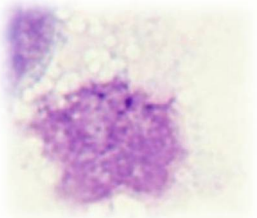
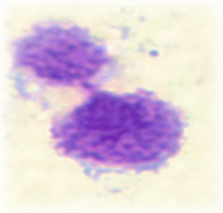
Characteristic of a binucleated lymphocyte cell	Photomicrograph
1. The cells should be binucleated. 2. The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary. 3. The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining intensity. 4. The two nuclei within a BNC 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 BNC should be intact and clearly distinguishable from the cytoplasmic boundary of adjacent cells.	

Contd.

Characteristics of Mono and multinucleated cells	Photomicrograph
Mono and multinucleated cells are viable cells with intact cytoplasm and normal nuclear morphology containing one or more nuclei, respectively. They may or may not contain one or more MN or NBUDs.	
Characteristics of Micronuclei (MN)	**Photomicrograph**
MN is morphologically identical to but smaller than the main nuclei and may be observed and scored in MNC and BNC. They 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 BNC cell, respectively. 2. MN is round or oval in shape and is not linked or connected to the main nuclei. 3. MN is non-retractile and can therefore be readily distinguished from artefacts such as staining particles. 5. MN may touch but not overlap the main nuclei and the micronuclear boundary should be distinguishable from the nuclear boundary. 6. MN usually has the same staining intensity as the main nuclei.	 Micronuclei in a BNC  Micronuclei in a MNC

Contd.....

Characteristics of Nucleoplasmic bridges (NPB)	Photomicrograph
NPB are sometimes observed in BNCs following exposure to clastogens and are thought to originate from rearranged chromosomes with more than one centromere, e.g. dicentric chromosomes. 1. NPB is a continuous nucleoplasmic link between the nuclei in a BNC. 2. The width of a NPB may vary considerably but usually does not exceed one-fourth of the diameter of the nuclei within the cell. 3. NPB should have the same staining characteristics of the main nuclei. 4. On rare occasions more than one NPB may be observed within one BNC. 5. A BNC with a NPB may or may not contain one or more MN. NPB are preferably scored in BNC with clearly separated nuclei because it is usually difficult to observe a NPB when the nuclei are touching or overlapping.	  Nucleoplasmic bridge in a BNC
Characteristics of Nuclear Bud (NBUD)	**Photomicrograph**
The NBUD may be measured in MNC and BNC. NBUD is morphologically similar to micronuclei with the exception that they are clearly joined to the nucleus and having a continuous connection between the nucleoplasmic material in the nucleus and the nuclear bud. 2. They usually have same staining intensity as MN 3. Occasionally buds may appear to be located within a vacuole adjacent to the nucleus. If it is difficult to determine whether it is a MN touching the nucleus or a NBUD, it is acceptable to classify it as the latter.	 Nuclear bud in BNC  Nuclear bud in BNC Contd….

Characteristics of Apoptotic lymphocyte	Photomicrograph
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.	 
Characteristics of Necrotic lymphocyte	**Photomicrograph**
1. Early necrotic cells can be identified by the presence of pale cytoplasm with numerous vacuoles (mainly in the cytoplasm and some in the nucleus) and a damaged cytoplasmic membrane with a fairly intact nucleus. 2. Late necrotic cells exhibit loss of cytoplasm and a 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.	 

Abbreviations: MN: micronuclei; NPB: nucleoplasmic bridges; NBUD: nuclear buds, BNC: binucleated lymphocyte cells, MNC: mononucleated lymphocyte cell

4.3 Applications: Lymphocyte CBMN-Cyt assay has been well validated and is being currently employed in assessment of *ex vivo/in vitro* genetic instability or DNA damage. Some of applications include:

- Ecotoxicology to measure the genotoxic effect of radiation and chemical genotoxin exposure (377,541)
- Measurements of the DNA damaging effects of micronutrient deficiency and its prevention by dietary recommendations (430,530,531,542,543)
- Radiation sensitivity testing both for cancer risk assessment (544-547) and optimization of radiotherapy protocol to maximise killing of tumour cells and minimising normal tissue DNA damage (548)
- Biomonitoring of human populations with greater attention towards infants and young children with the aim to understand early origins of diseases (306,315,326,328,330-334,549-556)
- Bio-monitoring of human populations exposed to genotoxic chemicals (557-559) and testing of new pharmaceuticals and other chemicals (560,561) and to determine the safety of chemicals and pharmaceuticals (560,562).

It is also being currently investigated for proposed utilization as a biomarker for pregnancy associated complications such as pre-eclampsia (PE) (118), and Alzheimer's disease (563,564) Among all genome instability biomarkers, MN frequency has been the most sensitive marker used in the bio monitoring of cord blood, newborns and children (113,330,331,400,537,565-577) because of its potential to detect clastogenic and aneugenic effects in human genome (578). The available data for CBMN-Cyt biomarkers, primarily MN frequency measured in binucleated cells collected from lymphocytes in cord blood and children among various populations has been summarized in **(Table 4.3).**

Table 4.3: Frequency of CBMN-cyt biomarkers as assessed in lymphocytes collected from cord blood of infants

Author (year) country	Participants	Age of infants (months)	CBMN-Cyt biomarkers
Merlo *et al* 2014 Greece, Spain, United Kingdom, Norway, Denmark	Cord blood samples were collected (n=623) from infants born to healthy women (age ≤ 27≥ 36 years)	0	Country(n) Mean MN/1000 BNC MN/1000 MNC UK (143) 0.55 0.04 Greece (232) 1.79 0.62 Denmark (142) 0.70 0.10 Spain (70) 1.00 0.20 Norway (36) 1.16 0.11
Stayner *et al* 2014 Greece	Lymphocytes collected from 214 mothers and 223 newborns from the Rhea mother–child cohort in Crete, Greece	0	Mean MN/1000 BNC in cord blood lymphocytes=1.80 (1.51) Mean MN/1000 MNC in cord blood lymphocytes=0.62 (0.72)
Moreno-Palomo *et al* 2014 Spain	cord blood from 74 newborns	0	Frequency of BNC with MN was 2.93 (2.26) (range: 0-11) per 1000 BNC
Witczak *et al* 2014 Poland	Pregnant women with type 1 Diabetes (n=17) and their newborns (n=17). The control group consisted of pregnant women with-out type 1 Diabetes (n=40) and their newborns (n=40). The control-positive group pregnant women without type 1 Diabetes (n=10) and their newborns (n=10).	0	The mean (SD) of MN per 1000 BNC= 2.35 (1.07) for type 1 Diabetes mothers, 1.42 (0.60) for their newborns, 0.86 (0.90) for mothers without type 1 Diabetes and 0.67 (0.79) for their new-borns. The Mean MN/1000 BNC was significantly higher in newborns of mothers with type I (333) Diabetes compared with newborns of mothers without type I Diabetes ($p < 0.05$).
Fucic *etal* 2013 Greece	Rhea mother child cohort of pregnant women in Heraklion, Greece (n=92)	0	The Mean (SD) CBMN-Cyt biomarkers in cord blood lymphocytes were MN/100 BNC= 4.51 (3.29) MN/1000MNC=2.09 (1.54) NPB/1000 BNC=0.12 (0.36) NBUD/1000 BNC=0.27 (0.63) NDI/1000 BNC=1.57 (0.12) There was a significant correlation between NBUD in mothers and in newborns ($r = 0.29$, $p = 0.005$), but no correlation between NPB in mothers and newborns ($r = -0.05$, $p = 0.636$). The NDI in the mothers was significantly higher than in newborns ($p < 0.001$). There was a significant correlation between NDI of mothers and their newborns ($r = 0.32$, $p = 0.002$).

Author (year) country	Participants	Age of infants (months)	CBMN-Cyt biomarkers
Vande-Loock *et al* 2011 Greece	Peripheral blood samples from the mothers (n=251) and umbilical cord blood samples (n=182)	0	Mean (SD) for MN frequency/1000 BNC in cord samples=1.77 (1.41); MN frequency/1000 MNC in cord samples=0.67 (0.74); NDI=1.59 (0.20). Median MNBNC were significantly higher in mothers than in newborns ($p < 0.001$). In newborns, MN frequencies per 1000 MNC and MN per 1000 BNC were positively correlated ($r = 0.346$). A significant positive correlation between the MN per 1000 MNC from newborns and mothers ($r = 0.263$).
Lope *et al* 2010 Spain	Cord blood lymphocytes (n= 110 newborns), Peripheral lymphocytes (136 pregnant women, and 134 fathers	0	Mean micronucleated cells per 1000 BNC in cord blood lymphocytes=3.94 Mean micronucleated cells per 1000 MNC=0.70 6.4% Infants were observed to have cells with 1 NBUD per 1000 BNC 0.9%nfants were observed to have cells with 2 NBUD per 1000 BNC 16.4% infants were observed to have cells with 1 NPB and 1.8% had 2 NPB Mean NDI=1.7 per 500 cells
Witczak *et al* 2010 Poland	Cord blood lymphocytes collected from mothers exposed to anti-epileptic drugs (n=37) Negative controls (n=30 newborns of healthy mothers not exposed to any medication) Positive controls (n=10 newborns of healthy mothers not exposed to any medication during pregnancy but the known mutagen chlormethine hydrochloride was added to lymphocyte samples *in vitro* at the doseof0.25 g/mL).	0	For negative control group Mitotic index =0.059 (0.032); NDI=1.6 (0.18); MN/1000 BNC=0.53 (0.67)
Pederson *et al* 2010 Denmark	Maternal and cord blood was collected from healthy pregnant women (n=98,	0	MN frequency (median) in newborns was 3.2 (range: 0-9) and was significantly different from maternal MN measured per 1000 BNC ($p< 0.001$)

	median age 33 years) with planned singleton at delivery,		
Das and Karuppaswamy 2009 India	Human umbilical cord blood samples were collected from a 271 healthy newborns (61 from Normal Level Radiation Areas and 210 from High Level Natural Radiation Areas), born to healthy mothers (mean maternal age: 24.08+4.23 years).	0	Mean frequency of BNC with MN in lymphocytes collected from cord blood in Normal level radiation areas (1.5 mGy/year)=1.23 (0.07) per 1000 BNC
Author (year) country	**Participants**	**Age of infants (months)**	**CBMN-Cyt biomarkers**
Milosevic-Djordjevic *et al* 2005 Serbia	Cord blood collected from 41 healthy newborns (n=41, 20 M, 21 F) born to healthy mothers (mean age 28.71±4.96 years). 16 mothers were reported to smoke (<20 cigarettes a day)	0	Mean (SD) MN frequency =4.73 (3.38) per 1000 BNC
Zalacain *et al* 2006 Spain	cord blood of 143 newborns (102 from mothers who never smoked and 41 from mothers who smoked> 10 cigarettes per day during pregnancy	0	MN per 1000 BNC=4 (0.71) Apoptotic cells/1000 viable cells: Median= 61.5 (40, 5; 70.5) The median number of MN in cord blood samples from the mothers who smoked was 4 (1; 10.5), which was significantly higher than that of nonsmoking pregnant women, 3 (0; 8) (Kruskal-Wallis, p 0.016).
Levario-Carrillo *et al* 2005 Mexico	Cord blood from healthy newborns grouped according to residence of mothers: n=35 (urban cities, groups I and II); n=16 (agricultural area, group III); and newborns of mothers with high-risk pregnancy ((n=15, group IV). Mothers blood was also collected (Group I and III)	0	The mean (SD) frequency of BNC with MN was 3.7 (1.4) in mothers and 1 (0.9) per 1000 BNC in newborns from urban areas; 4.5 (2.4) in mothers and 2 (1.5) per 1000 BNC in newborns from the agricultural area. There was a significant correlation between the MN frequency in mothers and newborns ($r = 0.61$, $p < 0.01$)
Neri *et al* 2005 Multiple	13 studies selected after a systematic search in various databases. Only studies measuring MN frequency in lymphocytes with the cytokinesis block method and with at least 10 subjects in the referent group were included. Referent children exposed to genotoxic	0-18 years	MN frequency for children < 1 year of age (n=51) was 3.27 per 1000 BNC (95% CI, 2.22–4.82). Overall means of 4.48 [95% CI, 3.35–5.98] and 5.70 (95% CI, 4.29–7.56) MN per 1,000 binucleated cells were estimated by the meta- (n=440) and pooled analysis (n=332), respectively.

	agents or affected by any disease were excluded.		
Maluf SW& Erdtmann B 2001 Brazil	Peripheral blood samples were collected from 30 individuals with Down syndrome (DS), 14 with Fanconi anaemia (FAn), and 30 healthy individuals (controls, aged 0-17 years). DNA damage index obtained with Single cell gel electrophoresis.	Mean (SD) age (yr) DS=0.72 (1.80); FA=9.74 (6.10); Control=3.54 (4.86).	For controls, mean (SD) frequency of MN was 9.3 (1.31); and frequency of Dicentric bridges was 2.73 (1.31) per 2000 BNC.
Author (year) country	**Participants**	**Age of infants (months)**	**CBMN-Cyt biomarkers**
Shi *et al* 2000 China	Healthy phenotypically normal subjects (n=68, non smokers and non drinkers of alcohol, 37 M and 31 F)	Group I: 0–10 yrs., Group II: 20–30 yrs., Group III: 40–50 yrs Group IV: 60–70 yrs. FISH using chromosome-specific DNA probes on BNC used.	Mean (SD) age of group I (7 F and 13 M) was 5 (2.98). Mean (SD) frequency of BNC containing MN with or without chromosome 21 was 2 (2.31) per 7000 BNC in females and 1.54 (1.61) per 13000 BNC in males.
Fellay-Reynier *et al* 2000 France	Blood samples from healthy children (n=20, 14F and 6M, aged 4 months-18 years) and tumour affected children (n=21, 7F and 14M, aged 3 months - 15 years)	3-4 months	Mean (SD) of micronucleated cells per 1000 BNC was 5.1 (3.9) for the children with malignancies and 2.4 (2.3) for the control.
Barale *et al* 1998 Italy	Blood samples from healthy participants (n=1650, age range: 0-70 years).	0-19 years	Mean (SD) MN/1000 BNC for female participants (n=61) = 2.20 (2.41) and males (n=75)=2.20 (2.04) (aged 0-19 years).

Abbreviations: MN: micronuclei; NPB: nucleoplasmic bridges; NBUD: nuclear buds, BNC: binucleated lymphocyte cells, MNC: mononucleated lymphocyte cells; NDI: nuclear division index; SD: standard deviation; n=number of subjects; DS: Down syndrome (DS); FAn: Fanconi anaemia

5 Setting up and optimization of Microbiological assay for Red blood cell Folate

5.1 **Introduction**

Folate is the group name for a class of bioactive vitamers with a structure comprising of a parent pteroic acid that is conjugated with one or more L-glutamic acid molecules (77,579). Folic acid (FA) (pteroylmonoglutamate) is the partly oxidized stable pharmaceutical form used in food fortification and supplements. It consists of a 2-amino-4-hydroxy-pteridine moiety linked via a methylene group at the C-6 position to p-aminobenzoyl-glutamate moiety (77) (**Figure 5.1**). The different forms of folate (folypolyglutamates) are interconverted during metabolism in the human body and involve the reduction of the pyrazine ring of the pterin moiety to the coenzymatically active tetrahydro form (THF) (579,580). THF polyglutamates are the form of the vitamin present in cells and in food from natural sources. THF polyglutamates must be hydrolyzed to THF monoglutamates in the gastrointestinal tract before absorption across the intestinal epithelium (580). Intracellular THF monoglutamates are processed into functional metabolic cofactors through the re-establishment of the polyglutamate peptide (497). The glutamate polypeptide is essential to retain the vitamin within cells and to increase its affinity for folate-dependent enzymes (581). The intracellular metabolism of folates to polyglutamate derivatives is important for folate homeostasis as folylpolyglutamates serve as physiological substrates for the enzymes of one-carbon metabolism (OCM) and are required for normal cellular retention of folates (582). The identification and assaying of individual folate vitamers has been a challenge for the investigators due to the large number of folate derivatives, and the potential for some of them to interconvert chemically after extraction from biological samples (93,583). For example, 5-methyl THF a relatively stable form may be oxidized to 5-methyldihydrofolate (5-methyl DHF) at different pH values, with and without heat treatment. Also, THF can oxidize to FA under heat and/or low pH conditions (584). Many different folate vitamers with diverse level of oxidation state of pterin and glutamate chain length can thus be found in biological samples. Therefore, the measurement of folate is considered a complex

process (585,586). Additionally, the polyglutamate forms of the vitamin need to be converted to monoglutamates prior to analysis (77,579).

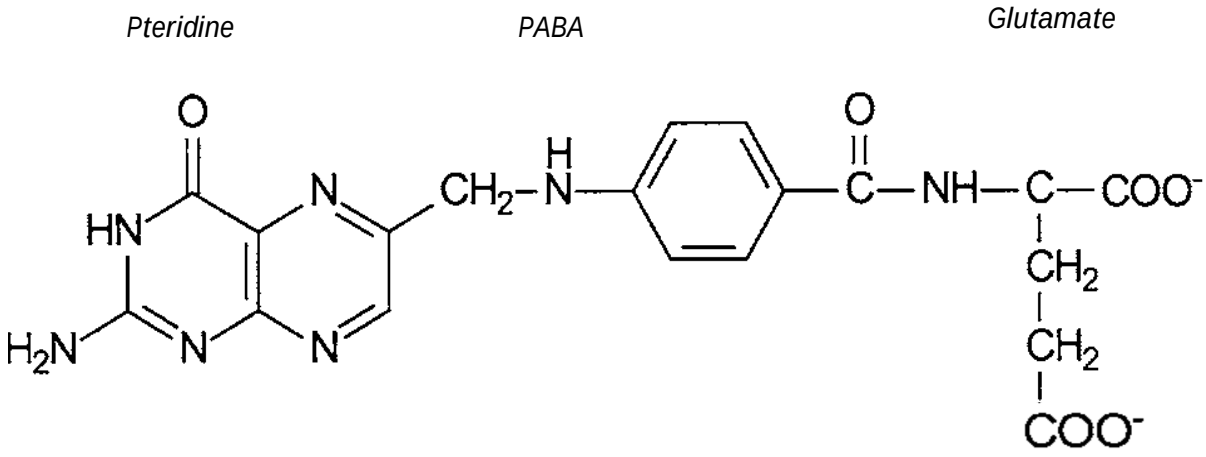


Figure 5. 1: Structure of Folate consisting of a pteridine base attached to para aminobenzoic acid (PABA) and glutamic acid (587)

5.2 **Folate measurement in humans**

Measuring folate in biological fluids is complicated due to its presence in multiple forms, lower stability, and lower concentration in biological systems that warrant complex extraction and detection techniques (588). Folate measurements have evolved along with constant issues of comparability across laboratories and methods (93) owing to various biochemical and public health aspects of folate metabolism in humans. Firstly, while plasma almost totally contains only folate monoglutamates- the 5-methyltetrahydrofolate (5-methyl THF) form, red blood cells (RBC) have long chain polyglutamates of 5-methyl THF. Secondly, there is evidence that presence of common genetic polymorphisms in the methylenetetrahydrofolate reductase gene (MTHFR, C677T variant) (36,174) may possibly result in redistribution of one-carbon- folate forms in RBC (589) and other tissues (590). Also, the investigators have addressed the need for revising baseline biomarkers for folate status to allow for mandatory folate food fortification and folic acid (FA) supplement use in the population (96,591). In addition whether this introduction of folic acid fortification of cereals and cereal products (81,268,592-596), as well

as high dose of folic acid supplement use by pregnant women (5 mg/ d) to prevent occurrences of neural tube defects (NTD) (203,214,597-602) may result in extreme increase in concentrations in blood leading to folate toxicity (596,603,604) requires careful investigation in diverse population groups (74). Further there are safety concerns for the presence of unmetabolized form of folate that may have detrimental effects (268,590,603,605) and hence warrants development of techniques to measure all forms of folate.

At present, various assays are employed in laboratories all over the world to assess folate in serum, RBC and whole blood; principally, protein-binding assays, chromatographic assays and a microbiological Assay (MA) (580,591). The protein assay is preferred by some investigators owing to easy availability and use of commercial kits while mass spectrometry methods are employed for their potential to measure individual folate one-carbon forms (93,95,584,586). Microbiological assay is considered the "gold standard" for folate analysis and is the simplest and most easily interpretable method for assessment of overall folate status in large population groups (93,96,591).

Hence, for the present study, the Microbiological Assay for folate was established at CSIRO Genome Health and Personalised Nutrition Laboratory and optimized for analyzing folate in packed cells from the cord and infant blood samples in the DADHI and INFACT study.

5.3 **Microbiological assay of folate**

The microbiologic assay (MA) was one of the first approaches used to quantify total folate in biological materials (584). The assay relies on the fact that a specific organism cannot grow in the folate-free medium and hence responds proportionally to the folate present in the sample under analysis (606). There is a folate 'standard' of known concentration and a 'sample' whose folate concentration is to be determined. The amount of growth of the folate dependent microorganism in sample/standard is proportional to the amount of folate in the sample/standard. The folate dependent organism used is *Lactobacillus rhamnosus* which

responds to various types of folate derivatives including 5-methyl-tetrahydro folate (5-methyl THF) in plasma (607), and RBC (588). After incubation at 37ºC, the growth of bacteria is observed as a change in the turbidity and is measured by light transmission in the sample/standard solution by the spectrophotometer. The optical reading thus obtained is extrapolated on a standard curve determined using different known concentrations of folate standard (96,586,588).

A decade ago, MA was a highly tedious process, however, now since the advancement of using an inoculum that is prepared in advance and cryoprotected in glycerol (608), development of a chloramphenicol-resistant strain of *L. rhamnosus* (93,606), combined with adaptation into a 96 well micro titre plate method (609), MA has evolved as a technique of choice for folate analysis in blood and food (588) and is given official of analysis status by the approved method of analysis by the association of official analytical chemists [(AOAC Method 992.05 (2002) and AACC (AACC Method 86-47)] (610,611). However, the assay is time consuming (incubation of sample/standard tubes with the bacterial inoculum for 18-22 hrs is required). This assay does not discriminate between the different folate forms and therefore 'total folate' is quantified (612). It demands proper sterilization procedures to prevent contamination of non-folate substances that may affect the organism growth during the assay (580,613). Nevertheless, as the assay is relatively inexpensive and does not require sophisticated instrumentation, it is being used for assessment of folate status in serum, whole blood, plasma and RBC collected from diverse population groups with reliability (94,96,580,586,591,604,614,615).

5.4 **Measuring folate in red blood cells**

Folate in blood represents the sum of several folate vitamers circulating in the blood stream, often referred to as "total folate" that includes primarily 5-methyl THF polyglutamates (93) and very small concentrations of other reduced folate vitamers such as THF and formyl-folates (5- or 10-formyl THF, sometimes 5,10-methenyl THF) (586). Measuring folate in RBC is clinically more relevant because mature RBC accumulate their folate stores during

erythropoiesis through the life span of the cell and thus are a better indicator of long-term folate status (77,93,616). Also, RBC folate correlates strongly and positively with hepatic concentrations (93,617,618) and has been investigated to study long term change in folate concentrations in different population groups utilizing microbiological as well as High Performance Liquid Chromatography (HPLC) or mass spectroscopy (591,614,619-621). Moreover, the blood samples for analyzing folate in RBC can be stored at -70ºC with minimal loss of folate content (93-95,580,586,622,623). Hence, in the present study, RBC folate concentration was measured. However recent findings of differential associations of serum and red cell folate with BMI of pregnant women raises concerns over appropriateness of red cell folate as an indication of adequate folate stores (624).

Conjugase

The accurate measurement of total folate necessitates hydrolysis of folylpolyglutamates in biological samples such as RBC to triglutamate or shorter glutamate chain length in microbiological assay. Conversion of polyglutamates to mono-or diglutamates requires γ glutamyl carboxypeptidase, commonly referred to as conjugase (611). Some of the frequently used conjugase enzymes in folate analysis are listed in **Table 5.1**.

Table 5. 1: Sources of Conjugase available for Microbiological assay of folate (611,625)

Source	Optimum pH	Folate end product based on glutamate residues
Chick pancreas	7.8	Two
Hog kidney	4.5	one
Rat pancreas	5.5-6.0	-
Human Plasma	4.5	one and three
Liver	5.0	one and two
Cabbage	5.0	three

In humans, γ glutamyl carboxypeptidase is present in lysosymes or in the intestinal brush border or plasma (626). Folate concentrations in plasma (entirely monoglutamate) are much lower than

those in RBC. Hence, human plasma is usually used to hydrolyse polyglutamates and is achieved by the lysing of whole blood followed by incubation of the lysate for 1–2 h that allows hydrolysis of polyglutamates to mono or di glutamates by γ glutamyl carboxypeptidase. One unit of enzyme activity corresponds to that amount of enzyme that releases 1ng of folic acid in 1hr at 37°C (625). This intestinal intracellular enzyme is a heat-labile endopeptidase and has optimum activity at a pH of 4.5 (627). In the present study, human plasma was used as the source of conjugase for deconjugating the folate (495,626) because it was easily available and was required in small amounts (0-2 ml). Also, Piyathilake *et al* reported 24 % lower RBC folate concentrations when rat plasma compared to human plasma ($p = 0.03$) was used to convert RBC folate polyglutamates to monoglutamates in human RBC samples (94) indicating lower efficiency of rat plasma in converting human RBC polyglutamates to monoglutamates. Further the low folate content of human plasma (626) could be stripped by applying a simple charcoal treatment which is explained in the following section 5.4 (step III) (628).

Calibrator

5-methyl THF was used as a calibrator in the present study (96,584) based on reliable method validated by Pfeiffer *et al* for MA (584,586,614,629). The choice of calibrator by different laboratories while assessing folate has evolved from using folic acid or 5-methyl THF or 5-formyl THF with the aim to assess total folate status of population that has shifted from consuming only 'food folates' to FA as food fortificant and/or as supplemental form (612,620,621,630-633). Different folate calibrators have been reported to produce slightly different calibration curves and 5-methyl THF standard curve shows lower response curve when compared with THF or other folate forms (614,632,634-636). The *Lactobacillus* was also reported to grow less at low concentration of 5-methyl THF (607). Three laboratories participated with their laboratory-specific MA in the National Health and Nutrition Examination Survey (NHANES) 2007–2008 to assess distributions of serum and RBC folate in USA. The data demonstrated that the folate results were 22–32% higher with FA as calibrator

and 8% higher with 5-formyl THF compared with 5-methyl THF, regardless of the matrix (96). The majority of folate in blood is in the form of 5-methyl THF. A large dose of FA as supplements or fortificant may cause appearance of unmetabolized FA in serum but its concentration in fasting individuals is usually small compared with the total folate (637). Thus, use of 5-methyl THF as a calibrator is expected to give more accurate results than the FA calibrator and hence has been recommended by the Biomarkers of Nutrition for Development (BOND) project (580) and NHANES (96,591,615). Additionally, there is discussions on public health platform of changing fortificant form to 5-methyl-THF on the basis of recent studies using labelled folates that indicated different plasma response kinetics to FA than to natural (food) folates, especially in population group with MTHF polymorphism (584,630,632,633,636,638-640).

5.5 **Method for microbiological assay of folate in red blood cells**

The method of MA for folate was established with direction and training from Associate Professor Jayashree Arcot (Nutrition, Food Science and Technology), School of Chemical Engineering University of New South Wales (641). The protocol was then modified for assessing folate in RBC using 5-methyl-THF calibrator as per laboratory protocol developed by Pfeiffer *et al* (629) for assessing folate status of DADHI and INFACT blood samples.

Apparatus/equipment required

- Autoclave set at 15 psi and 121 ºC to 123ºC
- Analytical balance to weight atleast up to four decimal places
- Automatic pipettes (100 µl ,200 µl, 500µl, 1ml, 5 ml)
- Aluminum foil
- Vortex mixer
- Centrifuge (Model ROTANTA 460, Benchmark Benchtop, Hettich Instruments, LP)
- Disposable plastic tubes (1ml, 2ml, 5 ml, 10 ml)
- Disposable syringes (1ml, 5ml)
- Eppendorf tubes (1ml)
- Glass beakers (250 ml, 200ml, 100 ml, 50 ml, 25 ml)
- Glass rods
- Incubator set at 37 ºC
- Inoculating loops and straight wires
- Micron filter (Millex filter unit, 0.22 µm, Millipore, Ireland Ltd, Lot: SLGV033RS)
- Disposable tips
- Measuring cylinders (100 ml, 50 ml, 20 ml)
- Petri dishes
- Para film
- Refrigerator (set to 2-8 ºC), freezer (set to-18 ºC)
- Reagent bottles with plastic caps (100 ml, 200 ml, 500 ml, 500 ml, 1000 ml)
- Sealing tape (clear polyolefin, Thermo Scientific, Australia, item number:232702)
- Test tube racks
- UV visible spectrophotometer (Varian, CARY, Agilent, Victoria, Australia)
- Vortex mixer
- Visible spectrophotometer (UV MAX 250, multi-mode micro plate reader, Molecular devices, USA)
- Wash bottle
- 96 well microplates (200 µl, Thermo scientific, Australia Nunc Cat no: 167008, flat bottom)

The setting-up and optimization of MA in a laboratory was carried out as per the following steps:

Step I- Preparing Cryopreserved stock culture according to laboratory protocol by Arcot and Shrestha 2008 (641)

Chemicals/material required

- Lyophilized culture of *Lactobacillus casei* subspecies *rhamnosus* (ATCC 7469) (Cryosite Granville, NSW, Australia).
- A broth (Lactobacillus broth AOAC, Difco) was prepared in a 100 ml sterilized bottle as per manufacturer's instruction: 1.9 g broth powder was dissolved in 50 ml ultrapure water from a Milli q system (18.2Ω resistivity) followed by filtering through 0.22 micron filter and further autoclaving at 121°C for 20 min. The broth was allowed to cool in a water bath at room temperature.
- Folic acid (FA) casei medium (Difco) was prepared by dissolving 4.7 g of medium along with 25 mg ascorbic acid (Sigma, Sydney, Australia) in 100 ml water from a Milli q system (18.2Ω resistivity).
- 0.5ml of working solution of FA standard (Sigma-Aldrich, New South Wales, Australia) (100 ng/ml) 0.5ml of working solution of FA standard (100 ng/ml)
- 100 ml of 80% glycerol solution (Sigma-Aldrich, New South Wales, Australia)

Method: 10 ml of sterilized and cooled lactobacillus broth was inoculated with the lyophilized bacterial culture (*L. rhamnosus,* ATCC 7469) [1.95* 10^9 colony forming units (cfu)/vial] from the glass vial aseptically. The solution was vortexed and incubated in a water bath at 37 °C for 22-24 hrs. Next day, culture medium was prepared by adding 0.5ml of working solution of FA standard (100 ng/ml) to the FA medium. This culture medium was autoclaved at 121°C

for 10 min followed by immediate cooling in running water bath for 30 minutes. The culture medium was then inoculated with 0.5 ml of bacterial culture in the broth. The solution was further incubated in a water bath at 37°C for 20-22 hrs. The appearance of white mucilaginous cottony mass in the medium was indicative of the end of incubation period. The culture medium was cooled in an ice bath. 100 ml of cooled 80% glycerol was then added to the 100 ml of mucilaginous mass. This inoculum of bacteria was then stored in 1 ml sterilized Eppendorf tubes at -80 °C.

Serial dilution method and streak-plate procedure (642,643) was used "to estimate the concentration (number of bacterial colonies) in the inoculum prepared by counting the number of colonies cultured from serial dilutions of the sample, and then back track the measured counts to the unknown concentration" (643). The accuracy of this estimation may be limited by sampling and counting errors (644).

Chemical needed

- Peptone (Merck, Germany) water was prepared as per manufacturer's instruction in a sterilized beaker (1.5 g peptone in 100 ml Milli Q water).
- Cryopreserved bacteria/inoculum (stored in Eppendorf tube) from step I
- Agar solution (MRS agar, Oxod ltd, Hampshire, England) was prepared by dissolving 15.5 g agar in 250 ml Milli Q water-). Mixture was heated for 1 minute and allowed to cool.

Method: 9 ml of peptone water was added individually to 10 sterilized test tubes and autoclaved at 121°C for 20 minutes. The tubes were allowed to cool in a water bath at room temperature. 1ml of inoculum was added to the 1st tube to prepare 1:10 dilution or 10^{-1} dilution. Next, 1 ml from test tube 1 was added to test tube 2 to get 1:100 dilutions (10^{-2}) and so on to finally obtain 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilutions of bacterial culture.

For plating the serial dilutions, agar solution was poured in the six petri dishes. The petri dishes were carefully labeled upside down (10^{-1} to 10^{-6}). 0.1 ml of 10^{-1} solution from test tube 1 was dropped into the 1st agar plate using a sterile hockey stick. The remaining bacterial dilutions

were also plated into subsequent petri dishes and incubated at 37^{o}C for 24 hours (Appearance of creamy white line on the surface of the agar ensures the growth of *L casei*) (641). The streak-plate procedure thus used allowed isolated pure cultures of bacteria to form colonies during incubation. It is assumed that single bacterium initially deposited on the plate will cluster as colony forming units that are visible to naked eye and can be counted manually (645,646). An average bacterial count obtained by manually counting the colony forming bacteria from each petri dish was $3.7x10^5$ bacteria per ml of solution.

Step II: Optimizing the size of inoculum

Various inoculum dilutions (1:50, 1:99, 1:200, 1:400, 1:500, 1: 600, 1:700, 1:800, 1:900, 1:1000, and 1:2000) (in medium) were tested to have a inoculum size to be used for the assay (**Figure 5.3**-**5.5**). The 1:99 dilutions (**Figure 5.6**) of inoculum gave the shape of dose response in accordance with the findings of Scott *et al* (613). A standard curve of 5-methyl THF concentration based on bacterial growth was established by adding inoculum to the microplate containing increasing concentrations of a standard 5-methyl THF solution (as described in step IV and V)

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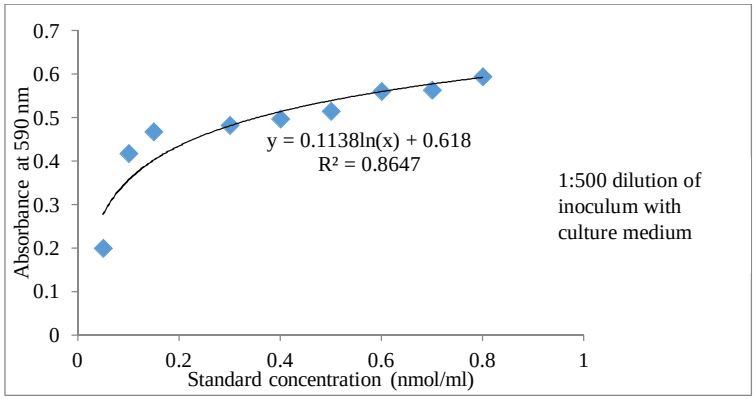

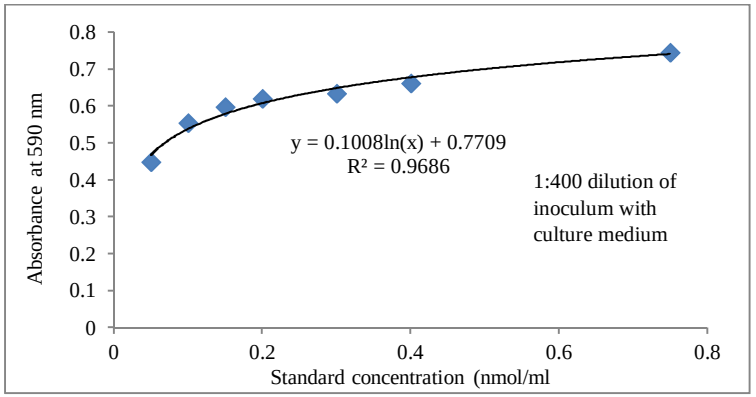

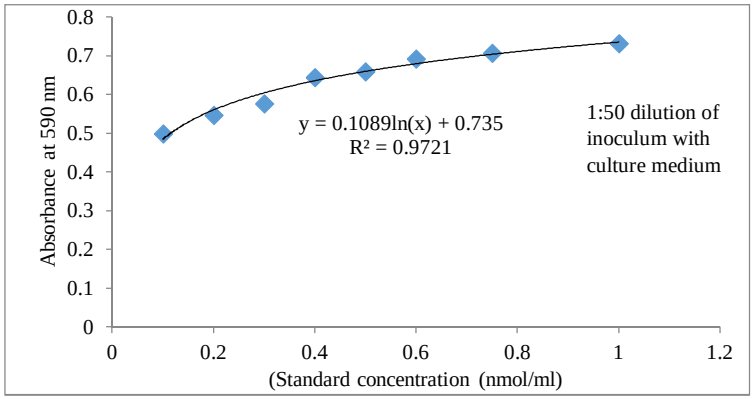

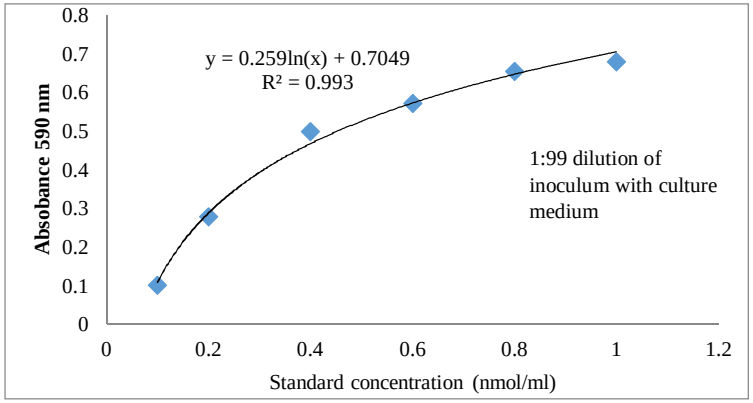

Figure 5. 2: Dose response of bacterial growth with respect to 5-methyl THF standard using different inoculum dilutions

The regression equation [y = a ln (x) + c] and R-square value of the calibration curve were computed in MS Excel, R value below 0.98 was discarded

Step III: Charcoal treatment of plasma used for conjugase activity according to Piyathilake et al 2007 (94)

Chemicals/material required

- Activated charcoal: (Sigma-Aldrich, New South Wales, Australia)
- Human plasma
- Micron filter (0.22µm)

Method: Pooled human blood was collected from volunteers and plasma was separated by centrifugation at 3000 rpm for 20 minutes at 4 ºC (647). To strip plasma of any folate, different amounts of activated charcoal were tested. 1 ml plasma was stirred with 0.05 g, 0.075 g and 0.1 g charcoal. The charcoal treated plasma was then tested for folate content. 0.1 g charcoal per 1 ml human plasma was found to be sufficient to make plasma folate free. Hence, 0.1 g of charcoal per 1 ml of plasma was stirred very gently with a sterile glass rod for 60 minutes on ice and centrifuged at 3500 rpm at 4 °C for 5 minutes. The supernatant was filtered through a 0.22 µm micron filter. After the charcoal treated human plasma was tested for folate to make sure that it was folate free, 100µl aliquots of folate free plasma were prepared and stored at -70 °C (94).

Step IV: Preparing the RBC Samples collected from cord and heel prick blood collected from infants according to Piyathilake et al 2007 (94)

Chemicals requires

- 1% ascorbate solution (A1): 10 g ascorbic acid (Sigma-Aldrich, New South Wales, Australia) dissolved in 1000 ml Milli Q water
- Folate free plasma (treated with charcoal from step III)
- Cord blood and heelprick blood samples

Method: Whole blood was collected in lithium heparin tubes and centrifuged at 3000 rpm for 20 minutes at 4ºC to separate the plasma. The remaining RBC were separated and prepared for the MA using the method described by Piyathilake *et al* (94). The buffy layer was not removed. 712.5 µl of 1% ascorbate solution (A1) and 12.5 µl of folate stripped human plasma was added to 25 µl RBC. The samples were mixed well and then incubated at 37 ºC for 20 minutes (93).

Dilution of blood samples: Average concentration for folate in RBC is approximately 906 nmol/L [400ng/ml or 181 nmol/ 20 µl (8 ng/20 µl)] (648). A dilution factor was calculated so that the concentration of sample to be tested should fall within the range of standard curve (0-1nmol). As the concentration of folate in human blood is 181 nmol in 20µl but we want it to be about 0.018nmol/well, so dilution factor is calculated as =181/0.018=100 times. To achieve 100 times dilution, first, 25µl of sample was added to 225 µl ascorbate solution (AI) to make (1/10) dilution. Further, 25 µl of this first dilution solution was added to 225 µl ascorbate solution (AI) to make another 1/10 dilution so as to achieve the final dilution of 100 times.

Step IV: Preparing 5 methyl tetrahydrofolate (5-methyl-THF) standard according to Pfeiffer CM 2008 (629)

Chemical required

- 5-methyl THF (Sigma-Aldrich, New South Wales, Australia)
- 20 nM Phosphate buffer solution (2.497 g K_2HPO_4, 0.762 g KH_2PO_4, and 0.1% cysteine in 1L Milli Q water).
- Ascorbic acid (Sigma-Aldrich, New South Wales, Australia) was used to prepare two solutions (0.1 and 0.5 % concentration) as follows:

*1% ascorbic acid solution: 10 g ascorbic acid (as A1) dissolved in 1000 ml Milli Q water

*0.5% ascorbic acid solution: 5 g ascorbic acid (A2), dissolved in 1000 ml Milli Q water

- 0.5% sodium ascorbate solution: 5g sodium ascorbate (Sigma-Aldrich, New South Wales, Australia) dissolved in 1000 ml Milli Q water

Method: All glassware was autoclaved at 121 °C for 20 minutes before the start of the assay. All solutions were purged with Nitrogen to minimize oxidation of 5-methyl THF stock solutions.

Preparing stock I: To prepare 5-methyl-THF stock standard solution (I), 5 mg 5-methyl-THF was dissolved in 25 ml of degassed 20 mM phosphate buffer solution. A small aliquot of stock was checked for absorbance to determine the exact concentration of the stock standard by UV spectrophotometer (Varian, CARY, UV visible spectrophotometer, Agilent, Victoria, Australia), at 290 nm. A 1/20 dilution of 1 ml aliquot of standard I was prepared with phosphate buffer and absorbance was read at 290 and 245 nm. The ratio of A290/A245 should exceed 3.3; 0.25 g of ascorbic acid was then added to the remaining stock I to ensure that 5-methyl-THF has not oxidized to THF derivatives. The exact concentration of MTHF solution (I) was obtained using Beer Lambert's law (630)

A=ε*b*c, where: A=absorbance
ε=wavelength dependent molar absorbidity coefficient with units M^{-1} cm^{-1} (for MTHF =31.7 $mol^{-1}L$ cm^{-1} at 290nm)
b=path length (1cm)
and c is the concentration we wish to calculate.
Molecular weight (MW) of MTHF= 503 g/mol

Preparing stock II: An intermediate 25 ml of 5-methyl THF standard solution II (concentration= 100µg/ml) was made using 1% degassed ascorbic acid solution.

*Preparing stock solution III***:** Stock II was used for the preparation of a stock III (concentration= 1 µmol/L). 458.93 µl stock II was pipetted and the volume was made up to 100 ml with 0.5% ascorbic acid solution. This stock III may be stored in 1ml aliquots at -70 degrees for 6 months.

*Preparing working standard solution***:** On the day of the assay, a working standard solution of 5-methyl THF (solution A) was prepared by adding 100 µl of stock III (concentration=1µmol/L) to 400 µl of 0.5% sodium ascorbate solution. Lastly, to get the final

concentration of 1 nmol/L (working standard solution B), 250 µl of 'solution A' was taken and the volume made to 50 ml with 0.5% sodium ascorbate solution.

Step V: The Assay

Chemicals required

- 0.5% sodium ascorbate solution: 5g sodium ascorbate (Sigma-Aldrich, New South Wales, Australia) dissolved in 1000 ml Milli Q water
- Working standard solution B 5-methyl THF solution (concentration=1nmol/L)
- Folic acid casei medium (Difco): 9.4g media was added to 100 ml Milli Q water. The solution was boiled for 2-3 minutes and then filtered with a 0.22µm filter
- The bacteria inolculum (from step I) was thawed. 50 µl of the inoculum was added to 4950 µl of folic acid casei media and mixed well. This constitute the inoculated media.
- Blood samples (cord and heel prick bloods collected from the infants) of unknown folate concentration from step IV

The assay protocol is outlined in **Figure 5.3**

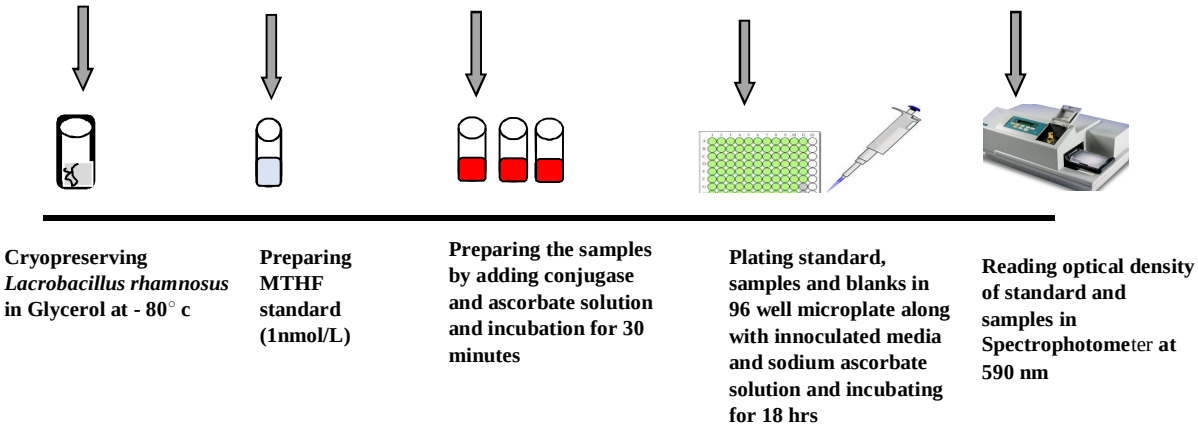

Figure 5.3: Outline for Microbiological assay for RBC folate for DADHI study and INFACT sub-study (495,608,629)

1. In a 96 well flat-bottom plate, firstly 0.5% sodium ascorbate was added in all the wells.

2. In the blank wells, 100 µl of 0.5% sod ascorbate solution and 100 µl inoculated media was added (**Table 5.2).**

Table 5. 2: Addition of solutions (µl) in 96 well microplate for MA folate

	0.5% sodium ascorbate solution	5-methyl-THF working standard solution B (µl)	Inoculated medium	Sample (µl)	Total (µl)
Blank	100	0	100	0	200
Standard (8 wells)	100-0	0-100	100	0	200
Sample	80	0	100	20	200
Recovery	60	20	100	20	200

3. In the standard wells, 100-0 µl (decreasing concentration from first to last well) of 0.5% sodium solution was added. Then the working standard solution of 5-methyl THF (1nmol/L) was added in the standard well in increasing concentration (0-100 µl) corresponding to the sodium ascorbate solution. Each concentration was achieved in triplicate.

4. In the sample wells, 80 µl of sodium ascorbate solution was added. Then 20 µl of blood sample was added in the sample well. The study ID was used as the label for each sample well to carefully define each well. Each concentration was achieved in triplicate.

5. Recovery wells were included for each sample to estimate percentage recovery of folate from the sample. Each recovery well had 60 µl 0.5% sodium solution, 20 µl of sample and 20 µl of standard solution.

6. Lastly, 100 µl of inoculum was added in standard and sample wells. Final volume in each well was 200 µl.
7. The plate was sealed and incubated for 18 hours in an incubator at 37°C.

8. After 18 hours, the bacteria were resuspended by shaking the plate which was covered with the seal to avoid cross-contamination. The plate was read at 590 nm on a spectrophotometer (UV MAX 250, multi-mode micro plate reader, Molecular devices, USA).

Quantification

The optical density values in triplicates were recorded for all wells (standard, sample and recovery). The average value was obtained for each well. Standard deviation and coefficient of variation (CV) was calculated for each point. If the CV values were > 10%, the readings were discarded and sample were re tested. A standard concentration response curve or calibrator curve was obtained by plotting average optical density value as ordinate and concentration of 5-methyl-THF standard as abscissa in logarithm scale utilizing MS Excel 2010 (**Figure 5.4** and a snap shot of calculation is included as **Appendix 4)**. The regression equation [y = a ln (x) + c] and R-square value of the calibration curve were computed in MS Excel (641). If the R value was below 0.98, the assay was repeated. The optical value of the sample and recovery was put in a regression equation (interpolate) to calculate the folate concentration in the sample well. The value was adjusted for the dilution factor (x100) to obtain the final folate content in nmol/L per sample.

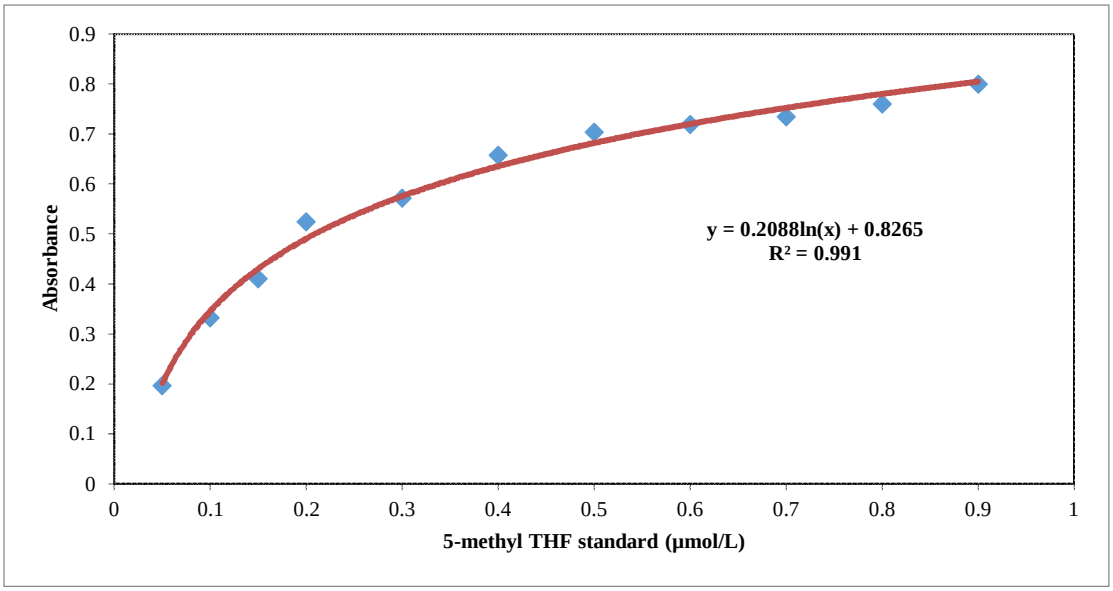

Figure 5. 4: The Standard curve using 5 methyl THF as a calibrator
Y axis has the absorbance (optical density) read from the spectrophotometer; X axis shows the corresponding concentration of standard 5 methyl THF solution.

Statement of authorship

Title of Paper	Infant birth outcomes are associated with DNA damage biomarkers as measured by the Cytokinesis block micronucleus Cytome assay –The DADHI study
Publication Status	Submitted to 'Mutagenesis' journal for publication

Mansi Dass Singh (Candidate)

Wrote manuscript, conducted experiment work (microbiological assay of folate: 100 %, scoring of sample slides: 20%), conducted all analysis and interpretation of the data and contributed to planning of the article.

Certification
This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.

Date---22.11.16---

Phil Thomas

Supervised study design, development of work, contributed to planning of the article and provided critical evaluation of the manuscript

Date---29/11/2016---

Signed by Prof. Michael Fenech on behalf of Dr. Phil Thomas

Maryam Hor

Recruited all the participants, collected all data and samples, conducted majority of the experimental work (setting, optimization, scoring and compiling of all data for cytokinesis block micronucleus assay for the study)

Date---22/11/16---

Theodora Almond

Conducted partial experimental work (50% scoring of slides in the study)

Date---22.11.2016---

Julie Owens

Provided critical evaluation of the manuscript

Date---------24/11/16---------

William Hague

Supervised study design, contributed to planning of the article and provided critical evaluation of the manuscript

Date---------23/11/16---------

Michael Fenech

Designed the study, development of work, conducted supervision of the study, assisted in data analysis and interpretation, contributed to planning of the article and provided critical evaluation of the manuscript

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6 DNA damage biomarkers in South Australian infants as measured by CBMN-Cyt assay and the influence of age, gender and mode of feeding during the first 6 months after birth

6.1 **Abstract**

Damage to the genome is recognised as an important fundamental pathological event that may lead to an increased risk for developmental and degenerative diseases, including cancer. Healthy infant development relies on accurate gene expression that is dependent on precise DNA replication and repair. DNA damage sustained during perinatal period and infancy may reflect epigenomic impact of maternal factors. Also, environmental factors that influences the integrity of the infant genome is nutrition through breast milk, formula or complementary feeds. The extent of DNA damage in the infants and the correlation of maternal factors during pregnancy with infant birth outcomes and DNA damage is not known. Further, there is yet no data whether mode of feeding may modulate these biomarkers in infants born in South Australia.

A prospective cohort study was designed; 'Diet and DNA damage in Infants', with the aim of collecting data on lymphocyte genome integrity in Australian infants (0, 3 and 6 months), as measured by the Cytokinesis block micronucleus cytome (CBMN-Cyt) assay. The secondary aim was to study associations of CBMN-Cyt biomarkers with infant birth outcomes and maternal demographic and lifestyle variables. Further, the objectives were to assess change in DNA damage biomarkers and gender differences from birth to six months after birth. Another aim was to test the effect of the type of feeding method adopted for infants on CBMN-Cyt biomarkers at three and six months.

Peripheral blood lymphocytes were isolated from the infants (born to healthy born at low risk of complications during pregnancy) at birth (cord blood) (n= 82), at 3 months (n=64) and 6 months (n=53) after birth. DNA damage biomarkers measured *ex vivo* in binucleated lymphocyte cells (BNC) and included: micronuclei (MN), nucleoplasmic bridges (NPB) and nuclear buds (NBUD). Apoptotic and necrotic cells were also scored and nuclear division index (NDI) was measured using the frequency of mono-, bi- and multinucleated cells. In addition, MN and NBUD were also scored in mononucleated lymphocyte cells (MNC) to assess genome damage that was already expressed *in vivo*. Mother-infant cohort's demographic variables were collected through

a health questionnaire. The information regarding mode of feeding for the infant was collected at three and six months.

The mean (± SD) frequency of MN, NPB and NBUD in BNC at birth (n = 82) was 2.0 (± 1.2), 5.8 (± 3.7) and 11.1 (± 5.7) per 1000 BNC respectively and tended to decrease significantly at three months ($p< 0.01$, $p<0.0001$, $p< 0.001$ respectively) and six months ($p <0.05$, $p<0.0001$, $p <0.0001$ respectively) after birth relative to cord blood when compared in the same cohort of infants (n= 48 at birth, 48 at three months and 39 at six months). The mean gestation age for infants at birth correlated positively with MN ($r = 0.38$, $p = 0.006$), NPB ($r = 0.30$, $p = 0.03$) and negatively with NDI ($r= - 0.29$, $p = 0.03$). Infants' birth weight was positively associated with MN, NPB and NBUD in cord blood ($r = 0.24$, $p = 0.08$, $r = 0.32$, $p = 0.02$ and $r = 0.28$, $p = 0.04$ respectively). Infant birth length was positively associated with NPB ($r = 0.32$, $p = 0.02$) and NBUD ($r= 0.27$, $p = 0.04$). Infant's birth head circumference was negatively associated with apoptotic lymphocyte cells ($r = - 0.27$, $p = 0.06$). APGAR score assessed at 1 and 5 min after birth was positively associated with NDI at birth ($r = 0.3$, $p = 0.05$, $r = 0.28$, $p = 0.06$ respectively). APGAR score recorded at 5 minutes was also negatively associated with NPB ($r= - 0.26$, $p = 0.09$). Mother's weight and body mass index (BMI) recorded at 8-16 week gestation was positively associated with NPB ($r = 0.38$, $p = 0.006$, $r = 0.32$, $p = 0.02$ respectively) and BMI was also negatively associated with APGAR score at 5 minutes ($r = - 025$, $p = 0.07$). The gestation age was also observed to be significantly associated with infant birth weight ($r = 0.33$, $p = 0.005$) and length ($r = 0.26$, $p = 0.03$). The birth weight, length and head circumference of the male infants was greater than that of the female infants ($p < 0.0001$, $p = 0.0003$, $p = 0.001$ respectively).

None of the CBMN-Cyt biomarkers measured at birth was associated with maternal smoking status, alcohol and folic acid intake during pregnancy. There was significant differences observed in NBUD BNC and NBUD MNC among male and female infants ($p = 0.08$ and $p= 0.07$ respectively) at birth.

At three months 68% of the cohort was being exclusively breast fed while only 9% were being exclusively formula fed. The percentage of infants that were exclusively breast fed at six months declined by half (to 34%) at six months while the frequency of formula feeding doubled at the end of six months (to 19.6%) relative to three months. Mode of feeding was not observed to be significantly associated with CBMN-Cyt biomarkers at three and six months after birth.

The significant positive associations of infant birth weight and length and maternal BMI with CBMN-Cyt biomarkers suggest the possibility of a genotoxic effect of metabolic processes that promotes excessive growth and high BMI. The study could not demonstrate substantial influence of type of feeding on DNA damage and cell death biomarkers in the first 6 months after birth. The non-association observed with the feeding score may be the result of the adequate complementary feeding regimens followed by the mothers in the study, of whom 68% and 34% were exclusively breast feeding their babies at 3 and 6 months respectively.

6.2 **Introduction**

The human genome is susceptible to genetic damage caused by exposure to various exogenous factors such as pollutants, ultraviolet radiation, smoking, etc., as well as endogenous factors

(free radicals) that result in oxidation, alkylation, hydrolysis and bulky adduct formation in DNA bases within human cells (98,289,292-294). It has been shown that DNA damage at the chromosomal; telomere and mitochondrial DNA level increases with age (119,649,650). Such DNA lesions are swiftly detected by DNA damage sensing molecules such as ataxia-telangiectasia mutated (ATM) protein kinase (651-653) and are subjected to the action of inherent DNA damage responses (654,655) involving an intricate web of signalling pathways (656,657). Such signalling results in the activation of cell-cycle checkpoints and the appropriate DNA repair pathways (658,659). However, when excessive oxidative damage exceeds the body's repair capacity, it may lead to unrepaired or mis-repaired DNA single and double strand breaks. This can lead to chromosome aberrations, chromosome malsegregation, micronucleus formation and gene mutation resulting in subsequent altered gene dosage and expression (99). These alterations in the genome may have particularly adverse consequences in early life, including developmental defects and immune system dysfunction (315,316). In Australia, the incidence of childhood cancer is estimated to increase (660). Insults to the genome in the perinatal period are likely to be very important relative to other life-stages because of the higher probability that mutated and genomically unstable cells could populate the rapidly growing tissues of an infant (313-316). Numerous studies have also shown a significant correlation between the frequency of DNA damage in mothers/fathers and their offspring suggesting a common environmental, nutritional or lifestyle insult (315,328,537,571,574,661-663). Detection and monitoring of DNA damage in human tissues at the earliest possible phase of life may enable timely intervention to prevent the further accumulation of cellular DNA lesions and their potential manifestation into chronic diseases, such as cancer, at a later stage of life (113).

The Cytokinesis block micronucleus-cytome (CBMN-Cyt) assay in peripheral blood lymphocytes (PBL) is one of the most comprehensive and best validated methods to measure

chromosomal DNA damage, cytostasis and cytoxicity (108). The ‘‘cytome’’ concept in the CBMN assay implies that every cell in the system studied is scored cytologically for its DNA damage, proliferation and viability status (108). In this assay, genome damage is measured by scoring:

(iv) Micronuclei (MN): biomarker of both chromosome breakage and/or loss;

(v) Nucleoplasmic bridges (NPB): a biomarker of DNA mis-repair and/or telomere end-fusions and

(vi) Nuclear buds (NBUD): a biomarker of gene amplification and /or the removal of unresolved DNA repair complexes (109,110).

DNA damage biomarkers expressed *ex vivo* (MN, NPB and NBUD) are measured in binucleated lymphocyte cells (BNC) because only cells that complete nuclear division can express molecular lesions in DNA and in the mitotic machinery as chromosome breakage or chromosome loss events respectively that lead to MN formation. Genome damage already expressed *in vivo* as MN and NBUD is measured in mononucleated lymphocyte cells (MNC) that fail to divide *in vitro* in the CBMN assay (325,326).

Among all the genome damage biomarkers the MN frequency has been one of the most sensitive biomarkers used in the bio-monitoring of cord blood, newborns and children (113,329-331,400,552,569,571-573) because of its potential to detect clastogenic and aneugenic effects in the human genome (578). The Human MicroNucleus project compiled prospective data on the association of MN frequency in lymphocytes of 6718 individuals (who were free of cancer at the time of testing) from 10 countries with cancer incidence and found a significant increase of all incidences of cancers in medium [relative risk (RR) 1.84; 95% CI: 1.28–2.66] and high MN frequency groups (RR 1.53; 95% CI: 1.04–2.25) (113,321,322) thereby showing that MN is a biomarker for early genetic effect and is predictive of cancer risk. DNA damage sustained during both the perinatal period and infancy may also reflect the epigenomic impact of maternal diet, life-style and genotoxin exposures (303-306,664,665)

because gene expression related to DNA damage and immune response among children is observed to correlate with MN as a consequence of exposure to environmental pollutants (664-667). Additionally, there is accumulating evidence that infant's birth weight and gain in weight during childhood is affected by maternal pre-pregnancy weight (312) and ambient exposures to PM 2.5 air pollutant (307,308), suggesting the possibility of an association of MN frequency in mother's and neonates blood with peri and postnatal maternal diet and lifestyle factors (309,311).

The available data for CBMN-Cyt biomarkers, primarily MN frequency measured in binucleated lymphocytes in cord blood among various populations has been summarized in **Figure 6.1.** A meta-and pooled analysis of 13 studies, conducted mainly in European countries, reported baseline frequency of 3.2 MN per 1000 BNCs in children <1 years of age (n=51) (555). Infants may be more susceptible to DNA damage induced by external factors because their cells are in a state of rapid proliferation and differentiation (330) and nutritional deficiency may lead to DNA replication stress and faulty DNA repair (294,498). There is increasing evidence that measure of DNA damage measured with CBMN-Cyt assay in lymphocytes collected from umbilical cord blood and from older infants (306,315,326,328-334), are higher among those with ailments such as malignancy (332), Down syndrome and Fanconi's anaemia (556), and also among those infants who are exposed to pollution (315,550) and radiation (575), compared with healthy infants (306,668). The findings of these prospective cohort studies are of significance because of the accumulating evidence that increased MN in lymphocytes predict risk of developing cancer (113,321,322,669). To date there have been no published data on baseline DNA damage biomarkers in infants born in Australia. Therefore, identifying and reducing exposure to risk factors that jeopardise genetic integrity is likely to be an important strategy in primary prevention of illness including malignant neoplasia. Hence, bio-monitoring of the foetal genome may be an important tool in assessing disease risk and genomic impact of dietary, lifestyle and environmental factors (326).

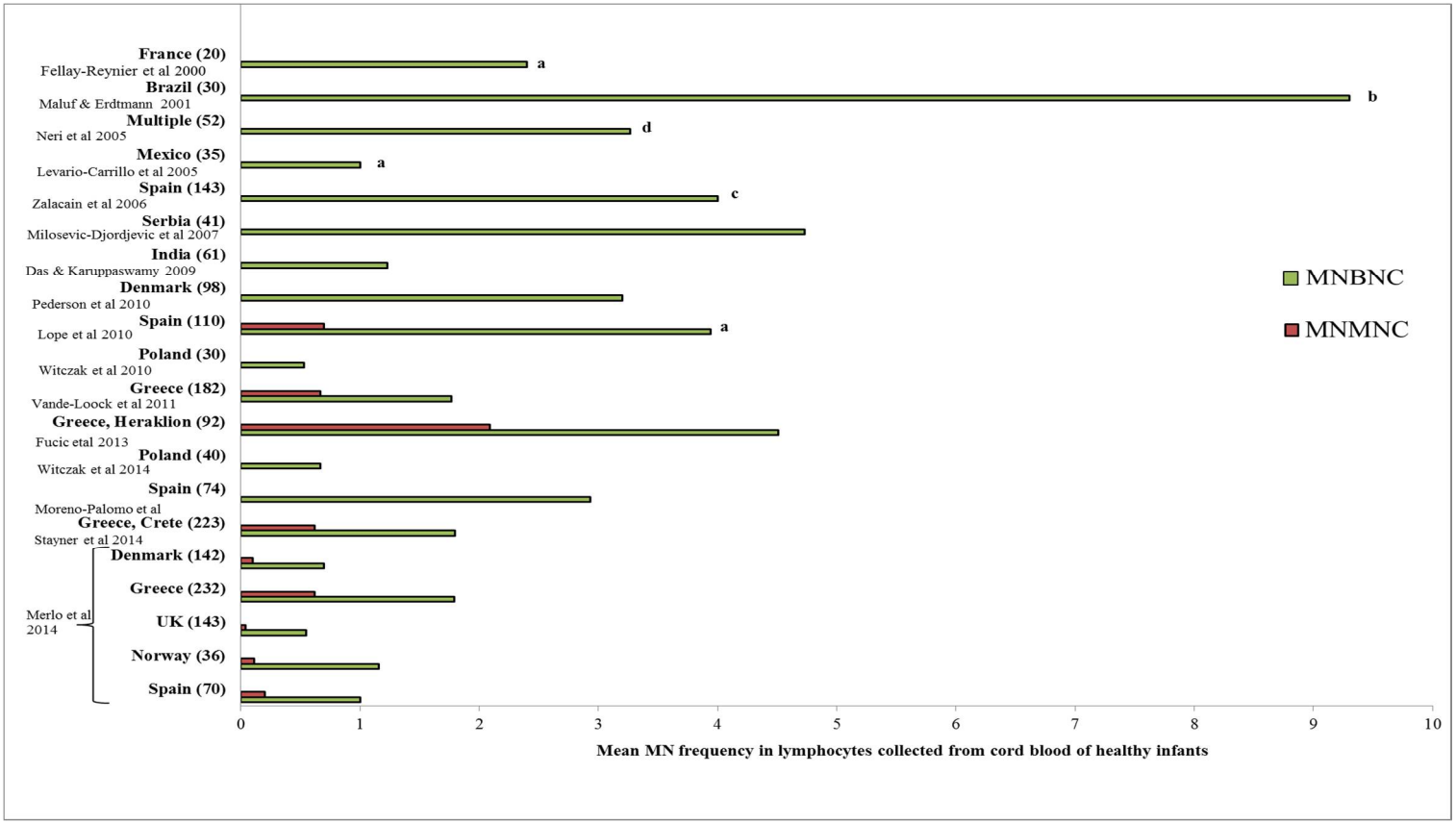

Figure 6.1: Summary of mean MN frequency measured in cord blood of healthy infants born to healthy women in various countries
(Number of subjects is shown in parenthesis, author name and year is included under the country's name)
Abbreviations: MN: micronuclei, BNC: binucleated lymphocyte cells, MNBNC represents micronuclei measured in binucleated lymphocyte cells; **a**: represents data as micronucleated cells per 1000 BNC; **b**: mean age =3.54 yrs and values per 2000 lymphocyte cells; **c**: medianvalue; **d**: mean age ≤ 1 year, data represents pooled estimates.

The CBMN-Cyt assay has emerged as a very reliable tool in measuring DNA damage in both adults and infants, which can be used to evaluate comprehensively DNA damage at the cytogenetic level together with cell death and proliferation capacity of cells (110,578). Preliminary studies during the 1980s showed that appearance of MN varies with age and gender (670). These findings have since been validated by other studies that have consistently shown that MN frequency increases steadily with age and is considerably higher in females compared with males in all age groups (**Figure 6.2**) (119,577,671-676). The increase in females is mainly due to malsegregation of one of the X chromosome (119,676).

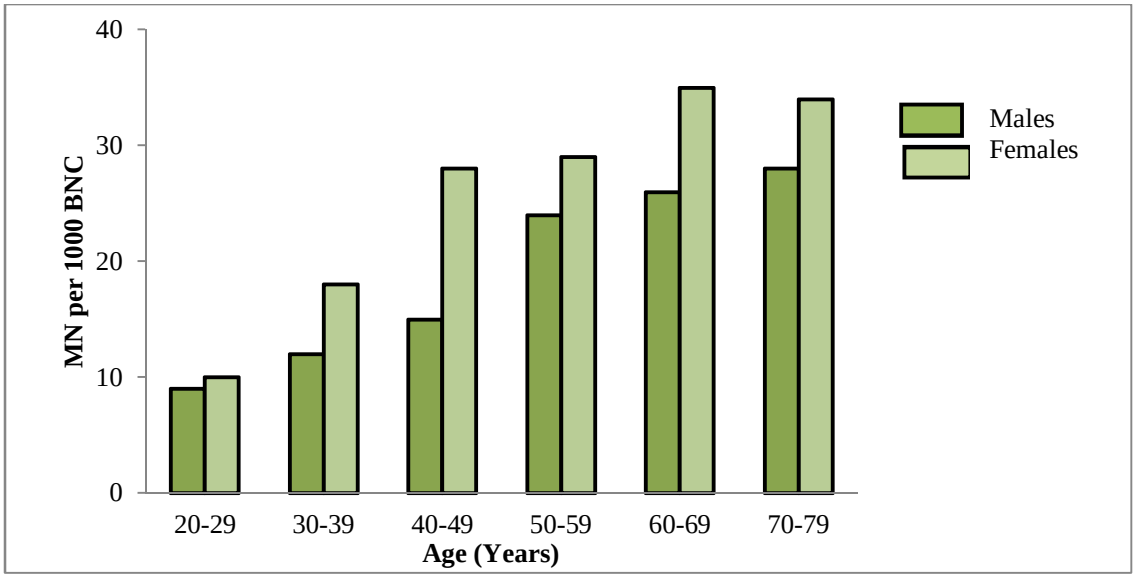

Figure 6.2: Baseline mean micronuclei (MN) frequencies (per 1000 binucleated lymphocytes (BNC) measured using the CBMN-Cyt assay) in peripheral blood of healthy, non-smoking, males and females, subdivided according to age-group in a South Australian cohort (n = 14–33 within each subgroup) (Adapted from Fenech M and Bonassi S 2011) (119).

Such data suggest the importance of exploring preventive strategies to reduce appearance of CBMN-Cyt biomarkers to its minimum during infancy. An important known modifiable environmental factor that contributes to increased DNA damage is deficiency of micronutrients, primarily folate but also zinc, iron selenium, vitamins B_{12}, A, C, and E, and β-carotene (242,409,413,414,435). An infant is dependent on optimal supplies of micronutrient from the

mother's breast milk, complementary feeds or other dietary sources. Data from the Longitudinal Study of Australian Children (406) show that the proportion of infants who are exclusively breast fed (BF) declines rapidly after birth (**Figure 6.3**). In those babies who are not exclusively BF, breast milk may be replaced, to varying degrees, with formula milk, cow's milk, soy milk and other drinks that differ significantly in micronutrient and macronutrient composition relative to human breast milk (**Figure 6.4**) (406).

Children who are breastfed for longer periods have lower infectious morbidity and mortality than do those who are breastfed for shorter periods, or not breastfed (379). Further, evidence also suggests that breastfeeding might protect against overweight (378,382) and shorter telomere length later in life (354).

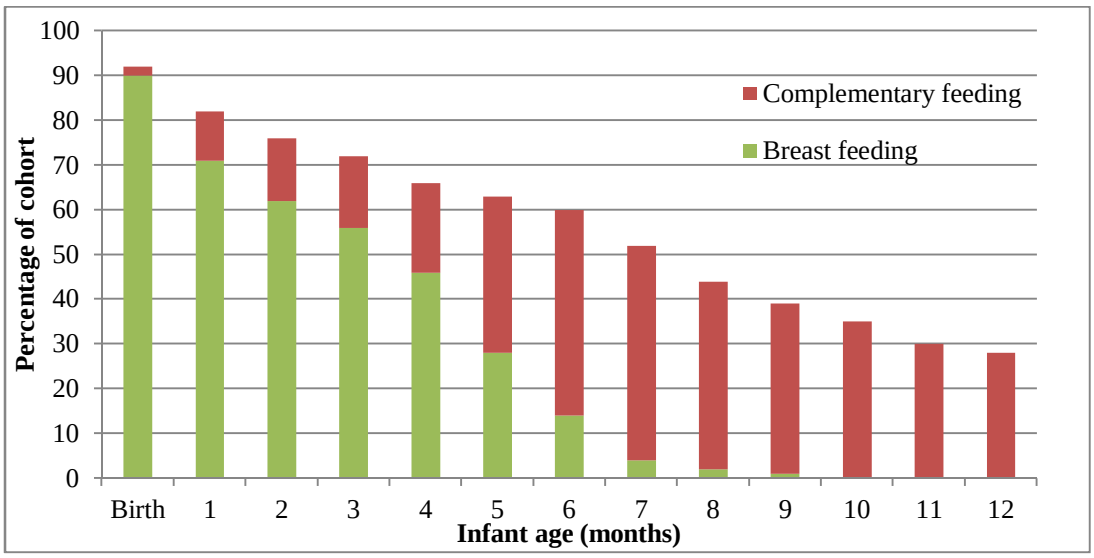

Figure 6.3: Growing up in Australia: The Longitudinal Study of Australian Children Annual report, Australian Institute of Family Studies 2006-7
(Growing Up in Australia, Waves 1 and 2)

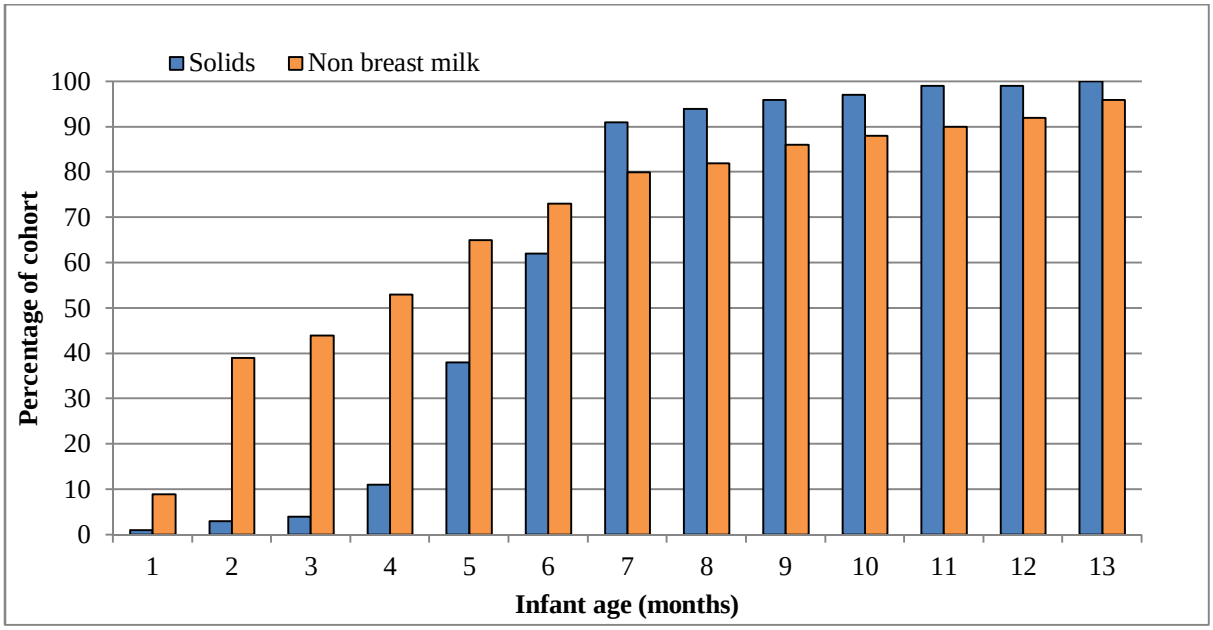

Figure 6.4: Growing up in Australia: The Longitudinal Study of Australian Children (Complementary feeds)
Annual report, Australian Institute of Family Studies, 2006-7
(Growing Up in Australia, Waves 1 and 2)

Previous studies on DNA damage in infants have shown CBMN-Cyt biomarkers in mainly European cohorts. However, there has been no study done yet using DNA damage, cell proliferation and cytotoxicity biomarker in Australian infants. Further, it is not clear whether there are any differences in the frequency of these biomarkers in infants with respect to gender and maternal factors, and whether mode of feeding may modulate DNA damage biomarkers in infants. A prospective study was therefore designed; 'Diet and DNA damage in Infants'-the DADHI study, with the primary aim of collecting comprehensive data on DNA damage biomarkers in South Australian infants (0, 3 and 6 months), utilizing the CBMN-Cyt assay with following hypotheses.

6.3 **Hypotheses**

- Genome damage increases from birth to 6 months after birth among infants in the cohort
- The CBMN-Cyt biomarkers measured in cord blood at birth are associated with infant's birth outcomes
- The CBMN-Cyt biomarkers measured in cord blood are associated with maternal demographic and lifestyle characteristics
- The genome damage as measured by CBMN Cyt assay is higher in female infants compared with male infants
- The CBMN-Cyt biomarkers are correlated at birth, three and six months after birth
- Genome damage is less in infants who are breast fed compared with those who are fed with complementary foods or formula milk.

6.4 **Aims**

- To measure CBMN-Cyt biomarkers in peripheral lymphocytes collected from infants at birth, three and six months

- To test whether the CBMN-Cyt biomarkers are correlated with infant's birth outcomes
- To test whether CBMN-Cyt biomarkers are associated with maternal demographic and lifestyle characteristics
- To test whether CBMN-Cyt biomarkers are different between male and female infants during first six months after birth
- To use the CBMN-Cyt assay to test whether genome damage biomarkers in peripheral lymphocytes are different at birth, and at three and six months after birth
- To test whether the CBMN biomarkers are modulated by the type of feeding adopted for the infants at both 3 months and 6 months after birth.

6.5 Material and Methods

6.5.1 Recruitment of participants

A prospective cohort study 'Diet and DNA damage in Infants' (DADHI) was conducted on healthy pregnant women and on their neonatal offspring. Pregnant women, attending the antenatal clinic at the Women's and Children Hospital (WCH), Adelaide and identified as being at low risk of pregnancy complications, were approached to participate in the study. Pre-determined inclusion criteria included a second viable pregnancy (naturally conceived) and having no more than two previous first trimester losses. Women with multiple and/or IVF pregnancy, or with any disease or complication (including hypertension, Type I and II diabetes mellitus, epilepsy, asthma, anaemia, inflammatory bowel syndrome, renal, liver or thyroid problems) or with a body mass index (BMI) $\geq$ 35 kg/m^2 were excluded from the study. Premature infants were also excluded. All eligible women were informed about the study aims and requirements using a detailed information sheet, before being asked to give informed and signed consent at between 8 and 16 weeks gestation. The study was approved by the Human Experimentation Ethics Committee of the Commonwealth Scientific and Industrial Research Organization (CSIRO) and the Human Research Ethics committee of the WCH, Adelaide.

Blood samples were collected at birth (cord blood), at 3 months (heel prick) and 6 months after birth (heel prick) from the baby The consort diagram for detailed information on recruitment of participants and their completion of the protocol is presented in **Figure 6.5** (n=82 at birth, n=64 at three months and n=53 at six months).

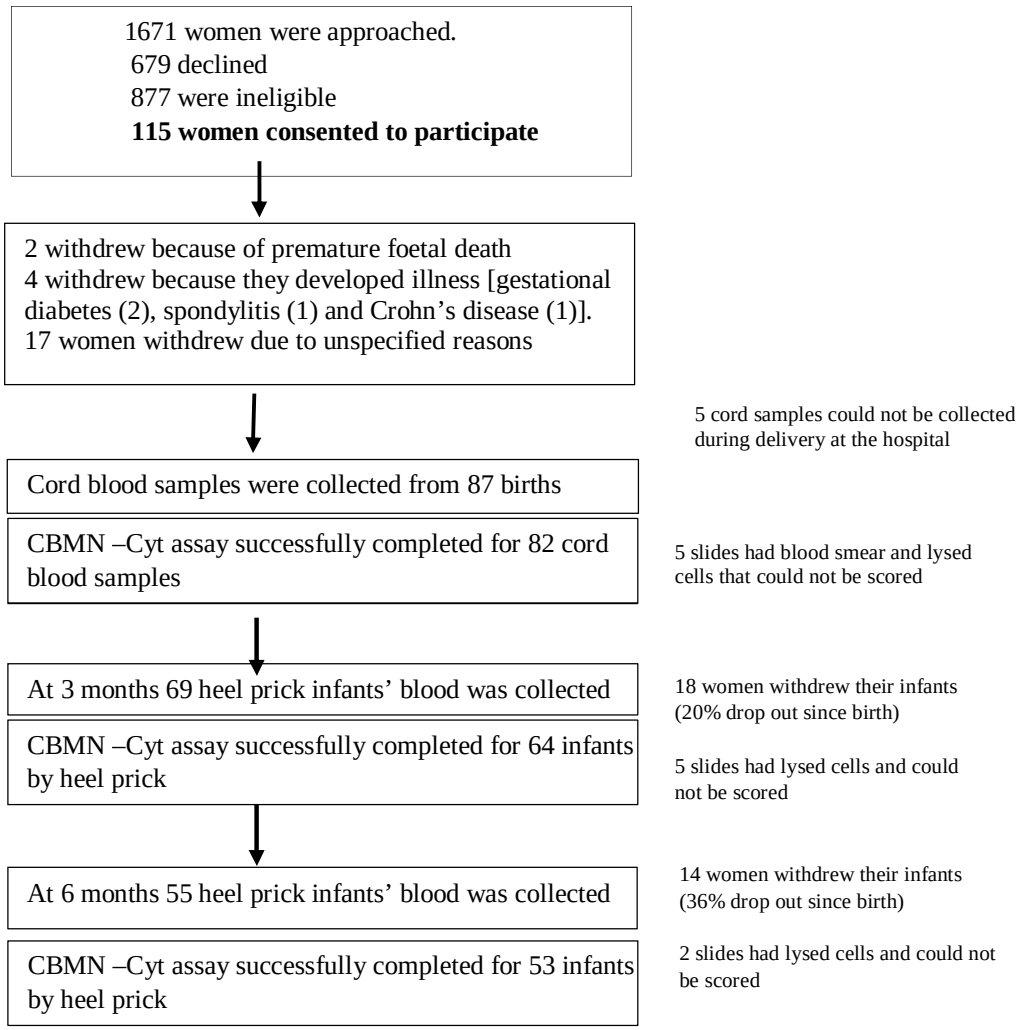

Figure 6.5: Consort diagram for DADHI study recruitment, blood collection and CBMN-Cyt assay completion
(CBMN-Cyt: Cytokinesis block micronucleus Cytome assay)

6.5.2 **General health and Food frequency questionnaire**

A general health questionnaire was administered to participating women at between 8 and 16 weeks gestation to collect detailed information about the mother's demographics, medical and

family history, lifestyle habits such as smoking, dose and duration of folic acid supplementation and other supplements and any medicines consumed during the pregnancy period. Mother's weight at recruitment was recorded using a digital balance accurate to within 100 g, and height was determined using a stadiometer accurate to within 1 cm of overall height. BMI was then calculated using the formula weight (kg)/ height (m) ^{2}. Type of labour and delivery (Caesarean/induced, normal/spontaneous) and any complications during labour was also recorded. A Food Frequency questionnaire (FFQ) (The Cancer Council, Victoria) was administered at 3 and 6 months postpartum to collect information about the mother's intake of macro and micro-nutrients (534)**.** Details regarding infant's birth weight, height, head circumference, gender, gestation age and APGAR score at 1 and 5 minutes post birth were also recorded from the hospital records. APGAR score was devised by Dr Virginia Apgar with the aim to standardize the assessment of newborns utilizing five signs: heart rate, respiratory effort, muscle tone, reflex irritability, and colour (677). A rating of zero, one or two, is given to each sign depending on whether it's presence or absence. A final aggregate score of ten indicates the best possible infant birth outcomes (678).

6.5.3 **Infant's feeding record**

During the first six months after birth, infants may vary significantly in their feeding history in terms of (i) the period that they were exclusively breast fed, (ii) the total cumulative duration of breastfeeding and (iii) the substitute or "complementary" foods used when the baby was not exclusively breast fed (406). The information regarding mode of feeding for the infants in the cohort was collected during months 1-3 and 4-6 (**Appendix 1**). Based on the data collected each infant was given a score of 1 to 4 (**Table 6.1**). The scores were then averaged for the first 3 months and for the period between 3- 6 months (**Appendix 1a**).

Table 6.1: Infant mode of feeding record

Mode of feeding	**Score**

Exclusive breast fed	4
Partially breast fed	3
Exclusive formula fed or other milk (soy or cow)	2
Partially formula fed or other milk	1

6.5.4 **CBMN-Cyt assay**

The blood samples were collected and processed as explained in chapter 3. The whole blood CBMN-Cyt assay was conducted in duplicate on all collected samples (cord blood, 3 and 6 month bloods) (108). The detailed protocol has been explained in chapter 4. Briefly, duplicate whole blood lymphocyte culture for each blood sample from a participant was prepared. On day 0, 100 µl of heparinised whole blood was cultured in 810 µl medium. The mitogenic activity in lymphocytes was initiated by adding 90 µl PHA to give a final concentration of 202.5 µg/ml. The cells were incubated at 37 ºC with loosened lids in a humidified atmosphere containing 5% carbon dioxide for 44 h.

At 44 hrs, the cell cultures were carefully removed from the incubator and 100 µl of cytochalasin-B solution was gently mixed. At 68 hrs, cultures were removed from the incubator, and the cells were mixed gently. The cell suspension was underlaid with 400 µl of Ficoll-Paque (Amersham Pharmacia Biotech, Sweden, cat no. 17144002) in a TV10 tube (Techno Plas, S9716VSU, Australia) using a ratio of 1 (Ficoll): 3 (cell suspension) without disturbing the interface. The tube containing cell suspensions overlaid on Ficoll was then centrifuged once at 400g for 30 min at 18 to 20ºC to separate the lymphocytes. Using a pipette with a 200 µl clear plugged tip, the 'buffy' lymphocyte layer at the interface of the Ficoll Paque and culture medium was removed carefully avoiding uptake of Ficoll. The lymphocyte suspension was washed in three times its volume of Hanks balanced salt solution (Hanks HBSS, Trace Scientific, Melbourne, Australia, Cat no. 111010500-V) by gently pipetting in 1320 µl HBSS solution and then centrifuging at 180g for 10 min at room temperature to remove any residual Ficoll and cell debris. The supernatant was gently removed, leaving approximately 200 µl cell suspension. Subsequently, 15 µl dimethyl sulfoxide (DMSO 7.5% v/v of cell suspension Sigma, Sydney, Australia) was added to prevent cell clumping and to optimize visualization of cytoplasmic boundaries. This was followed by harvesting of cells by cytocentrifugation onto cleaned slides. Microscope slides were cleaned by washing in absolute ethanol and then allowed

to dry for 10 minutes. The slides were then labelled and assembled with a filter card onto a cytocentrifuge cup utilizing a slide holder. The combined slide, filter card, and cytocentrifuge cup were arranged as per manufacturer's instruction and spun in a cytocentrifuge (Model Cytospin 3, Shandon Southern Products, Cheshire, UK).

One hundred microliters of cell suspension was added to the cytospin cup corresponding to the numbered slide in the rotor and spun at 600 rpm for 5 min. A spot was obtained at the end of centrifugation. The card and the slide were inverted and the above process repeated in order to obtain a second spot. The slides were air dried in a biohazard hood for 10 minutes followed by fixing in Diff Quick fixative (Lab Aids, Narrabeen, Australia) for 10 min. Then the slides were transferred directly into Diff Quick stain: 10 dips in the orange stain followed by 5 dips in the blue stain. The extra stain was washed off with tap water and slides were left to air-dry for 10 minutes. The slides were finally cover slipped using DePeX mounting medium (BDH laboratory, Poole, UK) in a fume-hood. A slide with two stained cytospin cell prepared from each of the duplicate cultures was thus prepared. A conventional light microscope (Model Leica DMLB2: Leica Microsystem, Wetzlar, Germany) was used to examine the cells at 1000 x magnification. Cytostatic and cytotoxic events were measured by scoring 500 lymphocyte cells including mono-, bi-, multinucleated cells, necrotic and apoptotic lymphocyte cells according to previously published classification criteria (108). This allowed calculation of nuclear division index (NDI) which provides a measure of the proliferative status of the viable cell fraction and thus indicates mitogenic response in lymphocytes (108,540). The CBMN-Cyt assay genome damage biomarkers (MN, NPB, NBUD) from each duplicate culture were averaged and presented for every 1000 BNC. An average of 500 mononucleated lymphocyte cells were also scored in each duplicate culture (539). The DNA damage biomarkers results in MNC were expressed as MN and NBUD per 100 MNC per subject. The HUMN scoring criteria recommends that the MN frequency be determined in a minimum of 1000 cells (539) but in 40% of our slides, there were insufficient MNC to score 1000 cells.

6.5.5 **Power calculations**

Based on previously published data on 408 newborns (328,333,334,533) the expected mean (± SD) of micronucleus frequency measured in lymphocytes using the CBMN Cyt assay is 1.20 (± 1.02). Using the SD value of 1.02 the study was powered to detect differences in micronucleus frequency between two groups ranging between 0.41 and 0.58 at 80% power and $p < 0.05$ (two-tailed) depending on the number of subjects per group (50-100) as indicated in **Table 6.2.**

Table 6.2: Difference in MN frequency in BNCs that can be detected at $p < 0.05$ depending on number of subjects per group and statistical power level

N per group	Statistical power			
	99%	**95%**	**90%**	**80%**
50	0.88	0.74	0.67	0.58
60	0.81	0.68	0.61	0.53
70	0.74	0.63	0.56	0.49
80	0.70	0.59	0.53	0.45
90	0.66	0.55	0.50	0.43
100	0.62	0.52	0.47	0.41

Note: Power calculations were made using GraphPad Statmate version 2.0
N = number of subjects

6.5.6 **Statistical analysis**

The data for each CBMN-Cyt assay biomarker was first analysed to test whether the distribution was Gaussian by using the D'Agostino-Pearson omnibus normality test which determined the choice of subsequent tests (parametric or non parametric). Degrees of association between continuous variables were evaluated by correlation analysis. Pearson correlation coefficients were calculated for Gaussian distributed data. Correlation analysis for non-Gaussian distributed

data was performed using the Spearman rank test. Differences between the CBMN-Cyt biomarkers at 0, 3 and 6 months were assessed using analysis of variance (ANOVA) for repeated measures. Analysed data are presented as mean ± [standard deviation (SD)]. Differences with $p < 0.1$ (two tailed) were considered statistically significant. For multiple comparisons of group at three time points, post hoc‘t test for linear trend’ and ‘Tukey’s test’ were also conducted. The effect of lifestyle and supplementation variables recorded for mothers during pregnancy (smoking, BMI, alcohol and folic acid intake) on CBMN-Cyt biomarkers measured in the cord blood were also assessed using ‘student t test’ for normal distributed data and ‘Mann-Whitney’s t test’ for non-Gaussian data. Graph Pad Prism version 6.04 for Windows (Graph Pad Inc., San Diego, Calif., USA) and SPSS 22.0 (IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) were used for all statistical analyses.

6.6 **Results**

6.6.1 **General demographics of the cohort**

The mean (± SD) data for general demographic characteristics for mother-infant cohort is presented in **Table 6.3**. 4.5% of the maternal cohort reported smoking during pregnancy, 59.6% reported alcohol consumption during pregnancy, one subject was on a vegan diet, and 94% reported taking folic acid supplements (400 µg/d). The mean (± SD) birth weight of infants (n = 82) was 3463 (± 420.8) g. The mean (± SD) infant weight at 3 months [n = 64, mean (± SD) age 12.7 (± 1.01) weeks] was 6207.8 (± 763.05) g. At 6 months (n = 53) [mean (± SD) age 23.7 (± 1.20) weeks] the mean (± SD) weight was 7896.1 (± 921.99).

Table 6.3: General demographic data for DADHI mother-infant cohort [mean (± SD)

Mothers (n=87)		**Infants (at birth) (n=87)**	
Age (years)	30.6 (± 5.3)	Gestation age (weeks)	39.77 (± 1.1)
BMI (kg/m^2)	25.3 (± 3.7)	Birth weight (gm)	3463 (± 420.8)
Height (m)	1.64 (±0.07)	Birth length (cms)	50.5 (± 2.9)
Weight (Kg)	67.3 (± 11.9)	Head circumference (cms)	35.2 (± 2.7)
Women who took Folic acid supplement (400 µg)*	93.9%	APGAR score at 1 minute	8.4 (± 0.91)
Women who smoked during pregnancy *	4.54%	APGAR score at 5 minutes	8.9 (± 0.29)
Women who consumed alcohol during pregnancy *	59.6%		

* Percentage of women

6.6.2 **Mean CBMN-Cyt biomarkers of the cohort at birth, three and six months**

The mean and standard deviation for each CBMN-Cyt biomarker for all the infants (even if they did not complete the study till six months) is presented in **Table 6.4.** At birth mean (± SD) for frequency of MN, NPB and NBUD measured in BNC was 2.0 (± 1.2), 5.8 (± 3.7) and 11.1 (± 5.7) respectively. The frequency of apoptotic and necrotic lymphocytes was 6.6 (± 4.1) and 35.9 (± 12.2) respectively. Mean (± SD) for NDI was 1.5 (± 0.16) and mean frequency of MN and NBUD in MNC was 0.19 (± 0.21) and 1.0 (± 0.80) respectively.

At three months, Mean (± SD) for MN, NPB and NBUD in BNC was 1.6 (± 1.1), 3.1 (± 1.6) and 7.9 (± 3.8) respectively. The mean frequency of apoptotic and necrotic cells was 7.1 (± 2.9) and 29.7 (± 7.9) respectively. The mean (± SD) for NDI was 1.7 (± 0.14), for MN and NBUD in MNC was 0.16 (± 0.15) and 0.64 (± 0.42) respectively.

At six months, mean (± SD) for MN, NPB and NBUD in BNC was 1.7 (± 1.2), 2.7 (± 2.5) and 7.3 (± 3.5) respectively.

Table 6.4: Mean (± SD) CBMN-Cyt biomarkers measured at birth, 3 and 6 months for DADHI cohort

CBMN-Cyt biomarker	Mean (± SD)		
	Birth (n=82)	3 month (n=64)	6 month (n=53)
MN BNC	2.06 (± 1.2)	1.6 (± 1.1)	1.7 (± 1.2)
NPB BNC	5.8 (± 3.7)	3.1 (± 1.6)	2.7 (± 2.5)
NBUD BNC	11.1 (± 5.7)	7.9 (± 3.8)	7.3 (± 3.5)
NDI	1.5 (± 0.16)	1.7 (± 0.14)	1.8 (± 1.1)
Apoptotic cells	6.6 (± 4.1)	7.1 (± 2.9)	7.1 (± 4.1)
Necrotic cells	35.9 (± 12.2)	29.7 (± 7.9)	27.9 (± 9.3)
MN MNC	0.19 (± 0.21)	0.16 (± 0.15)	0.17 (± 0.17)
NBUD MNC	1.0 (± 0.80)	0.64 (± 0.42)	0.73 (± 0.46)

Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD are presented per 100 MNC; n: number of subjects

6.6.3 Correlation between infants' birth outcomes and CBMN-Cyt biomarkers measured in cord blood

The summary for correlation analysis for infants' birth outcomes and DNA damage biomarkers is presented in **Table 6.5**. The mean (± SD) gestation age for infants at birth correlated positively with MN (r = 0.38, p = 0.006) and NPB (r = 0.3, p = 0.03) in BNC but no association was observed with other cytome biomarkers, except for an inverse trend with NDI (r = - 0.29, p = 0.03). Infant birth weight was associated positively with MN, NPB and NBUD in BNC (r = 0.24, p = 0.08, r = 0.32, p = 0.02, r = 0.28, p = 0.04 respectively). Infant birth length was positively associated NPB and NBUD in BNC (r = 0.32, p = 0.02, r = 0.27, p = 0.04). Infant head circumference was observed to be negatively associated with apoptosis (r = - 0.27, p = 0.06). A low score (5-6) was recorded for three infants at 1 minute after birth while at 5 minutes after birth all infants were assessed to have a normal score. APGAR score at 1 and 5 minute was positively associated with NDI (r = 0.3, p = 0.05, r = 0.28, p = 0.06 respectively) while with NPB it was observed to have a negative association (r = - 0.26, p=0.09) (**Table 6.5).**

Table 6.5: Correlation analysis of Infant Birth outcomes and CBMN-Cyt biomarkers measured in cord blood at birth

	MN BNC	NPB BNC	NBUD BNC	NDI	Apoptotic cells	Necrotic cells	MN MNC	NBUD MNC
Gestation age (weeks)	**r = 0.38 p = 0.006****	**r = 0.30 p = 0.03****	r = 0.22 p = 0.11	**r = - 0.29 p = 0.03****	r = 0.07 p= 0.59	r = 0.002 p = 0.98	r = -0.06 p = 0.65	r = - 0.09 p = 0.53
Birth weight (gm)	**r = 0.24 p = 0.08***	**r = 0.32 p = 0.02****	**r = 0.28 p = 0.04****	r = - 0.19 p = 0.16	r = - 0.08 p = 0.55	r = 0.09 p = 0.48	r = - 0.10 p = 0.44	r = 0.09 p = 0.48
Birth length (cms)	r = 0.21 p = 0.13	**r =0.32 p = 0.02****	**r = 0.27 p = 0.04****	r = - 0.20 p = 0.14	r= - 0.01 p= 0.89	r = 0.04 p = 0.77	r = 0.10 p = 0.46	r = 0.22 p = 0.11
Head circumference (cms)	r = 0.17 p =0.23	r = 0.17 p = 0.23	r = 0.09 p = 0.52	r = 0.06 p = 0.66	**r = - 0.27 p = 0.06***	r = 0.02 p = 0.84	r= - 0.07 p = 0.59	R = - 0.02 p= 0.85
APGAR score at 1 minute after birth	r = - 0.07 p =0.62	r = - 0.16 p = 0.30	r = - 0.25 p = 0.10	**r = 0.30 p = 0.05****	r = - 0.01 p = 0.94	r = 0.15 p = 0.35	r = 0.19 p = 0.21	r = - 0.17 p = 0.27
APGAR score at 5 minutes after birth	r = 0.005 p =0.97	**r = - 0.26 p =0.09***	r = - 0.19 p = 0.23	**r = 0.28 p = 0.06***	r = 0.02 p = 0.90	r = 0.08 p = 0.61	r = 0.23 p = 0.14	r = 0.001 p = 0.99

Each DNA damage biomarker was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution);

**: significant at $p \leq 0.05$, * $p \leq 0.1$ (All p value are two tailed)

Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD presented per 100 MNC

6.6.4 **Correlation between mothers' demographic characteristics with CBMN-Cyt biomarkers measured in cord blood and infant birth outcomes**

Mothers' weight and BMI at recruitment were found to be positively associated with NPB BNC in cord blood ($r = 0.38$, $p = 0.006$, $r = 0.32$, $p = 0.02$ respectively). Mother's age was negatively correlated with frequency of apoptotic cells ($r = 0.25$, $p = 0.07$) (**Table 6.6**). Mother's height was positively associated with infant birth weight ($r = 0.21$, $p = 0.09$) and BMI was negatively correlated with APGAR score at 5 minutes ($r = - 0.25$, $p = 0.07$) (**Table 6.7**). Gestation age was also positively associated with infant birth weight ($r = 0.33$, $p = 0.005$) and length ($r = 0.26$, $p = 0.03$) (**Table 6. 8**).

Table 6.6: Correlation analysis of Mother's demographic characteristics at recruitment and CBMN-Cyt biomarkers at birth

Mother's characteristics	**CBMN-Cyt biomarkers in cord lymphocytes at birth**							
	MN BNC	NPB BNC	NBUD BNC	NDI	Apoptotic cells	Necrotic cells	MN MNC	NBUD MNC
Age (yrs)	r = - 0.008 p =0.95	r = - 0.04 p =0.74	r = -0.02 p = 0.84	r = 0.13 p = 0.35	**r = - 0.25** **p = 0.07***	r = 0.05 p = 0.70	r= 0.19 p=0.17	r = 0.03 p =0.78
Weight (kg)	r = - 0.04 p =0.74	**r = 0.38** **p =0.006****	r = 0.08 p =0.55	r = - 0.10 p = 0.47	r = - 0.12 p = 0.37	r = 0.05 p = 0.7	r= - 0.02 p = 0.8	r = 0.11 p = 0.41
Height (m)	r = - 0.06 p = 0.68	r = 0.20 p = 0.18	r = 0.07 p = 0.64	r= - 0.20 p=0.17	r = 0.01 p = 0.9	r = - 0.12 p = 0.42	r = - 0.12 p = 0.40	r = 0.01 p =0.9
BMI (kg/m^2)	r = 0.01 p =0.93	**r = 0.32** **p =0.02****	r = 0.05 p =0.70	r = - 0.02 p =0.89	r = - 0.14 p =0.34	r = 0.12 p =0.4	r = 0.01 p = 0.91	r = 0.17 p = 0.26

Each DNA damage biomarker was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution); ***: significant at $p \leq 0.01$; **$p \leq 0.05$, * $p \leq 0.1$ (All p value are two-tailed)
Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells, MN, NPB and NBUD presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD presented per 100 MNC

Table 6.7: Correlation analysis of mother's demographic characteristics at recruitment and infant's birth outcomes

Mother's characteristics	**Infant birth outcomes**				
	Weight (gms)	Length (cms)	Head circumference (cms)	APGAR score at 1 min	APGAR score at 5 min
Age (yrs)	r = 0.02 p = 0.84	r = 0.05 p = 0.66	r = - 0.12 p = 0.34	r = - 0.21 p = 0.11	r = 0.00 p = 0.96
Weight (kg)	r = 0.14 p = 0.24	r = 0.10 p = 0.40	r = 0.02 p = 0.80	r = - 0.05 p = 0.67	r = - 0.15 p = 0.27
Height (m)	**r = 0.21** **p = 0.09***	r = 0.15 p = 0.23	r = 0.13 p = 0.32	r = 0.03 p = 0.79	r = 0.04 p = 0.76
BMI (kg/m^2)	r = 0.00 p = 0.99	r = 0.06 p = 0.60	r = 0.02 p = 0.88	r = - 0.07 p = 0.61	**r = - 0.25** **p = 0.07***

Table 6.8: Correlation analysis of gestation age and infant's birth outcomes

Gestation age (weeks)	**Infant birth outcomes**				
	Weight (gms)	Length (cms)	Head circumference (cms)	APGAR score at 1 min	APGAR score at 5 min
	r = 0.33 **p = 0.005****	**r = 0.26** **p = 0.03****	r = 0.16 p = 0.20	r = - 0.10 p = 0.45	r = 0.05 p = 0.69

Note: Gestation age was not associated with any of the mother's characteristics at recruitment (weight, height or BMI)
Each infant birth outcome was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution);
***: significant at $p \leq 0.01$; **$p \leq 0.05$, * $p \leq 0.1$ (All p value are two tailed)

6.6.5 **Correlation between mothers' lifestyle characteristics and CBMN-Cyt biomarkers measured in cord blood at birth**

To test the hypothesis that CBMN-Cyt biomarkers assessed in cord blood were different according to mother's smoking status and alcohol intake, student (independent) 't test' was performed. There was no difference among the CBMN-Cyt biomarkers for mothers who smoked (n=4) and those who did not smoke during pregnancy (n=43) **(Table 6.9)** (though the number of cigarettes smoked per day was not recorded) and in those who consumed alcohol (n=18) as compared to non alcoholic consumers (n=29) during pregnancy (although amount of alcohol consumed was not recorded) **(Table 6.10).** There was no difference among CBMN-Cyt biomarkers assessed in cord blood with respect to folic acid intake by the mothers during pregnancy (**Table 6.11)** but only 3 mothers did not have the folic acid supplement**.** The mean frequency of necrotic lymphocytes was lower in mothers who had spontaneous labour (n=22) in comparison to those who had induced labour (n=22), however Levene's test for homogeneity of variances could not validate the observed effect (**Table 6.12**).

Table 6.9: Group statistic for student t test for influence of mother's smoking status during pregnancy on CBMN biomarkers

CBMN biomarkers (cord blood)	Mean (± SD) CBMN biomarkers		t test for equality of means		
	Smoker (n=4)	Non-smokers(n=40)	t	df	p (two-tailed)
MN BNC	1.18 (± 0.55)	1.86 (± 0.93	-1.41	42	0.73
NPB BNC	5.06 (± 2.63)	7.52 (± 3.62)	-1.3	42	0.19
NBUD BNC	7.87 (± 5.37)	11.01 (± 5.41)	-1.1	42	0.27
NDI	1.62 (± 0.19)	1.47 (± 0.16)	1.7	42	0.08
Apoptotic cells	5.62 (± 1.79)	5.51 (± 3.22)	.06	42	0.94
Necrotic cells	22.31 (± 9.6)	32.7 (± 11.8)	-1.6	42	0.09
MN MNC	0.24 (± 0.24)	0.21 (± 0.24)	.19	42	0.84
NBUD MNC	0.74(± 0.8)	0.82 (± 0.71)	-.21	42	0.83

The independent 't' test represent pool t test (assuming equal variances for two groups). It is to be noted that the groups were unevenly distributed in numbers.

Table 6.10: Group statistic for student t test for influence of mother's alcohol intake during pregnancy on CBMN biomarkers

CBMN biomarkers (cord blood)	Mean (± SD) CBMN biomarkers		t test for equality of means		
	Alcohol consumers (n=18)	Non Alcohol consumers (n=29)	t	df	p (two-tailed)
MN BNC	1.74 (± 0.80)	1.83 (± 0.94)	-.34	44	0.73
NPB BNC	7.47(± 2.19)	7.63 (± 4.30)	-.14	44	0.88
NBUD BNC	11.3 (± 5.24)	10.77 (± 5.34)	.36	44	0.71
NDI	1.47 (± 0.16)	1.47 (± 0.18)	-.02	44	0.97
Apoptotic cells	4.76 (± 3.62)	5.7 (± 2.69)	-1.0	44	0.32
Necrotic cells	29.14 (± 13.8)	32.8 (± 10.5)	-1.0	44	0.31
MN MNC	0.19 (± 0.14)	0.23 (± 0.27)	-.55	44	0.58
NBUD MNC	0.82 (± 0.48)	0.80 (± 0.79)	.12	44	0.90

The independent 't' test represent pool t test (assuming equal variances for two groups). It is to be noted that the groups were unevenly distributed in numbers.

Table 6.11: Group statistic for student t test for influence of mother's Folic acid intake (400µg/d) during pregnancy on CBMN biomarkers

CBMN biomarkers (cord blood)	Mean (± SD) CBMN biomarkers		t test for equality of means		
	Folic acid consumers(n=44)	Non Folic acid consumers(n=3)	t	df	p (two-tailed)
MN BNC	1.75 (± 0.89)	1.58 (± 0.62)	.32	44	0.75
NPB BNC	7.53(± 3.58)	6.66 (± 5.86)	.39	44	0.69
NBUD BNC	11.0 (± 5.55)	8.0 (± 1.14)	.93	44	0.35
NDI	1.47 (± 0.17)	1.61 (± 0.18)	-1.38	44	0.17
Apoptotic cells	5.27 (± 3.1)	7.25 (± 2.78)	-1.07	44	0.29
Necrotic cells	31.66 (± 11.7)	27.7 (± 16.0)	.54	44	0.58
MN MNC	0.21 (± 0.24)	0.16 (± 0.16)	.35	44	0.72
NBUD MNC	0.81 (± 0.72)	0.63 (± 0.03)	.43	44	0.66

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Table 6.12: Group statistic for student t test for type of labour and CBMN biomarkers measured in the cord blood

CBMN biomarkers (cord blood)	Mean (± SD) CBMN biomarkers		t test for equality of means		
	Induced labour (n=22)	Spontaneous labour (n=22)	t	df	p (two-tailed)
MN BNC	1.8 (± 0.91)	1.77 (± 0.94)	.10	42	0.91
NPB BNC	7.15(± 4.13)	7.78 (± 3.42)	-.54	42	0.58
NBUD BNC	10.3 (± 6.02)	11.4 (± 5.03)	-.64	42	0.52
NDI	1.50 (± 0.17)	1.46 (± 0.16)	.92	42	0.36
Apoptotic cells	5.86 (± 2.8)	5.0 (± 3.3)	.88	42	0.37
Necrotic cells	35.2 (± 14.5)	27.6 (± 7.2)	2.1	42	0.03*
MN MNC	0.24 (± 0.22)	0.18 (± 0.26)	.75	42	0.45
NBUD MNC	0.85 (± 0.82)	0.79 (± 0.57)	.24	42	0.80

The independent 't' test represent pool t test (assuming equal variances for two groups). Significance of differences observed among necrotic cells assessed from induced and spontaneous labour need to be read with caution because assumption of homogeneity of variances by Levene's test was not satisfied (F=5.5, p=0.02)

Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNCs: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD are presented per 100 MNC

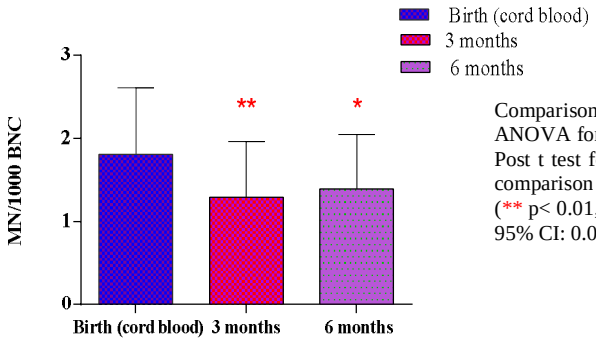
6.6.6 **Differences among CBMN-Cyt biomarkers in infants' lymphocytes at birth and at 3 and 6 months after birth**

In order to test the hypothesis that the age of the infant has any effect on the genome instability biomarkers measured in PBL, repeat measures ANOVA (one way) was performed. For this analysis, only data for those infants was included from whom blood was collected at all three time points (birth: n = 48, three: n = 48 and six months: n = 39). The ANOVA results along with test for the homogeneity of variances (F) and significance (p) is presented in **Table 6.13**. There were significant differences between all the CBMN-Cyt biomarkers at three time points. MN, NPB and NBUD in BNCs decreased significantly by 28.7 %, 52.6 % and 34.9 % respectively at 3 months and 22.6 %, 58 %, 35.9 % respectively at 6 months relative to cord blood. NDI and apoptotic cells increased significantly by 16.2 % and 42.8 % respectively at 3 months and 14.8 % and 30 % respectively at 6 months relative to cord blood (**Figure 6.6**). Necrotic cells were observed to significantly decrease by 16.3% at six months but no change was observed in MN and NBUD in MNC (**Figure 6.7**).

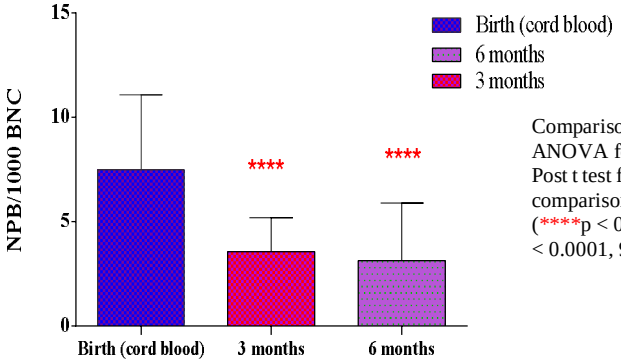
Table 6. 13: Comparison of CBMN-Cyt biomarkers measured at birth, 3 and 6 months for DADHI cohort

CBMN-Cyt biomarker	Mean (± SD)			ANOVA			Post-test for linear trend
	Birth (n=48)	3 month (n=48)	6 month (n=39)	F	p value	r square	(p)
MN BNC	1.81 (± 0.87)	1.29 (± 0.67)	1.40 (± 0.66)	6.9	0.001	0.09	0.007
NPB BNC	7.49 (± 3.65)	3.55 (± 1.65)	3.14 (± 2.77)	33.3	<0.0001	0.34	<0.0001
NBUD BNC	10.81 (± 5.37)	7.03 (± 3.59)	6.92 (± 3.51)	12.3	<0.0001	0.16	<0.0001
NDI	1.48 (± 0.17)	1.72 (± 0.16)	1.70 (± 0.13)	50.5	<0.0001	0.43	<0.0001
Apoptotic cells	5.42 (± 3.06)	7.74 (± 3.13)	7.05 (± 3.74)	6.1	0.002	0.08	0.02
Necrotic cells	31.50 (± 11.7)	28.65 (± 7.02)	26.35 (± 7.49)	3.5	0.03	0.05	0.009
MN MNC	0.21 (± 0.24)	0.16 (± 0.17)	0.15 (± 0.16)	1.5	0.2	0.02	0.09
NBUD MNC	0.80 (± 0.70)	0.63 (± 0.46)	0.70 (± 0.39)	1.0	0.3	0.01	0.3

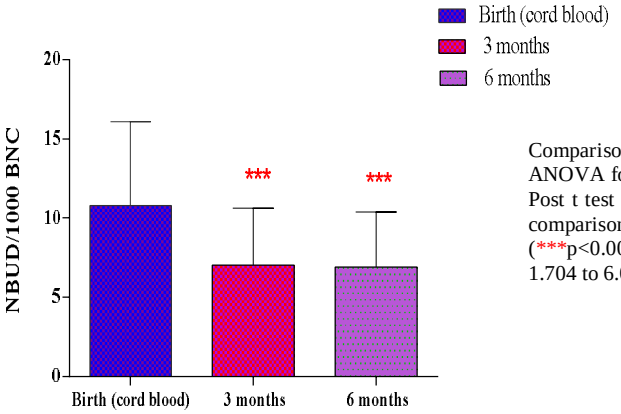
ANOVA for repeat measures was performed to compare each biomarker for the same cohort of infants at birth, 3 and 6 months.
Post-hoc test for linear trend was significant for MN, NPB, NBUD in BNC, NDI, apoptotic and necrotic lymphocytes but not for MN & NBUD in MNC.
Tukey's multiple comparison tests showed significant differences at birth & 3 months and birth & 6 months for all biomarkers except for those measured in MNCs. No significant difference was observed between biomarkers assessed at 3 and 6 months (These results are presented in **Figure 6.7 and 6.8**)
Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNCs: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD are presented per 100 MNC



Comparison between **MN BNC** at birth, 3 and 6 months using ANOVA for repeat measures (p = 0.001).
Post t test for linear trend significant at p = 0.007. Tukey's multiple comparison test showed significant differences at birth and 3 months (** p< 0.01, 95% CI: 0.16 to 0.87) and birth and 6 months (* p<0.05, 95% CI: 0.051 to 0.788) but not between 3 & 6 months.



Comparison between **NPB BNC** at birth, 3 and 6 months using ANOVA for repeat measures (p < 0.0001).
Post t test for linear trend significant at p = 0 <0.0001. Tukey's multiple comparison test showed significant differences at birth and 3 months (****p < 0.0001, 95 % CI: 2.56 to 5.32) and birth & 6 months (****p < 0.0001, 95% CI: 2.91 to 5.805) but not between 3 and 6 months.



Comparison between **NBUD BNC** at birth, 3 and 6 months using ANOVA for repeat measures (p < 0.0001).
Post t test for linear trend significant at p < 0.0001. Tukey's multiple comparison test showed significant differences at birth and 3 months (***p<0.001, 1.67 to 5.86) and birth & 6 months (***p<0.001, 95% CI: 1.704 to 6.096) but not between 3 and 6 months.

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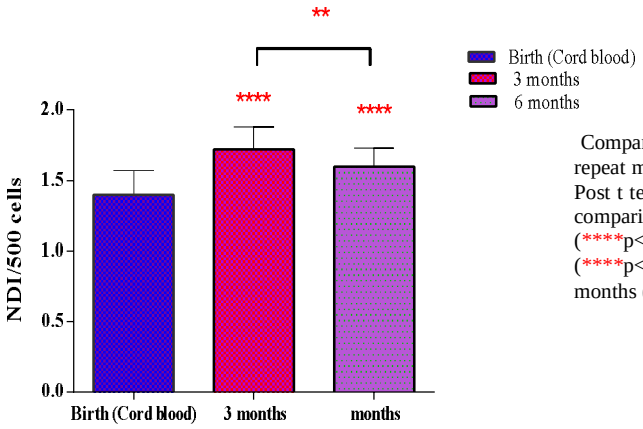
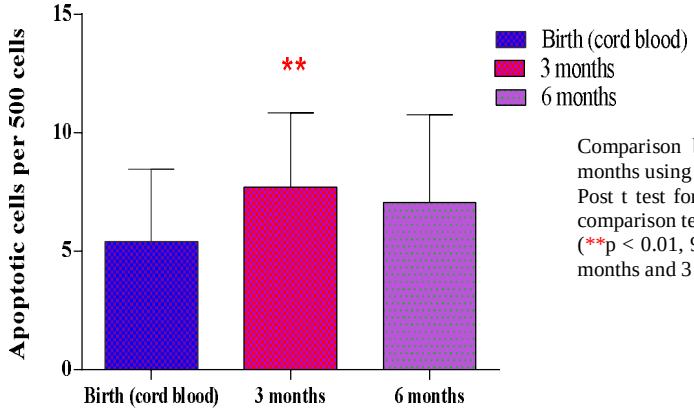
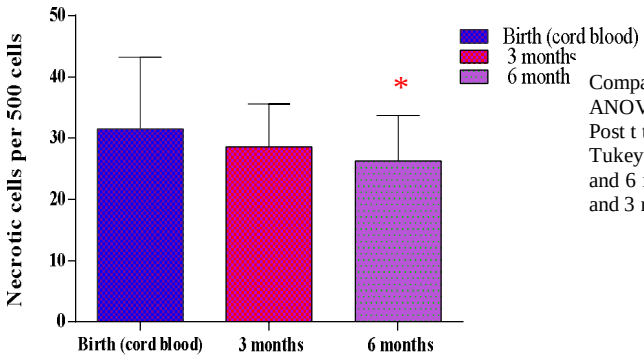


Comparison between **NDI** at birth, 3 and 6 months using ANOVA for repeat measures ($p < 0.0001$).
Post t test for linear trend significant at $p < 0.0001$. Tukey's multiple comparison test showed significant differences at birth and 3 months (****$p<0.0001$ 95% CI: -0.3964 to -0.2436), birth & 6 months (****$p<0.0001$, 95% CI: - 0.2799 to -0.1201) and between 3 and 6 months (** $p < 0.01$, 95% CI: 0.038 to 0.201)



Comparison between **Apoptotic lymphocytes** at birth, 3 and 6 months using ANOVA for repeat measures ($p = 0.002$).
Post t test for linear trend significant at $p=0.02$. Tukey's multiple comparison test showed significant differences at birth and 3 months (**$p < 0.01$, 95% CI: -3.905 to -0.6947) but not between birth & 6 months and 3 and 6 months.

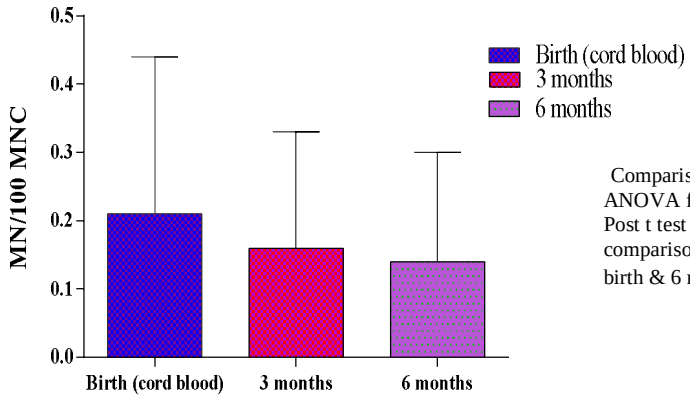


Comparison between **Necrotic lymphocytes** at birth, 3 and 6 months using ANOVA for repeat measures ($p = 0.03$).
Post t test for linear trend significant at $p = 0.009$.
Tukey's multiple comparison test showed significant differences at birth and 6 months (* $p<0.05$, 95% CI: 0.5180 to 9.882) but not between, birth and 3 months and 3 and 6 months

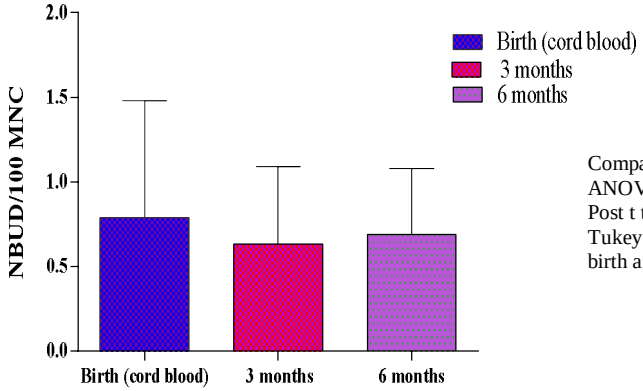
Figure 6.6: Comparison between CBMN-Cyt biomarkers measured in binucleated lymphocyte cells at birth, 3 and 6 months

[ANOVA used mean (± SD) values for infants whose data was available for all three time points: 48 at birth, 48 at three months and 39 at six months]

Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells.



Comparison between MNMNC at birth, 3 and 6 months using ANOVA for repeat measures (p = 0.2).
Post t test for linear trend not significant (p = 0.09). Tukey's multiple comparison test showed no significant differences at birth & 3 months, birth & 6 months and 3 and 6 months.



Comparison between NBUD MNC at birth, 3 and 6 months using ANOVA for repeat measures (p = 0.3).
Post t test for linear trend not significant (p=0.3).
Tukey's multiple comparison test showed no significant differences at birth and 3 months, birth & 6 months and 3 and 6 months

Figure 6.7: Comparison between CBMN-Cyt biomarkers measured in mononucleated lymphocyte cells at birth, 3 and 6 months
[ANOVA used mean (± SD) values for infants whose data was available for all three time points: 48 at birth, 48 at three months and 39 at six months]
Abbreviations: MN: micronuclei; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN and NBUD are presented per 100 MNC

6.6.7 Correlation between CBMN-Cyt biomarkers in Infants at birth and at 3 and 6 Months

Correlation analysis was conducted for the same cohort of infants at birth (n = 48), three months (n=48) and six months (n=39). The association between all CBMN Cyt biomarkers at birth, three and six months is presented in **Table 6.14** and correlation among DNA damage biomarkers (MN, NPB and NBUD) are also shown in **Figures 6.8**, **6.9** and **6.10**. A significant correlation was observed for NBUD in BNC at birth and at 3 months (r = 0.45, p= 0.001) (**Table 6. 14**). A similar relationship was evident for NPB in BNC at birth and at 3 months (r = 0.47, p=0.0006) but there was no correlation at birth and 3 months for MN frequency in BNC (**Figure 6. 9**). The frequency of apoptotic and necrotic cells assessed at birth did not correlate with their frequency measured at 3 months, however NDI at birth and 3 months was significantly correlated (r = 0.35, p = 0.01).

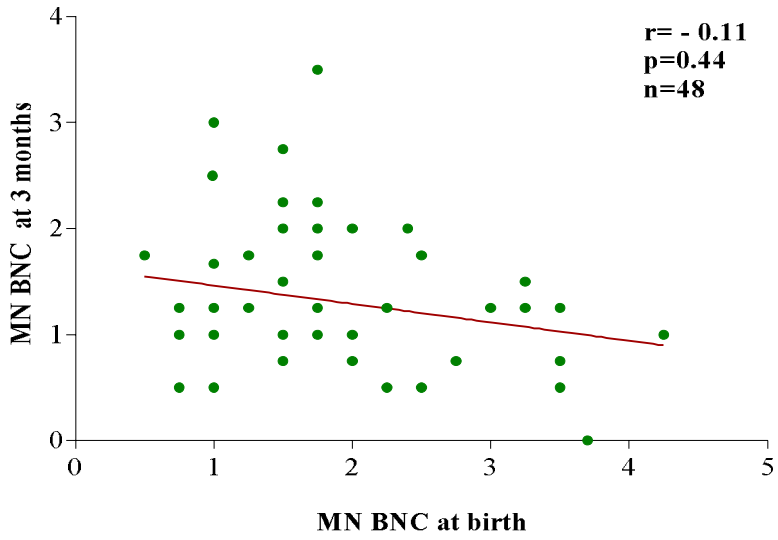
NPB measured at in BNC at birth correlated significantly with those measured at 6 months. (r = 0.36, p = 0.02) (**Figure 6. 10**).

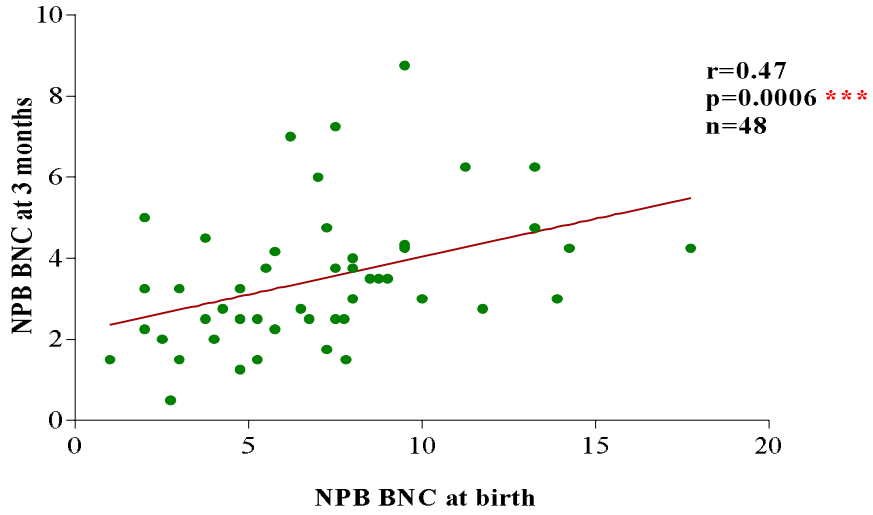
Among all CBMN-Cyt biomarkers measured at three months, MN, NPB and NBUD in BNC correlated positively with those measured at six months (r = 0.35, p = 0.01, r = 0.29, p = 0.03 and r = 0.24, p = 0.08 respectively) (**Figure 6. 11**). NDI at three and six months also correlated with each other (r = 0.24, p = 0.08) NBUD measured in MNC at three and six months was positively associated with each other (r = 0.26, p= 0.06) (**Table 6.14)**.

Table 6. 14: Correlation analysis between CBMN-Cyt biomarkers at birth & three months, birth & six months and three & 6 months

CBMN-Cyt biomarker	Birth and three months (n=48)		Birth and six months (n=39)		Three and six months (n=50)	
	'r' value	'p' (two tailed)'	'r' value	'p' (two tailed)	'r' value	'p' (two tailed)
MN BNC	-0.11	0.44	- 0.08	0.59	0.35	**0.01****
NPB BNC	0.47	**0.0006*****	0.36	**0.02****	0.29	**0.03****
NBUDBNC	0.45	**0.001*****	0.11	0.47	0.24	**0.08***
NDI	0.35	**0.01****	0.25	0.11	0.24	**0.08***
Apoptotic cells	0.07	0.6	0.04	0.7	- 0.06	0.6
Necrotic cells	0.04	0.7	0.14	0.3	0.15	0.26
MN MNC	0.15	0.29	0.05	0.7	0.11	0.45
NBUD MNC	0.22	0.14	0.2	0.21	0.26	**0.06***

Each DNA damage biomarker was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution);
***: significant at $p \leq 0.001$, **$p \leq 0.05$, * $p \leq 0.1$ (All p value are two tailed)
Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocytes are presented per 500 cells, MN and NBUD are presented per 100 MNC





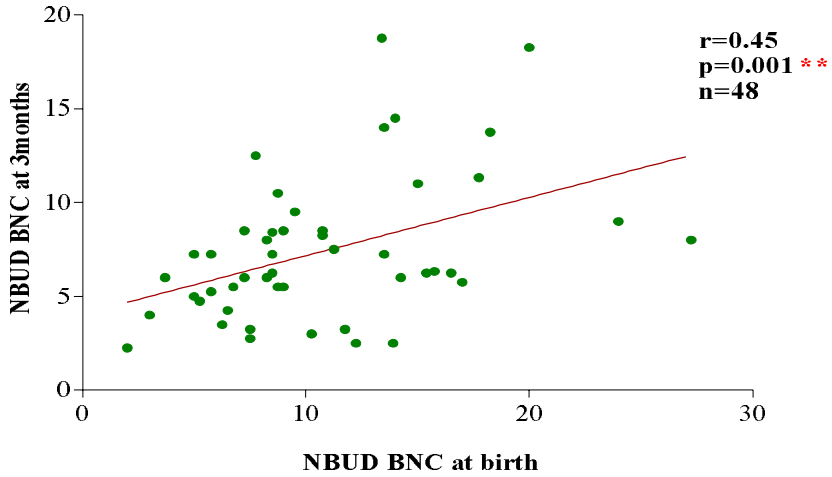
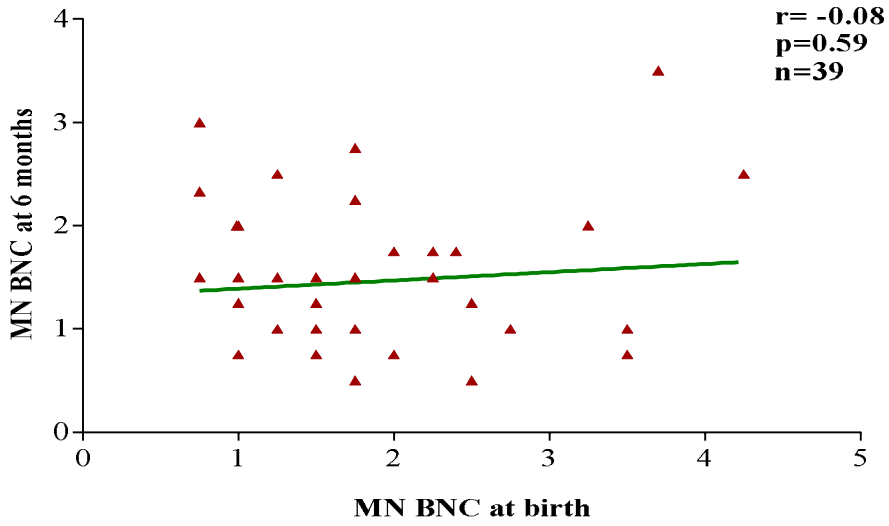
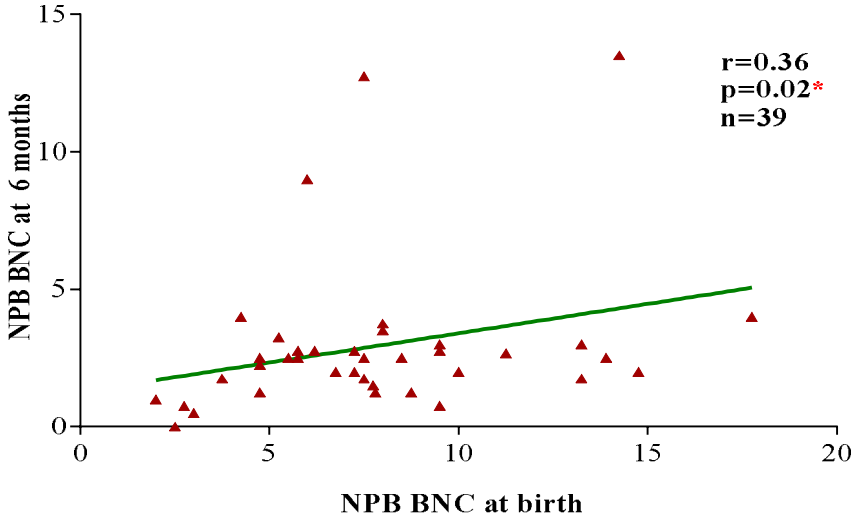


Figure 6.8: Correlation between MN, NBUD and NPB measured in BNC at birth and at three months[***: significant at $p \leq 0.001$, **$p \leq 0.05$, * $p \leq 0.1$ (All p value are two tailed) 'r': correlation coefficient; n = number of subjects; MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000

BNC, NDI, apoptotic and necrotic lymphocytes are presented per 500 cells, MN and NBUD are presented per 100 MNC]





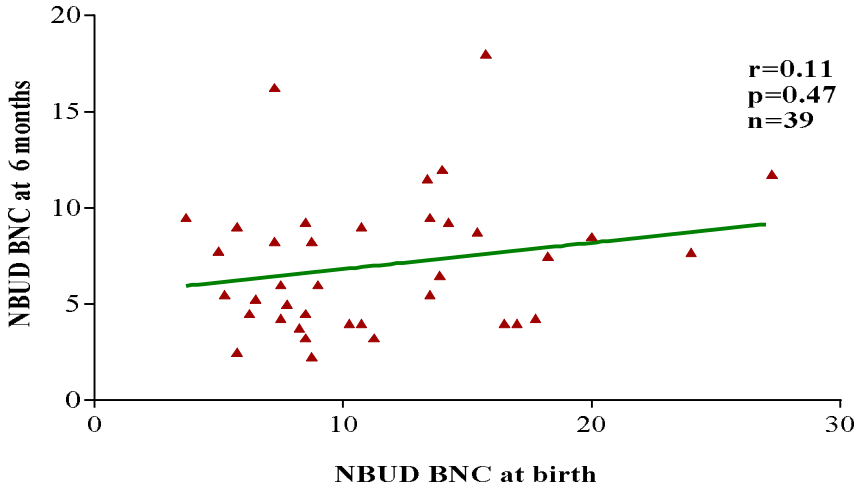
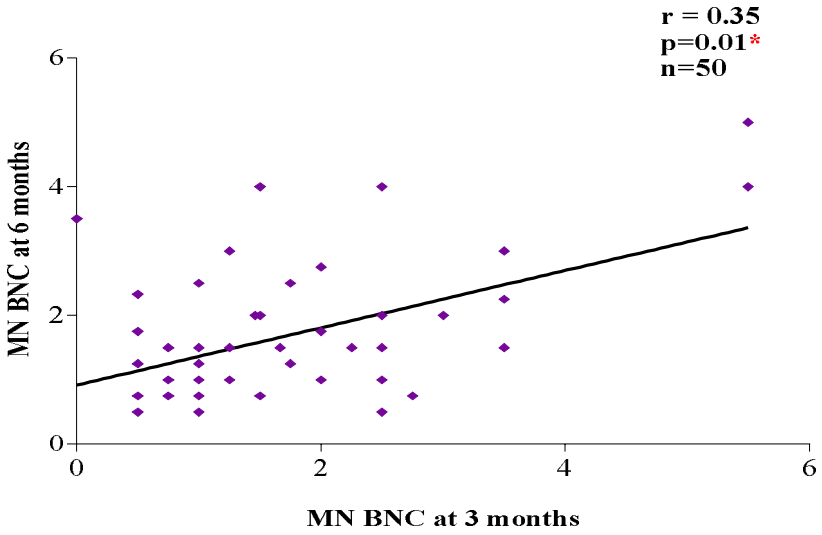
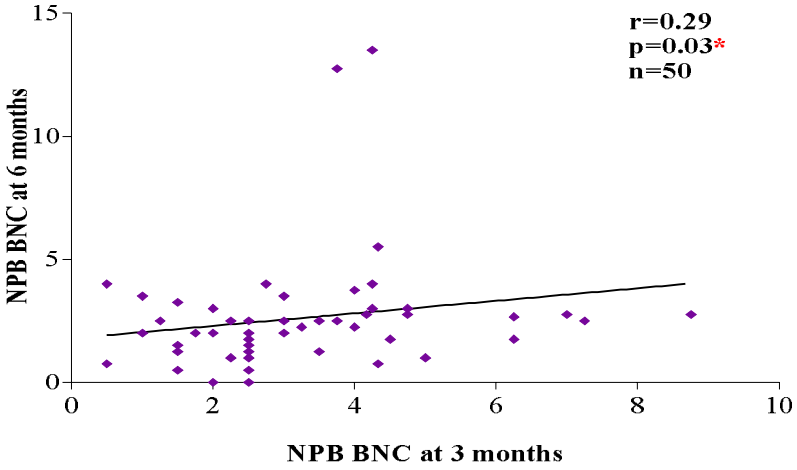


Figure 6.9: Correlation between MN, NBUD and NPB measured in BNC at birth and at six months [***: significant at p ≤ 0.001, **p ≤ 0.05, * p ≤ 0.1 (All p value are two tailed), 'r': correlation coefficient; n = number of subjects; MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge;

NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocytes are presented per 500 cells, MN and NBUD are presented per 100 MNC]





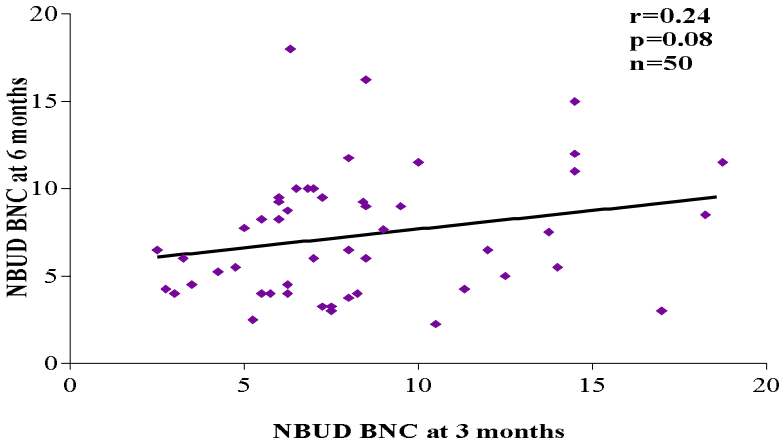


Figure 6.10: Correlation between MN, NBUD and NPB measured in BNC at birth and at six months

[*: significant at, $p \leq 0.1$ (All p value are two tailed)

‘r’: correlation coefficient; n = number of subjects; MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocytes are presented per 500 cells, MN and NBUD are presented per 100 MNC

6.6.8 **Correlation between NDI with other CBMN-Cyt biomarkers at birth, 3 and 6 months**

Because DNA damage in lymphocyte may impair cell proliferation and immune response, we investigated the correlation between NDI and MN, NPB and NBUD in BNC. At birth, NDI was negatively correlated with NPB in BNC ($r = -0.45$, $p < 0.0001$) and positively with necrotic cells ($r = 27$, $p = 0.01$) (**Table 6.15**). At 3 months, an inverse correlation was observed between NDI and NPB ($r = -0.31$, $p = 0.01$) and NBUD ($r = -0.36$, $p=0.002$) measured in BNC (**Table 6.16**) and NBUD in MNCs ($r = -0.22$, $p = 0.08$). At six months, NDI showed significant negative association with MN, NPB and NBUD in BNCs ($r= -0.24$, $p = 0.08$, $r = -0.22$, $p = 0.1$, $r = -0.31$, $p = 0.02$) (**Table 6.17**).

Table 6. 15: Correlation between NDI and CBMN-Cyt biomarkers at birth

CBMN-Cyt biomarkers	'r' value	'p'(two tailed)
MN BNC	-0.08	0.46
NPB BNC	-0.45	< **0.0001****
NBUD BNC	-0.17	0.11
Apoptotic cells	-0.008	0.93
Necrotic cells	0.27	**0.01****
MN MNC	0.02	0.81
NBUD MNC	0.06	0.57

Table 6. 16: Correlation between NDI and CBMN-Cyt biomarkers at 3 months

CBMN-Cyt biomarkers	'r' value	'p' (two tailed)
MN BNC	-0.03	0.8
NPB BNC	-0.31	**0.01*****
NBUD BNC	-0.36	**0.002*****
Apoptotic cells	0.16	0.18
Necrotic cells	0.17	0.17
MN MNC	-0.18	0.15
NBUD MNC	-0.22	**0.08***

Table 6. 17: Correlation between NDI and CBMN-Cyt biomarkers at 6 months

CBMN-Cyt biomarkers	'r' value	'p' (two tailed)
MN BNC	- 0.24	**0.08***
NPB BNC	- 0.22	**0.10***
NBUD BNC	- 0.31	**0.02****
Apoptotic cells	0.04	0.72
Necrotic cells	0.07	0.61
MN MNC	- 0.08	0.55
NBUD MNC	- 0.16	0.26

Each DNA damage biomarker was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution);
****: significant at $p \leq 0.0001$; *** $p \leq 0.01$; **$p \leq 0.05$, * $p \leq 0.1$
Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocytes are presented per 500 cells, MN and NBUD are presented per 100 MNC

6.6.9 **Correlation between micronucleus frequency in binucleated and mononucleated Lymphocyte cells**

The CBMN-Cyt biomarkers measured in BNC and MNC were found to be positively associated among infants at birth, three and six months. The frequency of MN scored in BNC were found to be positively correlated with MN in MNCs at 3 and 6 month ($r = 0.3$, $p = 0.01$, $r = 0.28$, $p = 0.04$ respectively). The frequency of NBUD in BNC was correlated with NBUD in MNC at birth ($r = 0.53$, $p < 0.0001$), three months ($r = 0.35$, $p = 0.004$) as well as at six months ($r = 0.3$, $p = 0.03$) (**Table 6.18)**.

Table 6. 18: Correlation between CBMN-Cyt biomarkers in BNC and MNC at birth, 3 months and 6 months

	Birth (n = 82)		3 months (n = 64)		6 months (n = 53)	
	MN BNC	**NBUD BNC**	**MN BNC**	**NBUD BNC**	**MN BNC**	**NBUD BNC**
MN MNC	r =- 0.03 p = 0.7		**r =0.30** **p = 0.01*** **		**r = 0.28** **p = 0.04** **	
NBUD MNC		**r =0.53** **p=<0.0001**** **		**r = 0.35** **p=0.004*** **		**r=0.30 p=0.03** **

Each DNA damage biomarker was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution);

****: significant at $p \leq 0.0001$; *** $p \leq 0.01$; **$p \leq 0.05$

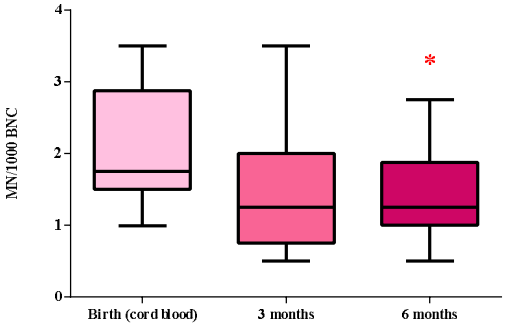
Abbreviations: n = number of subjects; MN: micronuclei; BNC: Binucleated lymphocyte cells; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN and NBUD are presented per 1000 BNCs, MN and NBUD are presented per 100 MNC, n: number of subjects

6.6.10 **Trend for CBMN-Cyt biomarkers in the female cohort from birth to six months**

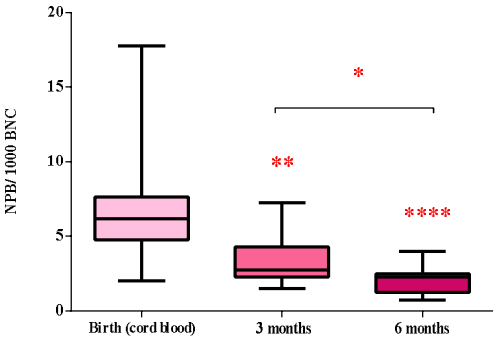
This section looks at changes in CBMN-Cyt biomarkers measured in the female cohort from birth to six months utilizing ANOVA that used number of female infants whose data for CBMN-Cyt biomarkers were available for birth, three as well six months (n = 24). There were significant differences in the MN BNC within the female cohort measured at birth, three and six months ($p = 0.03$, $F = 6.78$), with a linear trend towards a decrease with age (slope= - 0.36; $p = 0.007$). The tukey's multiple comparison showed that the difference was significant between birth and 6 months ($p < 0.05$, 95% CI: 0.01 to 1.4) but not for birth and three months or three months and six months (**Figure 6.11**). There were lower frequencies of NPB BNC at three and six months relative to birth (cord blood) in the female cohort ($p < 0.0001$, $F = 22.51$) and there was a linear trend showing a decline with age (slope= - 2.4, $p < 0.0001$). Multiple comparison tukey's test showed a significant difference between NPB measured at birth and at three months (95% CI: 1.22 to 6.0), at birth and six months (95% CI: 2.7 to 7.0) and at three and six months (95% CI: 0.24 to 2.3).

The ANOVA Friedman test was significant for NBUD BNC at birth, three and six months ($p =0.01$, $F = 8.6$) and there was a linear trend towards a decrease with age (slope = - 2.4, $p =0.0005$), but the mean frequencies were different at birth and six months only (95% CI: 1.7 to 7.9). The NDI was different at birth, three and six months ($p < 0.0001$, $F = 14.25$) and there was a linear trend towards an increase (slope = 0.09, $p < 0.001$). NDI was different between birth and three months (95% CI: - 0.3 to - 0.1) and between birth and six months (95% CI: - 0.3 to -0.07) but not between three and six months

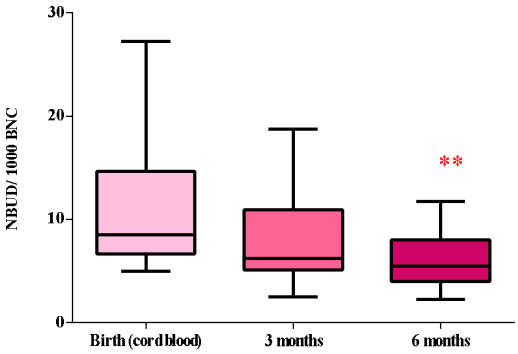
The apoptotic frequency was different among the female cohort at birth, three and six months ($p = 0.02$, $F = 7.1$), however no linear trend could be observed. No significant differences were observed in necrotic cell frequency, MN and NBUD (in MNC) **(Figure 6.11)**.



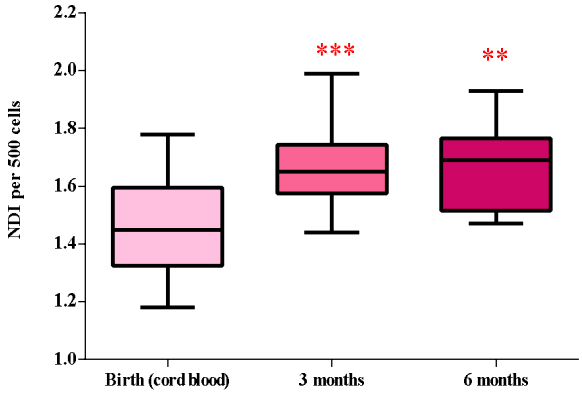
MN BNC: ANOVA Friedman statistic: 6.7, p = 0.03; post-test for linear trend significant at p = 0.07.
Tukey's multiple comparison test significant between birth and six months (p < 0.05)



NPB BNC: ANOVA Friedman statistic = 22.5, p < 0.0001; post-test for linear trend significant at p <0.0001. Tukey's multiple comparison test significant between birth and three months (p < 0.01), birth and six months (p < 0.0001) and three and six months (p < 0.05).

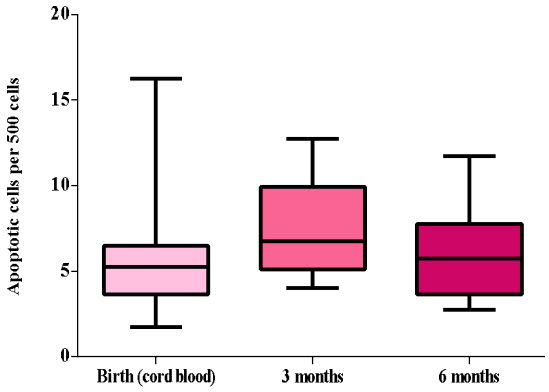


NBUD BNC: ANOVA Friedman statistic = 8.6, p = 0.01; post-test for linear trend significant at p =0.0005. Tukey's multiple comparison test significant between birth and six months (p< 0.01).

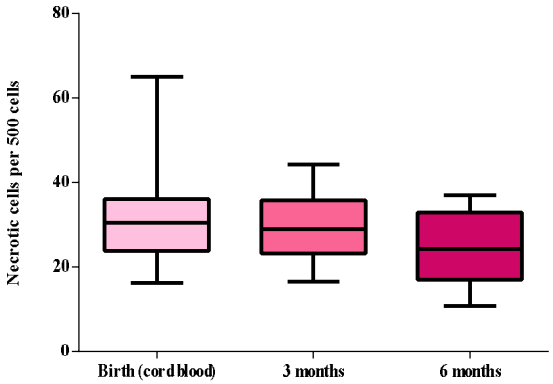


NDI: ANOVA Friedman statistic = 14.25, p <0.0001; post-test for linear trend significant at p <0.0001. Tukey's multiple comparison test significant between birth and three months (p< 0.001); birth and six months (p< 0.01)

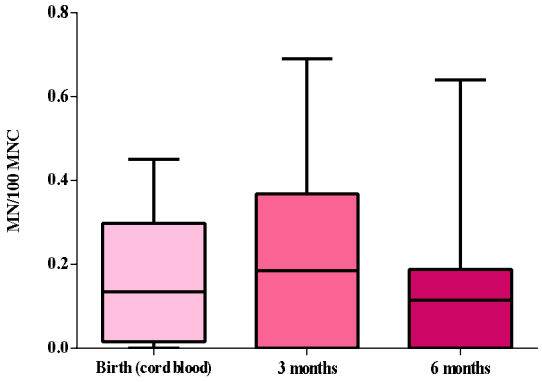
Contd Fig 6.11



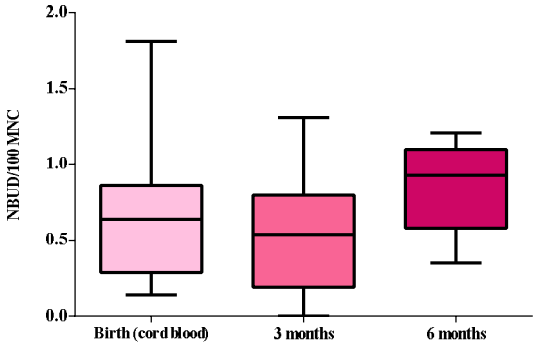
Apoptotic lymphocyte: Friedman statistic= 7.1, p = 0.02; post test for linear trend was not significant (p = 0.4). Tukey's multiple comparison test was non- significant



Necrotic lymphocyte: ANOVA Friedman statistic= 2.4, p =0.2; post test for linear trend was significant (p= 0.057). Tukey's multiple comparison test was non-significant.



MN MNC: ANOVA Friedman statistic= 1.03, p =0.5); post test for linear trend and Tukey's multiple comparison test was non-significant.



NBUD MNC: ANOVA Friedman statistic= 1.7, p =0.4; post test for linear trend and Tukey's multiple comparison test was non-significant.

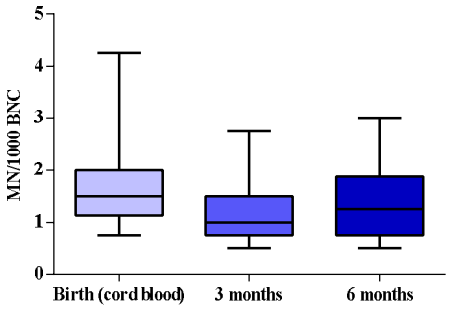
Figure 6.11: Comparison between mean (± SD) of CBMN-Cyt biomarkers for female cohort at birth, 3 and 6 months

[ANOVA used values for female infants whose data was available for all three time points, n= 24 at each time point;

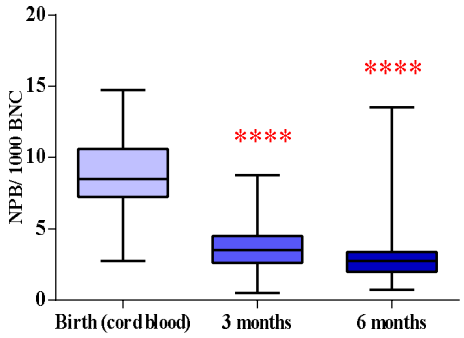
Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN and NBUD are presented per 1000 BNC, MN and NBUD are presented per 100 MNC

6.6.11 **Trend of CBMN-Cyt biomarkers in the male cohort from birth to six months**

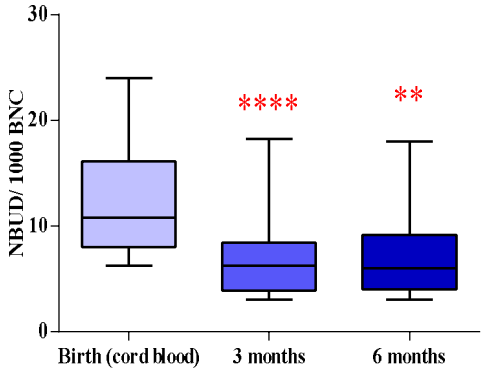
This section looks at changes in CBMN-Cyt biomarkers measured in the male cohort from birth to six months utilizing ANOVA that used number of male infants whose data for CBMN-Cyt biomarkers were available for birth, three as well six months (n = 29). There were no differences in the mean MN frequencies in BNC in the male cohort measured at birth, 3 months and 6 months (**Figure 6.12**). However, there were differences in mean NPB frequency at birth, three and six months ($p < 0.0001$, $F = 31.34$) with a negative linear trend indicating a decline with age (slope= -2.6, $p < 0.0001$). There were differences between NPB frequency in BNC at birth and at three months (95% CI: 3.6 to 6.5) and at birth and six months (95% CI: 3.0 to 7.5) but not between three and six months. Mean NBUD frequency in BNC was different at birth, three and six months ($p < 0.0001$, $F = 19.14$) in the male cohort with a negative linear trend indicating decrease with age (slope = -2.5, $p < 0.0001$). The mean frequency was different between birth and three months ($p < 0.0001$, 95% CI: 2.8 to 8.0) and between birth and six months ($p < 0.01$, 95% CI: 1.6 to 8.5). NDI assessed in the male cohort was different at birth, three and six months ($p < 0.0001$, $F=28.32$) with a linear trend towards an increase (slope 0.14, $p < 0.001$). NDI was different between birth and three months (95% CI: -0.3 to -0.1) and between birth and six months (95% CI: -0.3 to-0.17) with a linear increase (slope=0.14, $p < 0.001$). The apoptotic frequency did not differ among the male cohort at birth, three and six months ($p = 0.06$, $F = 5.5$). However, a linear trend towards an increase (slope = 1.2, $p = 0.02$) was observed that was only significant between birth and three months ($p < 0.01$, 95% CI: -5.5 to -0.7). No differences were observed in the frequencies of necrosis and of MN and NBUD in MNC, in the male cohort at birth, three and six months. A negative trend towards a decrease with age was seen for necrotic cell (slope = -3.1, $p = 0.03$) and for NBUD frequency in MNC (slope = - 0.18, $p = 0.03$) (**Figure 6.12**).



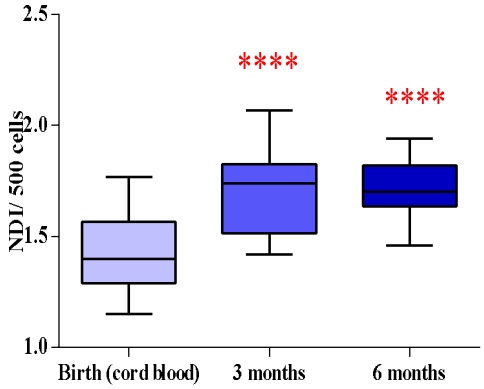
MN BNC: ANOVA Friedman statistic = 4.6, p = 0.09; post-test for linear trend was not significant.
Tukey's multiple comparison test was not significant



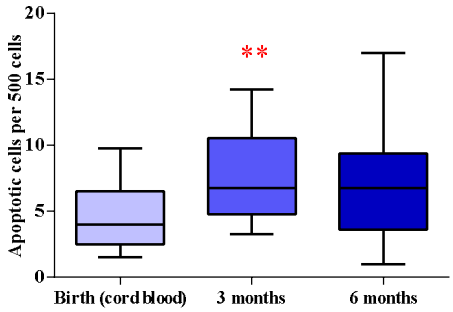
NPB BNC: ANOVA Friedman statistic = 31.3, p < 0.0001; post-test for linear trend significant (p < 0.0001).
Tukey's multiple comparison test significant between birth and three months and birth and six months (p < 0.0001).



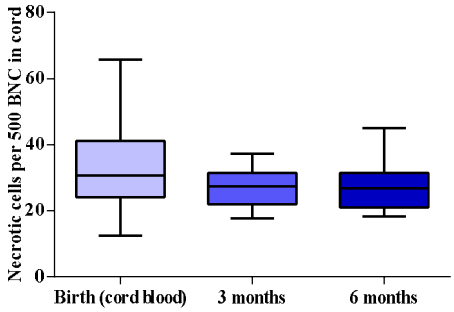
NBUD BNC: ANOVA Friedman statistic = 19.1, p < 0.0001; post-test for linear trend significant (p < 0.0001).
Tukey's multiple comparison test significant between birth and three months (p < 0.0001) and birth and six months (p < 0.01).



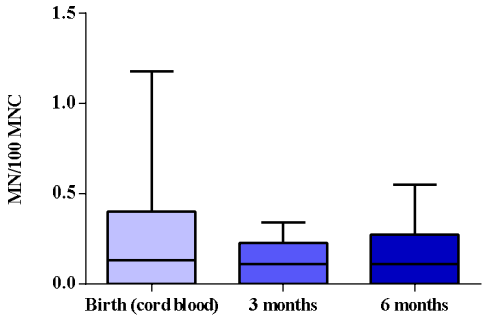
NDI: ANOVA Friedman statistic = 28.32, p < 0.0001; post-test for linear trend significant (p < 0.0001).
Tukey's multiple comparison test significant between birth and three months and birth and six months (p < 0.0001).



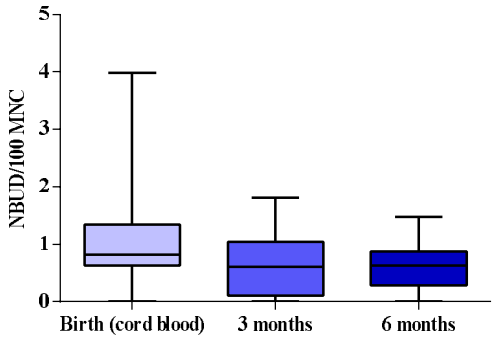
Apoptotic cells ANOVA Friedman statistic= 5.5, p =0.06;
post-test for linear trend significant (p = 0.02).
Tukey's multiple comparison test significant between birth and three months (p=0.01)



Necrotic cells: ANOVA Friedman statistic = 2.6, p =0.2;
post-test for linear trend significant (p = 0.03).
Tukey's multiple comparison test was non-significant.



MN MNC: ANOVA Friedman statistic = 1.2, p =0.5;
post-test for linear trend non-significant.
Tukey's multiple comparison test was non-significant



NBUD MNC: ANOVA Friedman statistic = 2.0, p =0.3;
post-test for linear trend significant (p=0.03).
Tukey's multiple comparison test was non-significant

Figure 6.12: Comparison between means (± SD) of CBMN-Cyt biomarkers for male cohort at birth, 3 and 6 months

[ANOVA used values for male infants whose data was available for all three time points, n= 29 at each time point; *Abbreviations*: MN: micronuclei; BNC: Binucleated lymphocyte cells; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN and NBUD are presented per 1000 BNC, MN and NBUD are presented per 100 MNC

6.6.12 **Gender differences in birth outcomes and CBMN-Cyt biomarkers at birth**

The birth outcomes and CBMN-Cyt biomarkers for the male and female infants in the cohort are presented in **Table 6.19**. The birth weight, length and head circumference of the male infants was greater than that of the female infants ($p < 0.0001$, $p = 0.0003$, $p = 0.001$ respectively). There was significant differences observed in NBUDS in BNC and NBUD MNC among male and female infants ($p = 0.08$ and $p = 0.07$ respectively).

Table 6. 19: Gender differences in the cohort at birth

	Male s (n=40) Mean (± SD)	**Female (n=37) Mean (± SD)**	**p-value**
Gestation (weeks)	39.8 (± 1.2)	39.6 (± 0.91).	0.34
Weight (g)	3656 (± 341)	3245 (± 398)	0.0001****
Length (cm)	51.0 (± 1.6)	50.0 (± 3.8)	0.0003***
Head circumference (cm)	35.9 (± 3.3)	34.3 (± 1.3)	0.001***
MN BNC	2.0 (± 1.1)	2.0 (± 1.2)	0.9
NPB BNC	6.2 (± 3.9)	5.6 (± 3.5)	0.5
NBUD BNC	12.0 (± 5.3)	10.3 (± 5.8)	0.08*
NDI	1.5 (± 0.17)	1.5 (± 0.16)	0.4
Apoptotic lymphocytes	6.7 (± 4.5)	6.3 (± 3.6)	0.8
Necrotic lymphocytes	36.6 (± 13.4)	34.5 (± 11.5)	0.4
MN MNC	0.20 (± 0.25)	0.18 (± 0.15)	0.4
NBUD MNC	1.1 (± 0.88)	0.8 (± 0.9)	0.07*

Each variable was tested for Gaussian distribution and student unpaired t test (parametric test for normal distribution data) and Mann Whitney test (non-parametric test for non-Gaussian distribution) were performed; ****: significant at $p \leq 0.0001$; *** $p \leq 0.001$; ** $p \leq 0.01$;, * $p \leq 0.1$

Abbreviations: MN: micronuclei; BNCs: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNCs: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD are presented per 100 MNC

6.6.13 **Gender differences in the cohort at three and six months after birth**

The infant's weight and height, CBMN-Cyt biomarkers and average feeding scores at three months after birth are presented for the male and female infants in the cohort, in **Table 6.20.** There was significant difference in the weight of male and female infants at three months ($p = 0.03$) with male being heavier by 8%. There was significant differences in MNMNC at three months between male and female cohort ($p = 0.05$). No gender differences were observed for feeding scores across the cohort at this time point**.**

There was no significant difference observed between birth and weight, CBMN-Cyt biomarkers and feeding scores between male and female cohorts at 6 months after birth (**Table 6.21).**

Table 6. 20: Gender differences in the cohort at three months after birth

	Male (n=31) Mean (± SD)	**Female (n=33) Mean (± SD)**	**p-value**
Age (weeks)	12.7 (± 0.97)	12.6 (± 1.04).	0.51
Weight (g)	6490 (± 677)	5968 (± 765)	0.003***
MN BNC)	1.5 (± 1.1)	1.7 (± 1.1)	0.44
NPB BNC	3.1(± 1.9)	3.1 (± 1.5)	0.8
NBUD BNC	7.7 (± 3.5)	8.1 (± 4.2)	0.8
NDI	1.7 (± 0.15)	1.7 (± 0.14)	0.6
Apoptotic lymphocytes	7.2 (± 3.1)	7.2(± 2.7)	0.9
Necrotic lymphocytes	29.2 (± 8.5)	30.0 (± 7.5)	0.6
MN MNC	0.11 (± 0.12)	0.2 (± 0.17)	0.05**
NBUD MNC	0.68 (± 0.48)	0.5 (± 0.37)	0.4
Average feeding score	3.5 (± 0.78)	3.4 (± 0.75)	0.81

Each variable was tested for Gaussian distribution and student unpaired t test (parametric test for normal distribution data) and Mann Whitney test (non-parametric test for non-Gaussian distribution) were performed; ***: significant at $p \leq 0.01$; **$p \leq 0.05$,

Abbreviations: MN: micronuclei; BNCs: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD are presented per 100 MNC

Table 6. 21: Gender differences in the cohort at six months after birth

	Male (n=29) Mean (± SD)	**Female (n=24) Mean (± SD)**	**p-value**
Age (weeks)	23.4 (± 1.14)	23.2 (± 4.36).	0.16
Weight (g)	7820 (± 1696)	7667 (± 838)	0.11
MN BNC	1.8 (± 1.3)	1.6 (± 0.9)	0.77
NPB BNC	3.2 (± 3.1)	2.1 (± 1.2)	0.21
NBUD BNC	7.7 (± 3.5)	6.9 (± 3.5)	0.43
NDI	2.0 (± 1.5)	1.6 (± 0.1)	0.19
Apoptotic lymphocytes	7.6 (± 5.0)	6.4 (± 2.6)	0.65
Necrotic lymphocytes	28.6 (± 10.2)	27.2 (± 8.4)	0.80
MN MNC	0.18 (± 0.16)	0.17 (± 0.19)	0.84
NBUD MNC	0.69 (± 0.5)	0.78 (± 0.43)	0.52
Average feeding score	3.08 (± 1.09)	2.9 (± 1.0)	0.58

Each variable was tested for Gaussian distribution and student unpaired t test (parametric test for normal distribution data) and Mann Whitney test (non-parametric test for non-Gaussian distribution) were performed;

Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNCs: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD are presented per 100 MNC

6.6.14 **Feeding trends**

The feeding scores for the infants at each month after birth were assessed to analyse the trend in feeding pattern for the cohort and are presented in **Figure 6.13**. At three months 68% of the cohort was being exclusively breast fed while only 9% were being exclusively formula fed. The percentage of infants that were exclusively breast fed at six months declined by half (to 34%) while the frequency of formula feeding doubled at the end of six months (to 19.6%) relative to three months. The most common formula milks given were S26 Gold, Nan, Farex and Aptami.

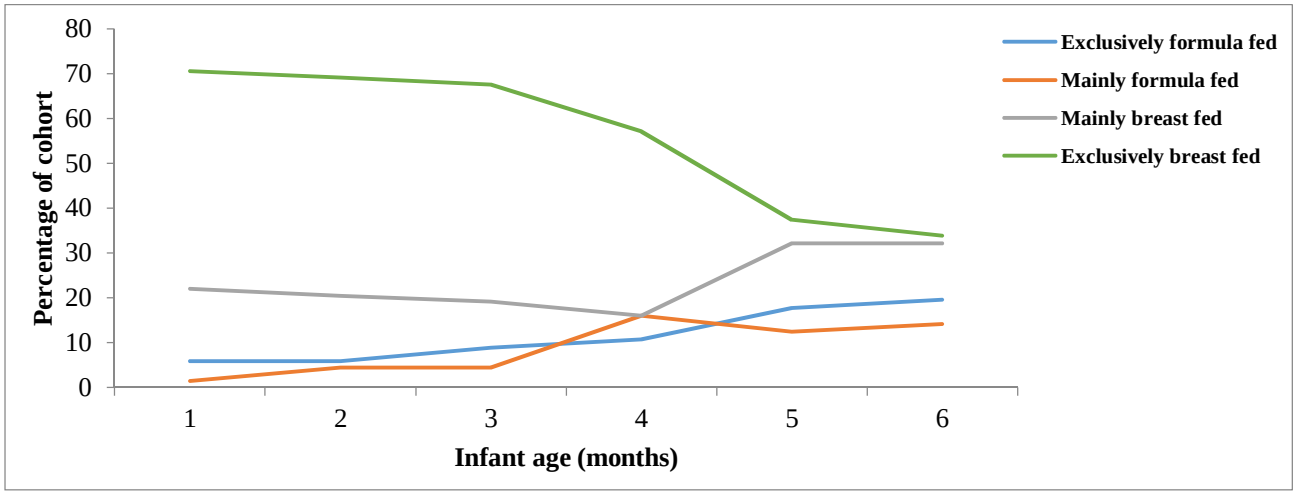


Figure 6.13: Feeding trends of infants in the cohort during six months after birth

The infants' nutrient intake in the cohort comprised of a variety of complementary foods and drinks that either replaced breast milk or were fed along with formula and are presented in **Figure 6.14.**

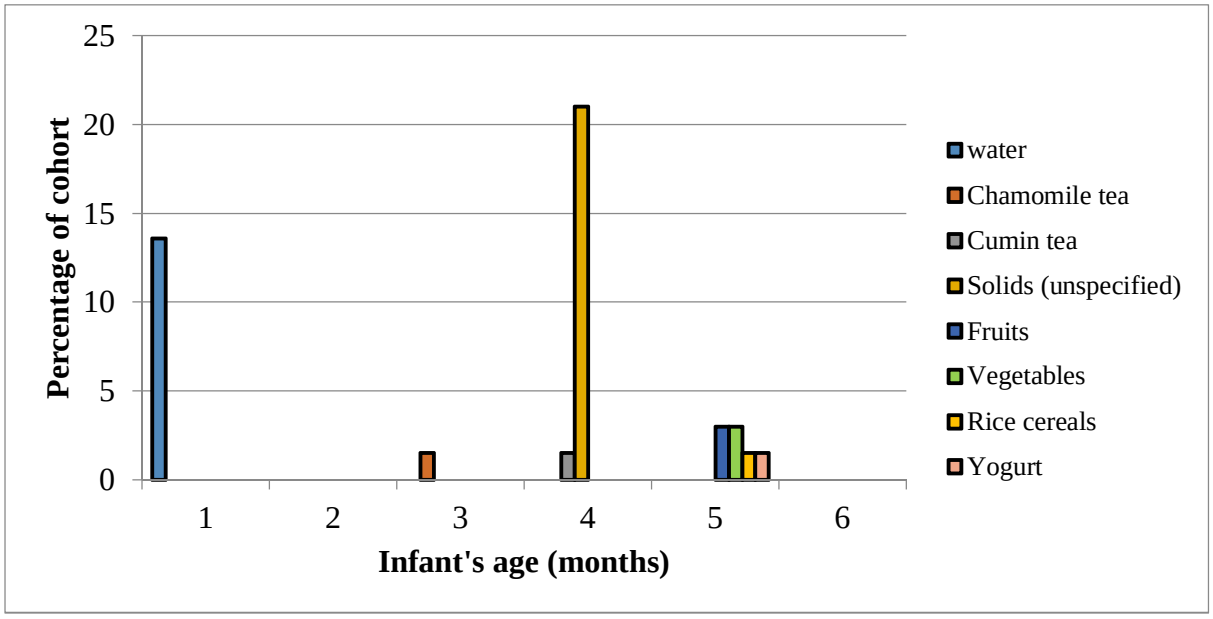


Figure 6.14: Type and time of introduction of complementary feed given to infants in DADHI cohort

6.6.15 **Effect of mode of feeding on genome damage biomarkers at three months**

To test the hypothesis that mode of feeding adopted for infants at three and six months may influence frequency of CBMN-Cyt biomarkers assessed in PBL collected from infants, correlation analysis was performed. We did not observe significant correlation between CBMN biomarkers and feeding scores for either male or female or combined infants in the cohort at three months (**Table 6.22).**

Table 6.22: Correlation analysis of CBMN biomarkers and average feeding scores at 3 months

	Total (n=64)		**Female (n=32)**		**Male (n=31)**	
	'r'	p-value	'r'	p-value	'r'	p-value
MN BNC	-0.01	0.91	- 0.05	0.7	0.11	0.5
NPB BNC	0.07	0.62	0.17	0.3	- 0.28	0.1
NBUD BNC	0.16	0.25	- 0.02	0.8	0.24	0.1
NDI	-0.06	0.67	- 0.21	0.2	0.11	0.5
Apoptotic lymphocytes	0.06	0.65	- 0.22	0.2	0.12	0.4
Necrotic lymphocytes	-0.001	0.99	- 0.16	0.3	0.02	0.8
MN MNC	-0.03	0.84	0.04	0.8	-0.09	0.6
NBUD MNC	-0.15	0.32	-0.20	0.2	-0.17	0.3

Each DNA damage biomarker was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution);
Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocytes are presented per 500 cells, MN and NBUD are presented per 100 MNC

6.6.16 **Effect of mode of feeding on genome instability biomarkers at six months**

At six months, combined cohort was not observed to have any association between average feeding scores and CBMN-Cyt biomarkers. The female cohort was observed to have significant association of NPB BNC with average feeding scores (r = 0.41, p = 0.05, 95% CI: - 0.01 to 0.7). In the male cohort NBUD BNC measured in was negatively correlated with average feeding scores (r = - 0.39, p = 0.03, 95% CI: -0.67 to-0.02 (**Table 6.23**).

Table 6. 2: Correlation analysis of CBMN biomarkers and average feeding scores at 6 months

	Total (n=53)		**Female (n=23)**		**Male (n=29)**	
	'r'	p-value	'r'	p-value	'r'	p-value
MN BNC	-0.13	0.41	-0.03	0.8	-0.25	0.1
NPB BNC	-0.03	0.83	0.41#	0.05*	-0.02	0.8
NBUD BNC	-0.23	0.14	- 0.02	0.9	-0.39# #	0.03*
NDI	0.04	0.80	0.00	0.9	0.08	0.6
Apoptotic lymphocytes	0.09	0.55	0.13	0.5	0.03	0.8
Necrotic lymphocytes	-0.03	0.82	- 0.11	0.5	-0.12	0.5
MN MNC	0.25	0.12	0.21	0.3	0.04	0.8
NBUD MNC	0.05	0.72	0.05	0.8	0.07	0.7

Each DNA damage biomarker was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution);
Significance: *$p \leq 0.05$; # 95%CI:-0.01 to 0.7; # # 95% CI: -0.67 to-0.02
Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNCs, NDI, apoptotic and necrotic lymphocytes are presented per 500 cells, MN and NBUD are presented per 100 MNC

6.7 Discussion

There is increasing evidence that the origin of certain diseases, such as cancer, may be attributed to the accumulation of cellular genetic damage during the human life span (88,113,332,661,679). Previous studies of MN frequency in cord blood (315,330,537,555,680) indicate that the mammalian genome may be susceptible to genotoxic insults during the prenatal period. The rise in incidences of cardiometabolic diseases and inflammatory conditions such as childhood asthma and cancers is a major public health (660,681) concern warranting innovative strategies to understand genetic and epigenetic modulations of DNA by our changing environment (306) and to detect any adverse clinical manifestation at the earliest phase of life. There are as yet no published data on the DNA damage biomarkers in infants born in Australia during the first 6 months of life.

6.7.1 CBMN-Cyt biomarkers in BNCs and MNCs and their association with each other at birth, three and six months in the DADHI cohort

More than one mechanism can explain the origin of MN, including terminal acentric chromosome fragments, acentric chromatid fragments, whole chromosome malsegragation, misrepair of DNA strand breaks, inappropriate base incorporation (e.g. uracil) or base damage (e.g. 8 oxoguanine that leads to transient DNA break (109). The mean (± SD) MN frequency in BNC for our Australian cohort at birth (n=82) was 2.0 (± 1.2) is similar to the mean MN frequency reported in the cord blood of healthy newborns born to Mexican mothers residing in a rural agricultural locality [n=16, 2.0 (± 1.5)] (330) and with results of a Greek cohort in the Newborns and Genotoxic exposure risks (NewGeneris) study [n = 232, 1.79 (± 1.5)] (537). The NewGeneris study was conducted on a large mother-child cohort from five European countries (n = 623), to investigate the relationship between biomarkers of exposure to carcinogenic compounds and MN frequency in cord blood lymphocytes and utilized semiautomated image analysis system (537). In the New Generis study, the highest mean MN frequency 1.79 (± 1.5) was observed in the Greek cohort (n = 232) and the lowest mean MN frequency 0.55 (± 0.74)

was observed in the British cohort (n = 143). Interestingly, a subgroup of the NewGeneris study; the Rhea mother-child cohort in Crete, (Greece), reported a higher cord blood mean MN frequency 4.51 (± 3.29) per 1000 BNC in the cord blood of infants (n = 92) (326) A possible reason for this observation may be that half of the mothers reported having smoked during pregnancy.

Lope *et al* reported a mean MN frequency of 3.94 (3.57 - 4.33 at 95% CI) in cord blood lymphocytes of newborns (n = 110), born to healthy mothers in Spain (328). A meta-analysis of MN frequency based on 13 field studies in children (n = 440) (0-18 years) and a pooled analysis of individual data (n = 332) reported an overall mean of 4.48 and pooled baseline estimate of 3.27 MN per 1000 BNCs for infants (555). These values are higher than the data for our cohort perhaps because their data resulted from pooling for 51 children of varying age groups (0-1 year), residing in different countries, such as China (576), Brazil (556) and France (332). The MN frequency is usually reported to increase in response to exposure to pollutants (315,551,571,574,575,664), disease state (331,334,554,556,682), and deficiency of micronutrients especially folate, B_{12}, vitamin E, and iron (145,242,435). The possible reasons for a difference in the frequency of MN measured in our study and the cohort from European countries may therefore be attributed to diverse environmental factors (119,145,683) that may modulate MN through epigenetic mechanism (306,664-668) and requires further investigations. Also, our current cohort included women at low risk of complications during pregnancy, of which only 4.5% reported smoking during pregnancy that may possibly account for a lower baseline MN frequency. The efficacy of the CBMN-Cyt assay to detect genotoxic effect of smoking in pregnancy was demonstrated in a South Australian study showing that the lymphocyte MN frequency was 42% greater at 18 weeks gestation in pregnant women who were smokers compared to women who were non-smokers (118). We did not find any observable genotoxic effect of mother's folic acid status and alcohol intake during pregnancy (recorded at the time of recruitment) on CBMN-Cyt biomarkers in cord blood. However these

observations were limited by the very small numbers of mothers abstaining from folic acid and the lack of quantitative data on the amount of alcohol consumed.

The MN frequency in mononucleated cells has not been frequently investigated as part of the CBMN-Cyt assay. A MN expressed in a mononuclear cell (MNC) prior to *ex vivo* mitosis provides additional information regarding MN that were already expressed *in vivo* due to DNA damage induced in the precursor cells (325). The mean (± SD) frequency of MN in MNC observed in cord blood in our study was 0.19 (± 0.21) per 100 MNC. The equivalent mean value of MN per 1000 MNC assessed in our study would be 1.9 (± 2.1) which is similar to mean MN frequencies in MNCs observed in cohort from Greece 2.09 (± 1.54) (326) but much higher than those reported in the NewGeneris study in cord blood from the Spanish 0.20 (± 0.45), Norwegian 0.11 (± 0.42) and Danish 0.17 (± 0.58) cohorts (537). The difference in MN frequency could be due to use of semiautomated image analysis system which tends to underestimate MN frequency relative to visual scoring but the reason for the higher MN frequency in MNC in our cohort is not known and would require a careful analysis of diet, lifestyle and environmental exposure factors to deduce and confirm causality.

We observed that MN in BNC and MNC were correlated at birth and three months as well as at three and six months and a similar observation was also reported in the NewGeneris Rhea mother-child cohort in Crete ($r = 0.35$, $p < 0.001$) study (333) suggesting that common factors *in utero* may impact MN frequency expression *in vivo* and *ex vivo* in the lymphocytes of infants.

Nucleoplasmic bridges (NPB) may be accumulated in a cell following misrepair of DNA breaks and the formation of dicentric chromosomes (679). NPB originate during anaphase in mitosis when the centromeres of dicentric chromosomes are pulled towards opposite poles in the cell (109). In the current study a NPB frequency [mean (± SD)] of 5.8 (± 3.7) was observed in infants at birth. The Rhea mother child cohort study reported much lower mean (± SD) values of NPB [0.12 (± 0.36) per 1000 BNC] at birth (326). The bio-monitoring study in Spain reported that in neonatal lymphocytes, 16.4% were observed to have 1 NPB and 1.8% to have 2 NPB

(328). These data indicate a high variability in NPB frequency between cohorts that may be explained not only by differences in exposure factors but also laboratory and scorer variability in the efficiency of scoring NPB which has been observed to be much higher for scoring NPB than scoring MN (562).

NBUD are nuclear projections often observed in aneuploid cells that remain transiently attached to the main nucleus by a strand of DNA of variable size (684). Some experiments have demonstrated that budding is a mechanism inherent in cells to 'bud out' any extra DNA due to hyperdiploidy or unresolved DNA repair complexes, some of which may be extruded as MN (685-687). In mammalian cells, amplified genes or small fragments of extra chromosomal DNA have been shown to localise selectively to specific sites at the periphery of the nucleus and subsequently be eliminated via nuclear budding during the S phase of interphase in the cell cycle (688). NBUD may also be formed from a nucleoplsmic bridge following a break in the bridge (109). NBUD may contain centromeric DNA material, and vary in size with ploidy of the cell, rather than the extent of DNA damage (689). The increase in NBUD in a cell has been associated with increased risk of cancer, Alzheimer's disease and also low folate status (242,690,691). The mean (± SD) frequency of NBUD observed in cord blood in our study was 11.1 (± 5.7) and is much higher than the values reported in a study conducted in Greece 0.27 (± 0.63) (326). A low frequency of NBUD was reported in the Bio Madrid study in which only 7% of the newborns registered one or two buds (328). The strong association of NBUD in BNC and MNC at three and six months suggest a possibilty that the cells observed in our study till 6 months were survivors from birth and were in the process of eliminating extra DNA material accumulated 6 months earlier (684). Recently, a bio-monitoring study, designed to assess the association between prenatal lead exposure and fetal development using three biological samples (maternal and paternal blood lead levels at around 34 weeks of gestation as well as cord blood lead levels) and genome damage biomarkers in cord PBL, reported that maternal and cord blood lead levels were not associated with newborn measurements or DNA damage biomarkers (MN, NPB and NBUD). However, increases in paternal blood lead concentrations

were associated with an increased risk of the presence of NPB (OR, 1.03; 95% CI, 1.00 to 1.06) and NBUD (OR, 1.02; 95% CI, 0.99 to 1.04) in newborn cord blood lymphocytes (550) showing close association between parental environment and infant genome. Also, as the appearance of all DNA damage biomarkers including NBUD has been associated with low micronutrient status, such as folate (109,692), it is important that the relationship between the micronutrient status of the infant and CBMN-Cyt biomarkers is assessed and is discussed in chapter 7. Our study also observed a decline in NBUD frequency three months after birth but a subsequent fall at 6 months was small. It is possible that the lymphocytes measured in cord blood differ from matured lymphocytes collected from infants at 3 and 6 months (693) with respect to T cell subtypes and proportion of each subtypes (694,695) which was not assessed in our manual scoring process. Further, the total T lymphocytes, CD4/CD8 (696) and Th1/Th2 ratio is reported to decrease with age and disease status in infants (697) that could explain the decrease of DNA damage biomarkers at six months relative to birth in the present cohort.

Whether the decline we observed in NPB (35%, 36%) and NBUD (52.6%, 58%) at 3 and 6 months respectively, relative to mean values at birth in our cohort, may be credited to a healthier *ex vivo* environment, requires further investigation. The apparent significant positive correlation of MN, NPB and NBUD between cord blood and three month and between cord blood and 6 month may suggest that long-lived lymphocytes in cord blood with DNA damage are persisting up to 6 months.

The Nuclear Division Index (NDI) in human cells is indicative of the regenerative capacity and immune responsiveness of lymphocyte and has become one of the standard cell proliferation tests for genetic toxicology testing when using the CBMN-Cyt assay (558,687,698). It has also been associated with colorectal (699) and lung cancer risk (401). The mean (± SD) for NDI observed in cord blood in our cohort was 1.5 (± 0.16) and is similar to that reported by Vande-Loock *et al* (n=182, 1.59 ± 0.20) in cord blood collected from a Greek cohort (333). Another mother-infant cohort study, investigating the impact of the intrauterine environment on health

risks in adult life, observed a similar mean NDI (1.57 ± 0.12: n=92) (326), suggesting that differences in DNA damage biomarkers between cohort could not be explained by differences in cell culture conditions or replication stress factors. Our study also found strong inverse association between CBMN-Cyt biomarkers and NDI implying that DNA damage leading to NPB and NBUD formation causes DNA replication stress and cell cycle delay (109,700-705) and that these effects may initiate *in utero* (706).

Apoptosis plays a significant role in the removal of inappropriately responding lymphocyte in T-cell ontogeny and also in the regulation of immune responses (707-709). The T cells in cord blood spontaneously apoptose to a greater degree when compared with adult peripheral blood *ex vivo* (710,711). The majority of neonatal T cells have a naive phenotype (712) that may indicate their functional immaturity with regard to proliferative response to mitogens and antigens (713). It is possible that the frequency of apoptotic lymphocytes of neonates were immature T cells that were using programmed cell death mechanisms to prevent further mutations/MN in daughter nuclei (558) as we found significant correlation between frequency of apoptotic cells and DNA damage biomarkers (MN, NPB, NBUD) at three but not with NDI in our study.

A cell may undergo necrosis, rather than apoptosis, depending on the intracellular oxidant/antioxidant status, the level of adenosine triphosphate (ATP), and the degree of induced membrane damage (536,558,714). We observed a wide range of frequencies of necrotic cells per 500 BNC at birth, 3 and 6 months (range: 10 to 65) with mean (± SD) values of 35.9 (± 12.2), 29.7 (± 7.9) and 27.9 (± 9.3) respectively. However, there was no correlation among the frequencies of necrotic cells measured during the three time points. But we find significant positive correlation of necrotic cells measured in cord blood with NDI, apoptotic lymphocyte and NBUD MNC at birth. To our knowledge, necrotic cells have not been previously reported in cord blood lymphocytes. Oxidative stress owing to smoking (715) and other factors such as deficiency of membrane antioxidants and ATP production (714) and folic acid deficiency have been previously reported to increase necrosis *ex vivo* in lymphocytes collected from adults

(690). Our observations hence require further investigation in a larger cohort to understand whether higher DNA damage via formation of MN, NPB and NBUD leads to necrosis of a cell or whether necrosis is a cell mechanism to get rid of cellular mutations to promote cytostasis and cell proliferation.

6.7.2 Association of infant birth outcomes with mother's demographic variables and CBMN-Cyt biomarkers

We found gestation age of infants to be correlated positively with MN and NPB at birth ($r = 0.38$, $p = 0.006$ and $r = 0.30$, $p = 0.03$). The previous observations have been contradictory with this regard; where studies did not find any effect of gestation age on MN BNC in cord blood in a Greek cohort (326) and a negative association in a subgroup og Rhea cohort with MN MNC (533). We also found a negative association of gestation age with NDI. The positive association of MN, NPB and NBUD with gestation age are not easy to interpret with respect to biological significance or mechanism. However, a positive association of MN, NPB and NBUD with infant birth weight, correlation of NPB and NBUD with birth length and negative association of birth head circumference with apoptotic cells suggest that a larger infant size may be consequential to more DNA damage possibly due to relaxation of cell cycle checkpoints to allow cell division and tissue growth. Higher DNA damage measured by CBMN-Cyt assay has been observed in over-weight adults (n =21, 40.52 ± 10.69 years) compared to normal-weight subjects (n =21, mean age ± SD, 34.81 ± 11.56 years) (716). We also observed that NPB measured in infants at birth increased significantly with mother's weight and BMI suggesting the possibility of an effect related to metabolic processes that promote a higher BMI (344,352,375,717-724). In this regard, we checked the association of maternal anthropometry data with infant birth outcome but found significant correlation of mother's height with infant birth weight only. Though our cohort was of appropriate birth weight for gestation age as per WHO classification (345) (**Appendix 5**), but positive correlations of gestation age with infant's

weight and length at birth further supports effect of neonatal anthropometrics on adult metabolic programming (719,720,725-730).

This finding is supported by recent findings of a prospective Boston-Birth cohort study where childhood z scores for BMI was observed to be positively associated with maternal pre-pregnancy body mass index. The risk of childhood overweight or obesity (measured at 6 years of age) was significantly increased in overweight (RR=1.3[95% CI: 1.2, 1.6]) and obese (RR=1.6 [95% CI: 1.3, 1.8]) mothers' children compared to the risk of childhood overweight and obesity in children of normal-weight mothers (based on maternal pre-pregnancy body mass index). Additionally, the risk of childhood overweight increased significantly by 30% with each unit increase in maternal pre pregnancy BMI (RR=1.3[95% CI: 1.1, 1.4] (312). And in the NewGeneris cohort, maternal serum vitamin D (<50 nmol/L recorded at 14-18 weeks of gestation) was associated with increased MN BNC frequency in cord blood [incidence rate ration (IRR= 1.32 (95%CI: 1.00, 1.72)]. This increase was higher for newborns with birth weight above the third quartile [≥ 3.5 kg; IRR = 2.21 (1.26, 3.89)] (310) indicating epigenetic influence of maternal factors on infants' metabolic profile.

We also observed that mother's BMI was negatively associated with APGAR scores assessed at 5 minutes after birth (r = - 0.25, p = 0.07). APGAR score is a routine measure of comprehensive health at birth with respect to breathing effort, heart rate, muscle tone, reflexes and skin colour (731). The score is usually assessed twice at 1 and 5 minutes to determine the neonate's tolerance to the birthing process and as an adaptation to the extra-uterine environment (339). Low APGAR score at 5 minutes has been associated with increased infant mortality (339), however the tool is not clinically proven to provide any predictive association with an infant's neurological or cognitive development (340). The positive association of APGAR score with NDI and negative correlation with NPB suggests a beneficial impact of improved cell division and lower chromosomal instability in immune system cells during very early stages of life after birth. Though we did not find any significant association between type of delivery

(induced/spontaneous) and genome damage biomarkers measured at birth, it is possible that the transition to extra-uterine life and/or neonates' exposure to the birthing process and mother's anthropometry may contribute to genomic stress through hypoxia or inflammation (678,722). But again, biological relevance and mechanism of association are difficult to explain unless a higher NDI and lower NPB happen to be indicators of stress resistance given the high metabolic stress of birthing process. Hence, the novel findings of an association of APGAR score with CBMN biomarkers needs further investigation in a larger cohort along with adjustments for intra and/or extra-uterine factors.

6.7.3 **Gender differences in relation to CBMN-Cyt biomarkers**

When compared with WHO weight charts, our male cohort were at $50^{th,}$ and above 97^{th} percentile for weight for age at birth and at 3 and 6 months respectively (**Appendix 7**). Similarly, female infants were at 50^{th} and above 97^{th} percentile at birth and 3 and 6 months respectively (**Appendix 8**). We observed that the changes in CBMN biomarkers in male and female cohort were similar from birth to six months. There was a decline in frequency of NPB and NBUD from birth to six months in both the male and female groups. The decrease in the MN frequency was observed only in the female cohort. Both the groups had an increase of NDI and apoptotic lymphocyte frequency from birth to six months indicating good proliferation capacity of infant's lymphocytes. At birth, the male infants were heavier and longer and had a larger head circumference. They also had significantly higher frequency of NBUD measured in BNC and MNC at birth compared to female subgroup. At three months, the male subgroup was heavier than the female cohort. The MN MNC were observed to be different among the two groups but there were no gender differences in the frequency of other DNA damage biomarkers or in the measures of cytotoxicity (apoptotic and necrotic lymphocytes). To our knowledge, gender differences for CBMN biomarkers have not been reported in a cohort of infants at less than 1 year of age. Previous findings have not reported any difference between frequency of MN among male and female infants (326,555). However, the studies conducted to assess DNA

damage in both younger (7-39 years) and older (40-80 years) individuals (732) provides evidence of the presence of at least one sex chromatin positive MN (733) and indicate that the X chromosome is preferentially lost in older adult women (aged >39 years) (610,734) as it tends to lag behind in female lymphocyte anaphase (675). Hando *et al* found an X chromosome to be present in 72.2% of the MN scored from lymphocytes collected from cord blood of 8 female newborns and 38 adult females (735) suggesting that X chromosome may be micronucleated more efficiently than autosomes (19-77 years), hence, the frequency of MN in PBLs collected from females have been observed to be 19% higher than in males (119,672,735-737). As one of the origins of micronuclei is known to be 'budding' (686), it is plausible that higher NBUD observed in our male cohort could be potential MN. Further, co-observations of male being heavier, longer and with more head circumference suggests effect of metabolic stress on DNA health (716,719,720) that requires investigations in a larger cohort.

6.7.4 Correlation of mode of feeding and CBMN-Cyt biomarkers measured in infants at three and six months

We next tested the hypothesis that the frequency of DNA damage biomarkers seen over time in the present cohort of infants was associated with mode of feeding adopted for the infants.

First of all, our study, in a South Australian cohort of infants, found that the frequency of exclusive breast feeding declined by 50% during the first six months of life. 68% and 34% of babies were being exclusively breast fed at 3 and 6 months respectively. This finding is similar to that in a previous longitudinal study of Australian infants (**Figure 6.1**) (406). The decline in exclusive feeding in an Australian cohort, and the introduction of 'other feed' methods are contrary to the recommendations of the World Health Organization, which promote exclusive breast feeding for the first six months of infant life because of the immune-supportive properties of breast feeding (379,738).

Next, the findings of our study are contrary to the current evidence in literature that breast fed infants have lower DNA damage, as measured by urinary excretion of 8OHdG (397) and the

Comet assay (398), as none of the DNA damage biomarkers measured in our study, utilizing a more comprehensive CBMN-Cyt assay, was observed to be associated with mode of feeding received by the infants in our cohort. The protective effect of human milk against the development of malignancy, either during childhood or later in life, has been emphasized in a number of retrospective studies (739-741). With respect to DNA health of infants, a cohort study conducted by Shoji *et al* compared the degree of DNA damage in breast-fed (n=15) versus formula-fed (n=14) very low birth weight infants at 2, 7, 14, and 28 days of age by measuring urinary 8-OHdG. The study, although performed on a small number of infants, reported that formula-fed babies had higher urine 8-OHdG concentrations than the breast fed infants ($p < 0.01$).

Another study investigated oxidative stress levels in healthy one month old infants (n=41) according to the type of feeding. These infants were divided into four groups according to type of feeding: the breast-fed group (n=10), who received >90% of their intake as breast milk; the breast milk dominant mixed-fed group (n=10), who received 50% to 90% of their intake as breast milk; the artificial milk dominant mixed-fed group (n=11), who received >50% to 90% of their intake as formula; and the formula-fed group (n=10), who received >90% of their intake as formula. The study reported significantly lower urinary excretion of 8-OHdG in the breast-fed group compared with that seen in the artificial milk dominant mixed-fed group ($P<0.05$) or the bottle-fed group ($p <0.01$) (397). However this data needs to be interpreted with some caution because an increase in urinary 8-OHdG may not reflect induced DNA damage but may rather be due to more efficient excision of 8-OHdG by DNA repair processes (536).

In another study group of infants aged 9-12 months, who were either being formula fed or fed with cow's milk (n=35 in each group), DNA damage was assessed in the peripheral blood lymphocytes by the Comet assay. An increase was reported in those infants fed with cow's milk of both limited DNA-damaged ($p < 0.001$) and extensively DNA-damaged ($p < 0.001$) cells (398). In our study, none of the infants were fed cow's milk so a comparison could not be made for the effect of feeding cow's or mother's breast milk on CBMN-Cyt biomarkers.

The lack of an effect of feeding choice for the infant on the CBMN-Cyt biomarkers could be due to good nourishment from the alternative feeding used for majority of infants in our cohort so that differences in micronutrients that increases MN, NPB, NBUD (e.g. folate and Zn deficiency) was avoided (431,470,499,686,742). Alternative explanation might be that malnourishment during the first six months of life might induce DNA lesions that are better detected by Comet assay or OHdG assay. However, our studies and those of others show that CBMN-Cyt assay responds to similar extend as comet and OHdG assays to oxidative stress and DNA strand breaks (401,558,743-745). The lower MN frequency in our cohort relative to that reported in a meta analysis (555) indicates that adequate nourishment is the more plausible explanation.

We did not observe any gender difference in the effect of mode of feeding on the modulation of DNA damage biomarkers at 3 months, though, at three months of age, the male infants, who were heavier than their female counterparts, were observed to be marginally more breast fed. However, at six months the female cohort was observed to have a significant association of NPB BNC with average feeding scores ($r = 0.41$, $p = 0.05$, 95% CI: - 0.01 to 0.7). In the male cohort NBUD BNC measured in was negatively correlated with average feeding scores ($r = -0.39$, $p = 0.03$, 95% CI: -0.67 to -0.02). The confidence intervals were wide that indicates the results were observed in a small sample.

There is accumulating evidence suggesting that nutrition during pregnancy and early postnatal life is one of the most important environmental cues that programs microbiological, metabolic, and immunologic development (746,747). Duration of breastfeeding has been associated with lower BMI and possible prevention from chronic lifestyle related diseases in adult life (398,748). A possible protective effect of breast feeding on DNA damage among neonates has also been reported (749). Human milk is known to contain enzymatic and non-enzymatic antioxidants, including superoxide dismutase, glutathione peroxidase, catalase, vitamins E and A, and β-carotene (738,750-752). The mechanism through which breast feeding provide protective effects on infant's health is now been understood through direct effect on the gut

microbiota that generates butyrate as a metabolic by product which is then utilized by gut epithelium to maintain its integrity and thereby protecting/strengthening gut lymphoid tissue (378,379,753-759). A possible explanation for findings of our pilot study, which has shown no observable significant correlation between frequency of breast feeding and CBMN-Cyt biomarkers in s small size cohort, may be that the majority of infants were exclusively breast fed and that the alternative feeding strategies were adequate to meet nutritional requirement for genome maintenance.

6.8 **Limitations**

One of the limitations of the study was that our samples were drawn from a small cohort that may not adequately represent the entire population. Furthermore, it is to be noted that, out of 794 eligible women, only 115 consented which indicates a difficulty in recruitment which may possibly lead to bias in relation to the study outcomes. In addition, a further limitation is that only mothers with low risk of pregnancy complications were recruited so that the data may not represent those with higher risk of DNA damage given that a high MN frequency at 18 weeks gestation was predictive of risk for pre-eclampsia or intrauterine growth restriction (118). Further, different subtype of lymphocytes were not assessed (693). And we used visual scoring process in contrast to semiautomated image analysis in other studies.

Also, the cohort may have been too well nourished to distinguish genome affects between exclusively breast feeding and alternative feeding. Further, the feeding data was self reported and might not be robust. Moreover, we did not collect the information on the amount and content of the breast milk. With evidence of possible genotoxic effects of breast milk (760), further research is required to understand effect of mother's breast milk on infants' genome. Furthermore, we donot report actual nutritional status in blood for all micronutrients relevant for genome maintenance which is necessary to test whether feeding choice produced substantial differences in the micronutrient status of infants.

6.9 **Conclusion**

In conclusion the current study provides a comprehensive measure of genome damage and cytotoxicity biomarkers in cord blood and infant blood at 3 and 6 months in a South Australian cohort measured by CBMN-Cyt assay. These data may provide a useful baseline reference to assist in the design of further studies aimed at monitoring changes in the human life cycle, caused by exposure to environmental genotoxin, poor lifestyle and malnutrition. Additionally, the study also shows significant associations of infant birth outcomes with DNA damage biomarkers suggesting the possibility of an effect of metabolic process that promotes higher BMI on DNA health of infants. Furthermore, the reduction in DNA damage at 3 and 6 months relative to cord blood suggests the possibility of a beneficial effect on genome integrity by feeding methods used in this cohort or alternatively indicates a genotoxic stress *in utero* as a consequence of the birth process that may have elevated DNA damage in cord blood. The non-association observed with the feeding score may be the result of the good feeding regimens followed by the mothers in the study, of whom 68% and 34% were exclusively breast feeding their babies at 3 and 6 months respectively.

7 The association of blood micronutrients status of South Australian infants with birth outcomes, feeding methods and genome damage during first six months after birth

7.1 **Abstract**

An optimal balance of dietary micronutrients is essential for the maintenance of human genome integrity. Dietary deficiency of specific micronutrients, such as folate, vitamin B_{12}, zinc, iron, copper and manganese at any stage of development may result in DNA damage and epigenetic changes. The present study was designed to test if plasma micronutrient concentrations vary significantly during first six months after birth and determine their correlation with maternal demographic data (weight, height, body mass index), infant's birth outcomes (gestational age, weight, length, head circumference and APGAR scores) and DNA damage biomarkers, as measured by the Cytokinesis Block Micronucleus-Cytome (CBMN-Cyt) assay in peripheral blood lymphocytes (PBL). PBL were isolated from a cohort of healthy Australian infants at birth (cord blood) (n= 82), at 3 months (n=64) and 6 months (n=53) after birth. DNA damage biomarkers, including micronuclei (MN), nucleoplasmic bridges (NPB) and nuclear buds (NBUD) were measured per 1000 binucleated lymphocyte cells (BNC). Apoptotic and necrotic cells were scored per 500 cells. Nuclear division index (NDI) was measured using the frequency of mono-, bi- and multinucleated lymphocyte cells. MN and NBUD were also scored in 500 undivided mononucleated lymphocyte cells (MNC) to assess genome damage that was already expressed in vivo. The secondary aim was to test whether the extent of breast feeding or complementary feeding influence plasma micronutrient concentration and DNA damage in infants.

A significant decrease in the concentration of plasma iron, potassium and red cell folate and an increase in copper, magnesium, sodium and sulphur was evident in infant plasma from 0 to 6 months after birth.

Sulphur and calcium concentrations were positively correlated with feeding scores at six months ($r = 0.2$, $p = 0.05$, $r = 0.2$, $p = 0.03$ respectively) suggesting that the mode of feeding

(mother's milk or complementary feeds) could affect plasma micronutrient concentrations to a small extent.

Plasma copper, the ratio of plasma Ca to Mg, and vitamin B_{12} concentrations were observed to be positively associated with gestational age (r = 0.4, p = 0.0007, r = 0.28, p = 0.04, r = 0.3, p = 0.01 respectively), while plasma potassium was negatively associated with gestational age (r = - 0.28, p = 0.04). Plasma calcium was negatively associated with head circumference at birth (r = -0.3, p = 0.01) and sulphur was inversely associated with APGAR score at 1 minute after birth (r = -0.3, p = 0.04). At three months, infant weight was negatively associated with plasma calcium, sodium and phosphorus concentrations (r= - 0.37, p = 0.003; r = - 0.4, p = 0.001; r = - 0.2, p = 0.02 respectively).

At birth cord plasma iron was negatively correlated with NBUD MNC (r= - 0.28, p = 0.01). Magnesium was positively correlated with MN MNC (r = 0.23, p = 0.03). Ratio of calcium to magnesium was positively correlated with MN BNC (r = 0.28, p = 0.01). Red cell folate was positively correlated with necrotic lymphocytes (r = 0.22, p = 0.05).

At three months infant plasma iron was negatively associated with apoptotic cells (r = - 0.32, p = 0.01). While zinc was negatively correlated with NBUDMNC, (r = - 0.27, p = 0.05), ratio of Ca: Mg correlated positively with NBUD MNC (r = 0.3, p = 0.03). Zinc was also positively associated with NPB BNC (r = 0.29, p = 0.03) and apoptotic lymphocyte (r = 0.26, p = 0.05). Phosphorous was negatively correlated with NDI (r = - 0.3, p = 0.02) and red cell folate was positively associated with necrotic lymphocyte (r= 0.3, p = 0.01).

At six months, plasma copper was observed to be positively correlated with MN MNC (r = 0.34, p = 0.02), calcium was positively associated with necrotic lymphocyte (r = 0.3, p = 0.04), and magnesium was negatively associated with NBUD BNC (r = - 0.28, p = 0.05). The ratio of calcium and magnesium was associated positively with NPB BNC (r = 0.31, p = 0.03) and NBUD BNC (r = 0.32, p = 0.02). While red cell folate was positively associated with NDI (r =

0.44, p = 0.006), plasma magnesium, sodium, potassium, were negatively correlated with NDI (r = - 0.33, p = 0.02, r = - 0.28, p = 0.05, and r = - 0.32, p = 0.02 respectively).

It is evident from the result of the study the plasma micronutrient status varies significantly during first six months of life and is significantly associated with birth outcomes and DNA damage in lymphocytes. The micronutrients that showed significant variation with age and/or birth outcomes were iron, potassium, folate, copper, calcium, magnesium, sodium and sulphur. The results thus support the hypothesis that micronutrient deficiencies or excess may affect birth outcomes and genome integrity of infants during the first six months after birth.

7.2 **Introduction**

An optimal balance of dietary micronutrients is essential for maintenance of human cellular genome integrity (407). Dietary micronutrients such as folate, vitamins B_{12}, B_6 and B_2 (254,408,409), magnesium (410), carotenoids (411,412), zinc (413-415), niacin (416), manganese (417,418), iron (419), selenium (420,421), copper (422), vitamin C, vitamin E (423-427) and vitamin D (428) are variably required as substrates or enzymatic cofactors in metabolic reactions (416,424,429-433) The roles of some of the micronutrients in human biological functions, including DNA replication and repair, were summarized in Table 2.1 in the introductory chapter**.** As these micronutrients are required in DNA synthesis and repair, for prevention of oxidative damage to DNA as well as methylation of DNA (513,761-764), hence, dietary deficiency of micronutrients at any stage of human development may induce DNA damage and epigenetic changes (98,511) and accelerated telomere shortening or dysfunction (99,409,512). Cells are sensitive to both endogenous and exogenous insults during early phases of life. This is particularly evident *in utero* and during the early stages of infancy when cells are replicating DNA and dividing more frequently making them more sensitive to the damaging effects of micronutrient deficiency (513). The pregnant woman's body undergoes preparation for labour, parturition and lactation at the same time while providing nutrients for foetal growth (514). During pregnancy an elevation in inflammatory cytokines is required at foeto-placental interface for successful implantation and completion of pregnancy (515,516). This demands maximal output from endogenous antioxidant systems (glutathione peroxidase and superoxide dismutase) (517). The deficiency of trace minerals required for efficient free radical quenching (mainly selenium, copper, zinc, iron, magnesium) along with cofactors necessary for strengthening immune and energy pathways (vitamin B_3, B_2, B_6, magnesium, copper, zinc, iron) may increase oxidative stress (517). Further, imbalances in folate/methionine pathway owing to either genetic polymorphism (MTHFR) or deficiency of folate, B_2, B_6, folate and B_{12} may elevate homocysteine (Hcy): a marker of oxidative stress (192,217,254,255,494,518-524).

These imbalances are also associated with increased DNA damage (525,526). Micronutrient status of some of these dietary components has been studied for association with DNA damage utilizing Cytokineses block micronucleus cytome assay (CBMN-Cyt). The CBMN-Cyt assay of peripheral blood lymphocytes is one of the most comprehensive and best validated methods to measure chromosomal DNA damage, cytostasis and cytoxicity (108). In this assay, genome damage is measured by scoring: micronuclei (MN): biomarker of both chromosome breakage and/or loss; nucleoplasmic bridges (NPB): a biomarker of DNA mis-repair and/or telomere end-fusions and nuclear buds (NBUD): a biomarker of gene amplification and /or the removal of unresolved DNA repair complexes (109,110).

Folate deficiency causes increased appearance of MN in human lymphocytes (145,499). There is also evidence to suggest that folate deficiency increases risk of inflammatory condition during pregnancy such as pre-eclampsia (PE) (71,72,206-209,212,217,218,246,527,528). MN has also been observed in women at 20 week gestation to predict subsequent development of PE and/or IUGR (118). Further, folate supplementation along with other B vitamins (B_2, B_6 and B_{12}) during pre and peri conception stages may potentially provide protective effects from complications arising from PE among women and their infants (71,523).

There are few studies that have investigated plasma concentrations of trace minerals and its association with DNA damage biomarkers in infants and young children. Micronutrient status of iron in young subjects (434-436); calcium in children (529); zinc (413,470,530) in *in vitro* human cells; nicotinic acid, vitamin E, retinol, beta-carotene, pantothenic acid, biotin and riboflavin in adults have also been observed to influence CBMN-Cyt biomarkers (145). A cohort study comprising of young children (n=30, mean age 11.5 yrs) of poor economic status in Brazil, found a negative association between the presence of both MN and NPB with red cell iron status ($r = -0.9$, $p = 0.002$; $r = 0.9$, $p = 0.01$ respectively) (434). A cross sectional study in South Australia comprising of healthy children (3, 6 and 9 years, n=462) reported positive associations of plasma calcium with both MN ($p = 0.01$) and necrosis ($p = 0.05$) and no

association between vitamin B_{12} with DNA damage biomarkers (529). A biochemical and cytogenetic epidemiological study found negative association of B_{12} with MN index in young subjects (aged 20-40 years, n =29, r = 0.20, p = 0.29) (171,531). There has been no study investigating other important minerals such as magnesium, zinc, sodium, potassium, phosphorous copper and sulphur and their correlation with CBMN-Cyt biomarkers among infants born in Australia.

Thus in order to understand DNA damage in infants born to mothers with normal pregnancy or with complications, it is important the plasma mineral status is assessed in cord blood and in infant blood after birth. These micronutrient concentrations may be altered during infancy as a consequence of the increasing requirements of a growing foetus/infant and changes in the infant's physiology (532,765). Also, infants are born with an immature acquired immunity that can be influenced by nutrition (738). Exclusive human milk feeding for the first 6 months of life and up to 2 years of life or longer is recognized as a normal regime for infant feeding (766,767). Milk-borne cytokines may protect against infection and reduce inflammatory responses. Breastfeeding induces a gut microbiota rich in *bifidobacteria*, which contribute to strengthening of immune response and reduce gut inflammation (564,768). Furthermore, it has been shown that deficiency of micronutrients, such as iron and folate, may enhance human inflammatory responses (769-771). Pro-inflammatory cytokines may cause DNA damage, and subsequently persistent chronic inflammation-related DNA damage responses which may have an important role in carcinogenesis (772). Various bio monitoring studies, conducted in different geographical locations, have reported the frequencies of CBMN-Cyt biomarkers of DNA damage including MN, NPB and NBUD as measured in lymphocytes collected from cord blood of healthy infants (330,333,555,668).

Additionally, infants born to mothers with diabetes, or those exposed to environmental pollutants, have been shown to have higher frequencies of such CBMN-Cyt biomarkers (306,315,326,331,332,334,551,552,556,571,575,664).

However, it is not known what concentrations of micronutrients may be required to prevent DNA damage in infants. Further, it is also unclear whether supply of these nutrients, either through breast feeding or in complementary feeds, influences plasma concentrations of micronutrients and/or DNA damage in infants. Because micronutrient deficiencies and increases in DNA damage may influence cell growth and development, the present study investigated correlation of plasma micronutrient status with DNA damage as measured by CBMN-Cyt assay in lymphocytes, infant birth outcomes, mother's demographic profile and mode of feeding of Australian infants at birth, and at three and six months of age. The micronutrients that were investigated were: iron, copper, zinc, calcium, magnesium, sodium, potassium, phosphorous, sulphur, vitamin B_{12} and folate.

7.3 Hypotheses

- Blood micronutrients in cord blood are correlated with birth outcomes of infants
- Blood micronutrient concentrations change over the period from birth to six months in infants
- Blood micronutrients are associated with infants' gender, weight and feeding score at three and six months.
- Blood micronutrients are correlated with CBMN-Cyt biomarkers measured in lymphocytes collected from infants at birth, and at three and six months.

7.4 Aims

- To determine which blood micronutrients are associated with birth outcomes of infants
- To determine whether micronutrients measured in infants change during the first six months after birth
- To determine whether infants' gender, weight and feeding score influence the concentration of blood micronutrients at three and six months
- To assess the correlations between blood micronutrients and CBMN-Cyt biomarkers at birth (cord blood), and at three and six months after birth.

7.5 Methods

The prospective cohort study was designed to include South Australian infants born to mothers with a low risk of complications during pregnancy.

7.5.1 Recruitment of participants

A prospective cohort study 'Diet and DNA damage in Infants (DADHI) was conducted on healthy pregnant women and on their neonatal offspring. Pregnant women, attending the antenatal clinic at the Women's and Children Hospital (WCH), Adelaide and identified as being at low risk of pregnancy complications, were approached to participate in the study. Pre-determined inclusion criteria included a second viable pregnancy (naturally conceived) and

having no more than two previous first trimester losses. Women with multiple and/or IVF pregnancy, or with any disease or complication (including hypertension, Type I and II diabetes mellitus, epilepsy, asthma, anaemia, inflammatory bowel syndrome, renal, liver or thyroid problems) or with a body mass index (BMI) $\geq$ 35 kg/m^2 were excluded from the study. All eligible women were informed about the study aims and requirements using a detailed information sheet, before being asked to give informed and signed consent at between 8 and 16 weeks gestation. Infants born premature were excluded from the study. The study was approved by the Human Experimentation Ethics Committee of the Commonwealth Scientific and Industrial Research Organization (CSIRO) and the Human Research Ethics committee of the WCH, Adelaide. Blood samples were collected at birth (cord blood), at 3 months and 6 months after birth (heel prick) from the baby The consort diagram for detailed information on recruitment of participants and their completion of the protocol is presented in **Figure 7.1**.

1671 women were approached.
679 declined
877 were ineligible
115 women consented to participate

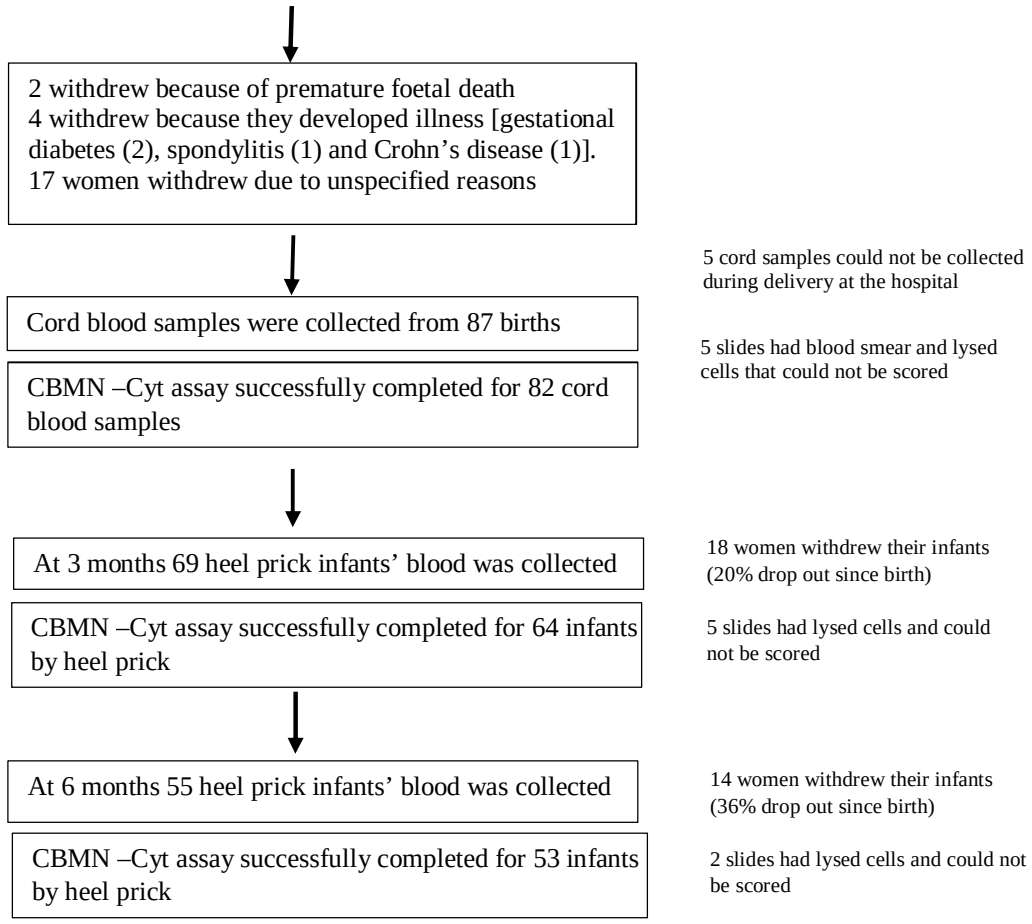


Figure7.1: Consort diagram for DADHI study recruitment, blood collection and CBMN-Cyt assay completion
(CBMN-Cyt: Cytokinesis block micronucleus Cytome assay)

7.5.2 **General health and Food frequency questionnaire**

A general health questionnaire was administered to participating women at between 8 and 16 weeks gestation to collect detailed information about the mother's demographics, medical and family history, lifestyle habits such as smoking, dose and duration of folic acid supplementation and other supplements and any medicines consumed during the pregnancy period. Mother's weight at recruitment was recorded using a digital balance accurate to within 100 g, and height was determined using a stadiometer accurate to within 1 cm of overall height. BMI was then calculated using the formula weight (kg)/ height (m) ^{2}. Type of labour and delivery (Caesarean/induced, normal/spontaneous) and any complications during labour was also recorded. A Food Frequency questionnaire (FFQ) (The Cancer Council, Victoria) was administered at 3 and 6 months postpartum to collect information about the mother's intake of macro and micro-nutrients (534)**.** Details regarding infant's birth weight, height, head circumference, APGAR score at 1 and 5 minutes post birth, gender and gestation age were also recorded.

7.5.3 **Infant's feeding record**

During the first six months after birth, infants may vary significantly in their feeding history in terms of (i) the period that they were exclusively breast fed, (ii) the total cumulative duration of breastfeeding and (iii) the substitute or "complementary" foods used when the baby was not exclusively breast fed (406). The information regarding mode of feeding for the infants in the cohort was collected during months 1-3 and 4-6 (**Appendix 1**). Based on the data collected each infant was given a score of 1 to 4 (**Table 7.1**). The scores were then averaged for the first 3 months and for the period between 3- 6 months (Appendix 1a).

Table7. 1: Infant mode of feeding record

Mode of feeding	**Score**
Exclusive breast fed	4
Partially breast fed	3
Exclusive formula fed or other milk (soy or cow)	2
Partially formula fed or other milk	1

7.5.4 **Blood collection**

Approximately 20 ml of cord blood was collected immediately after birth into two 9 ml sterile Lithium Heparin coated collection containers (green top; Greiner Vacuette 2 mL Cat.No. 454089). The tubes were kept at 4ºC before being transported to the CSIRO Nutrigenomics laboratory in a lab top cooler within 4-6 hours of collection. The cord blood was kept at room temperature (18-22ºC) and was prepared for the CBMN-Cyt assay within 8 hours of collection. At the 3 and 6 month time points, 1 ml of infant blood was collected in a Vacuette® Lith/Hep coated tube by an experienced nurse at CSIRO clinic using the tenderfoot heel prick method (535) and was stored in a labtop cooler (Nalgene 0ºC labtop cooler 3x4 tubes 17mm, Lot: 7111573010) at 18-22ºC and the CBMN-Cyt assay was performed within 8 hours of collection. After removing the blood required for CBMN-Cyt assay (2*100µl) and red cell folate (1*100 µl) from both cord and infant blood samples, the whole blood tubes were centrifuged at 3000 rpm for 20 minutes to separate the plasma. 2mL of cord plasma (100 µl plasma from infant blood) was isolated and stored for mineral/micronutrient analysis at -20°C, until analysis by SA pathology-
(http://www.sapathology.sa.gov.au/wps/wcm/connect/SA+Pathology+Internet+Content+New/Content/Home). An additional two tubes with 300 µl plasma (if remaining from cord and infant blood after isolation of CBMN and folate aliquots) were stored at -80 degrees till transported to SA pathology for serum folate and vitamin B_{12} by immunoassay method utilizing ADVIA

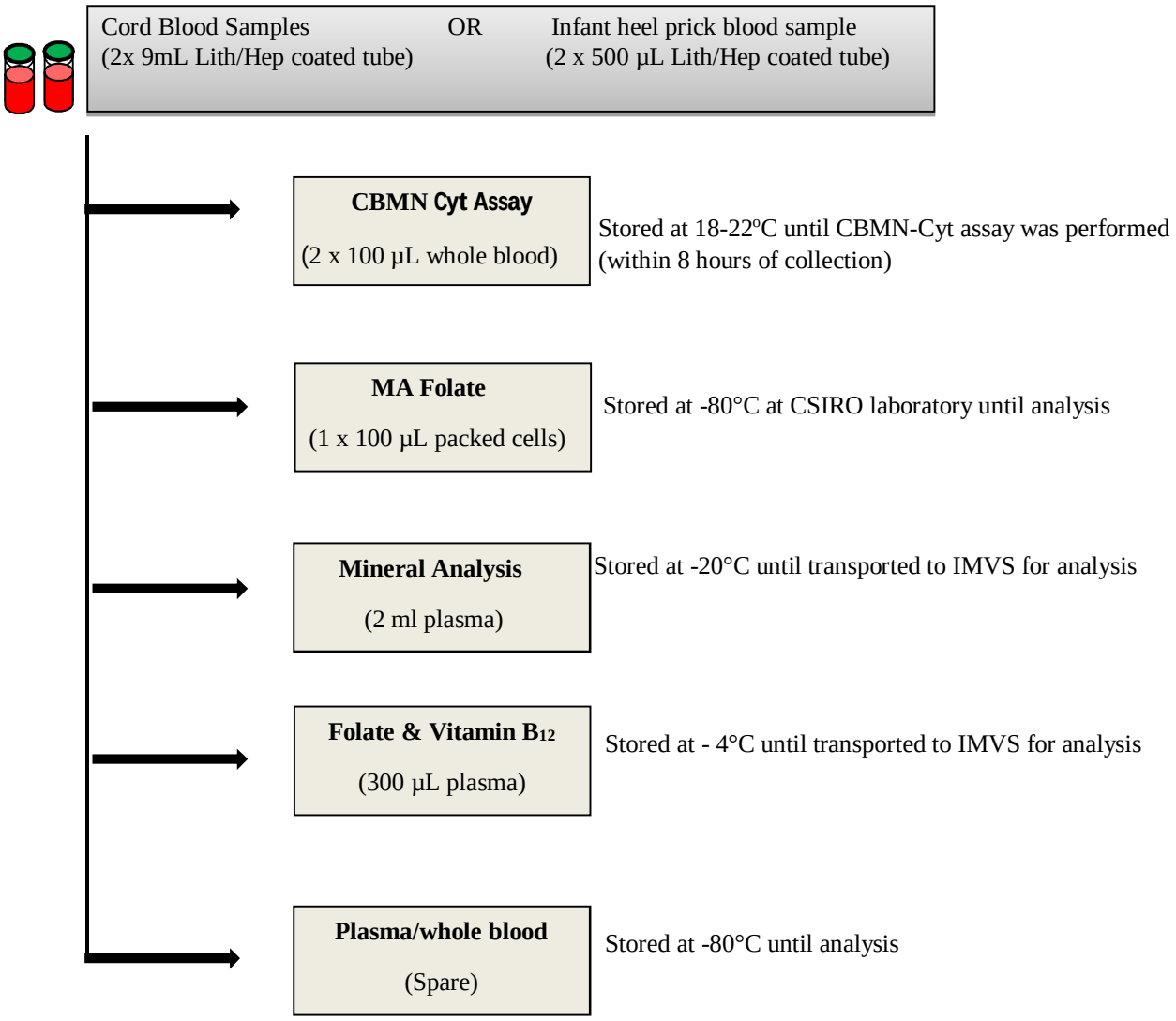


Figure7. 2: DADHI processing protocol for cord bloods and infant heel prick bloods

[Adapted from protocol designed by Maryam Hor (research assistant at CSIRO nutrigenomic laboratory)]
Abbreviations: MA Folate: Microbiological assay for Folate; IMVS: Institute of Medical and Veterinary Science

7.5.5 **CBMN-Cyt assay**

The whole blood CBMN-Cyt assay was conducted in duplicate on all collected samples (cord blood, 3 and 6 month bloods) (108). The detailed protocol of the assay has been explained in chapter 3 and 5. Briefly, duplicate whole blood lymphocyte culture for each blood sample from a participant was prepared. On day 0, 100 µl of heparinised whole blood was cultured in 810 µl medium. The mitogenic activity in lymphocytes was initiated by adding 90 µl PHA to give a final concentration of 202.5 µg/ml. The time of PHA addition was recorded. The cells were incubated at 37 ºC with loosened lids in a humidified atmosphere containing 5% carbon dioxide for 44 h.

At 44 hrs, the cell cultures were carefully removed from the incubator and 100 µl of cytochalasin-B stock solution was added and gently mixed to achieve a final concentration of 6 µg/ml. The cells were returned to the incubator for a further 24 hrs.

At 68 hrs, cultures were removed from the incubator, and the cells were resuspended by mixing gently. The cell suspension was underlaid with 400 µl of Ficoll-Paque (Amersham Pharmacia Biotech, Sweden, cat no. 17144002) in a TV10 tube (Techno Plas, S9716VSU, Australia) using a ratio of 1 (Ficoll):3 (cell suspension) without disturbing the interface. The tube containing cell suspensions overlaid on Ficoll was then centrifuged once at 400g for 30 min at 18 to 20ºC to separate the lymphocytes. Using a pipette with a 200 µl clear plugged tip, the 'buffy' lymphocyte layer at the interface of the Ficoll Paque and culture medium was removed carefully avoiding uptake of Ficoll. The lymphocyte suspension was washed in three times its volume of Hanks balanced salt solution (Hanks HBSS, Trace Scientific, Melbourne, Australia, Cat no. 111010500-V) by gently pipetting in 1320 µl HBSS solution and then centrifuging at 180g for 10 min at room temperature to remove any residual Ficoll and cell debris. The supernatant was gently removed, leaving approximately 200 µl cell suspension. Subsequently, 15 µl dimethyl sulfoxide (DMSO 7.5% v/v of cell suspension Sigma, Sydney, Australia) was added to prevent

cell clumping and to optimize identification of cytoplasmic boundaries. The assay was conducted in duplicate for each blood sample. This was followed by harvesting of cells by cytocentrifugation onto cleaned slides. The slides were air-dried for 10 minutes. Then the slides were transferred directly into Diff Quick stain: 10 dips in the orange stain followed by 5 dips in the blue stain. The extra stain was washed off with tap water and slides were left to air-dry for 10 minutes. The slides were finally cover- slipped using DePeX mounting medium (BDH laboratory, Poole, UK) in a fume-hood. A slide with two stained cytospin spot of cells were prepared from each of the duplicate culture. A conventional light microscope (Model Leica DMLB2: Leica Microsystem, Wetzlar, Germany) was used to examine the cells at 1000 x magnification. For each scoring analysis two scorers (MH and TA) individually determined cytostatic and cytotoxic events by scoring 500 cells including mono-, bi-, multinucleated cells, necrotic and apoptotic cells according to previously published classification criteria (108). This allowed calculation of nuclear division index (NDI).(108,540).

Both the scorers (MH and TA) independently counted the CBMN-Cyt assay genome damage biomarkers (MN, NPB, NBUD) in 1000 binucleated lymphocyte cells (BNC) from each duplicate culture to give an overall total for each biomarker of 4000 BNC scored per sample. The results were then averaged to obtain the frequency per 1000 BNC. A third scorer (MD) independently scored the frequency of genome damage biomarkers (MN and NBUD) in mononucleated lymphocyte cells (MNC), using criteria previously described (539). An average of 500 MNC were scored for MN and NPB in each duplicate culture. The results in MNC were expressed as MN and NBUD per 100 MNC per subject. The HUMN scoring criteria recommends that the MN frequency be determined in a minimum of 1000 cells (539) but in 40% of our slides, there were insufficient MNC to score 1000 cells which is why frequencies of MN and NBUD in MNC were reported per 100 cells.

7.5.6 **Measure of Red cell folate**

The method outlining the red cell folate measurement (94,629,641) is presented in chapter 5. A brief outlined is included in this section.

Chemicals required

- 0.5% sodium ascorbate solution: 5g sodium ascorbate (Sigma-Aldrich, New South Wales, Australia) dissolved in 1000 ml Milli Q water
- Working standard solution B of 5-methylTHF solution (concentration=1nmol/L)
- Folic acid casei medium (Difco): 9.4g media was added to 100 ml Milli Q water. The solution was boiled for 2-3 minutes and then filtered with a 0.22µm filter
- The bacteria inolculum was thawed. 50 µl of the inoculum was added to 4950 µl of folic acid casei media and mixed well. This constitute the inoculated media.
- Blood samples (cord and heel prick bloods collected from the infants) of unknown folate concentration.

The Assay

Briefly, in a 96 well flat-bottom plate, 0.5% sodium ascorbate was added in all the wells. In the blank wells, 100 µl of 0.5% sod ascorbate solution and 100 µl inoculated media was added. Lastly, 100 µl of inoculum was added in standard and sample wells. Final volume in each well was 200 µl. Secondly, in the standard wells, 100-0 µl (decreasing concentration from first to last well) of 0.5% sodium solution was added. Then the working standard solution of 5-methyl THF (1nmol/L) was added in the standard well in increasing concentration (0-100 µl) corresponding to the sodium ascorbate solution. Each concentration was achieved in triplicate. In the sample wells, 80 µl of sodium ascorbate solution was added. Then 20 µl of blood sample was added in the sample well. The study ID was used as the label for each sample well to carefully define each well. Each concentration was achieved in triplicate. Recovery wells were

included for each sample to estimate percentage recovery of folate from the sample. Each recovery well had 60 µl 0.5% sodium solution, 20 µl of sample and 20 µl of standard solution. Lastly, 100 µl of inoculum was added in standard and sample wells. Final volume in each well was 200 µl. The plate was sealed and incubated for 18 hours in an incubator at 37°C. After 18 hours, the bacteria were resuspended by shaking the plate which was covered with the seal to avoid cross-contamination. The plate was read at 590 nm on a spectrophotometer (UV MAX 250, multi-mode micro plate reader, Molecular devices, USA). The optical density values in triplicates were recorded for all wells (standard, sample and recovery). The average value was obtained for each well. Standard deviation and coefficient of variation (CV) was calculated for each point. If the CV values were > 10%, the readings were discarded and sample were re tested. A standard concentration response curve or calibrator curve was obtained by plotting average optical density value as ordinate and concentration of 5-methyl-THF standard as abscissa in logarithm scale utilizing MS Excel 2010 (a snap shot of calculation is included as **Appendix 4)**. The regression equation [y = a ln (x) + c] and R-square value of the calibration curve were computed in MS Excel (641). If the R value was below 0.98, the assay was repeated. The optical value of the sample and recovery was put in a regression equation (interpolate) to calculate the folate concentration in the sample well. The value was adjusted for the dilution factor (x100) to obtain the final folate content in nmol/L per sample (641).

7.5.7 **Plasma mineral/micronutrient analysis**

The cord blood sample and the infant blood samples were collected in EDTA tubes. The blood was centrifuged at 3000 rpm in order to separate the plasma from the red cells. The plasma was collected in Eppendorf tubes and stored at -20ºC and transported to SA pathology, Adelaide for mineral analysis.

The plasma mineral concentrations were determined by inductively coupled plasma analysis (ICP). Samples were first digested using 2.0 ml nitric acid and 0.5 ml hydrogen peroxide in a

50 ml polypropylene centrifuge tube with a lid to prevent contamination. Caps were hand-tightened and tubes were vortexed to ensure the entire sample was wetted, and then pre-digested overnight at room temperature (20–22°C). The digestion method gave good recovery of all the elements (773), achieving recoveries of between 94–113%. Sample solutions were then analysed using an inductively coupled plasma atomic emission spectrometry (ICPAES) method by either Axial circular optical systems (CIROS) or Radial CIROS. The limit of detection for the sample was calculated as 10 x the standard deviation of the calibration blank. The limit of detection (LOD) was automatically calculated by the Spectro software from the standard deviation of the calibration blank (CB) and slope of calibration curve (m) as

LOD = 3SD.CB ÷ m. Sample concentrations that were below method reporting limits (MRL) were calculated as MRL = 10SD.CB ÷ m ×Sample volume ÷ Sample mass.

The micronutrients analysed were: iron, copper, zinc, calcium, magnesium, sodium, potassium, phosphorous, sulphur. The ratio of calcium to magnesium was calculated because these two nutrients are known to compete for absorption and hypomagnesemia may often be present with hypocalcemia (774-776). Further calcium intake affects magnesium retention and vice versa which may influence risk of disease such as metabolic syndrome or cancer in humans (775,777,778). Also sodium and potassium ratio was calculated. Potassium and sodium are the major intracellular and extracellular cation respectively (477). Relatively small changes in the concentration of either greatly affect the transmembrane gradient and thereby neural transmission, muscle contraction and vascular tone (779). The interdependence of the two electrolytes can be attributed to biological mechanisms contributing to control of electrical potential of the cells and blood pressure (780).

7.5.8 **Statistical analysis**

Group statistics were calculated for each group of infants at birth, three and six months to obtain Mean (± SD) for CBMN-Cyt biomarkers and plasma micronutrients for each time point. All CBMN-Cyt biomarkers and plasma micronutrient concentrations for the infant population were first analyzed for normality utilizing the D'Agostino Pearson omnibus test. The concentrations of plasma micronutrients from birth to three and six months after birth were assessed with one way ANOVA for repeated measures to determine if the differences between the group means was greater than could be attributed to chance. For a non-Gaussian population, a Friedman test was performed. Post-test Tukey's test for multiple comparison test was performed to determine differences between group means (birth and three months, birth and six months, and three and six months). A post-test for linear trend was also performed. Gender differences for concentrations of plasma micronutrients were assessed by Student's unpaired t-test (two tailed) for Gaussian distributed data (using Mean ± Standard error of mean (SEM) values]. When the sample distribution was not normal, a Mann-Whitney test was performed. Degrees of association between continuous variables were evaluated by correlation analysis. Pearson correlation coefficients were calculated for Gaussian distributed data. Correlation analysis for non-Gaussian distributed data was performed using the Spearman rank test. For all analyses, differences were accepted as significant at a P-value of < 0.05. Graph Pad Prism version 6.04 for Windows (Graph Pad Inc., San Diego, CA, USA) and SPSS 23.0 (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY, USA: IBM Corp) were used for all statistical analyses.

7.6 **Results**

7.6.1 **Change in plasma micronutrients in infants at birth, three and six months**

The mean (± SD) values for micronutrient concentrations, as measured in plasma isolated from blood of infants born in South Australia at birth (cord blood), three and six months, are presented in **Table 7.2**. There were differences in mean values for most micronutrients: iron (p

= 0.009), sulphur (p = 0.02), copper, magnesium, calcium/magnesium ratio, sodium, potassium, sodium: potassium ratio and red cell folate (p < 0.0001) at birth, and at three and six months. There was a non-significant change in the concentration of zinc and calcium.

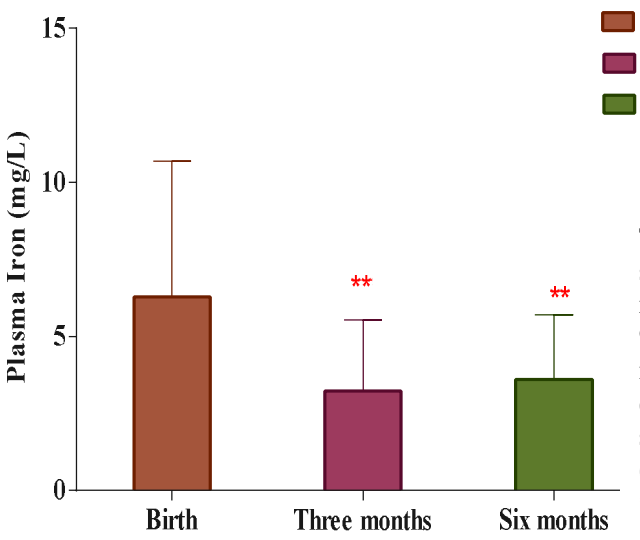
Table 7. 2: Comparison of Mean (±SD) of Blood micronutrients (mg/L) in infants at birth, three and six months

Blood Micronutrient	Mean (± SD)	Wilks' Lambda	F (df)	p	n^2
Iron					
Birth	6.29 (± 4.48)				
Three months	3.23 (± 2.30)	0.736	5.56 (2, 31)	**0.009**	0.264
Six months	3.60 (± 2.11)				
Copper					
Birth	0.41 (± 0.15)				
Three months	0.63 (± 0.39)	0.197	63.29 (2, 31)	**0.000**	0.803
Six months	1.04 (± 0.31)				
Zinc					
Birth	1.01 (± 0.15)	0.853	2.66 (2, 31)	0.08	0.147
Three months	1.49 (± 1.65)				
Six months	1.36 (± 1.04)				
Calcium					
Birth	105.7 (± 7.51)	0.847	2.79 (2, 31)	0.07	0.153
Three Months	110.9 (± 9.81)				
Six months	107.6 (± 8.95)				
Magnesium					
Birth	17.7 (± 2.13)	0.227	52.81 (2, 31)	**0.000**	0.773
Three months	20.8 (± 5.91)				
Six months	23.7 (± 2.64)				
Calcium/ Magnesium ratio					
Birth	6.05 (± 0.75)	0.224	50.24 (2, 29)	**0.000**	0.776
Three months	5.02 (± 0.30)				
Six months	4.55(± 0.39)				
Sodium					
Birth	3040 (± 117)	0.333	30.98 (2, 31)	**0.000**	0.667
Three Months	3280 (±287)				
Six months	3350 (± 238)				
Potassium					
Birth	402 (± 122)	0.311	34.5 (2, 31)	**0.000**	0.689
Three months	204 (± 38.3)				
Six months	216 (± 38.1)				
Sodium/Potassium ratio					
Birth	8.38(± 2.95)	0.193	64.7 (2,31)	**0.000**	0.99
Three Months	16.4 (± 2.19)				
Six months	15.8 (± 2.16				
Phosphorus					
Birth	104.7 (± 12.3)	0.213	57.3 (2, 31)	**0.000**	0.787
Three months	139.0 (± 16.9)				
Six months	138.6 (± 18.3)				
Sulphur					
Birth	987.7 (± 100.8)	0.788	4.18 (2, 31)	**.02**	0.212
Three months	1003 (± 96.0)				
Six months	1043 (± 74.2)				
Red Cell folate					
Birth	382.67 (± 58.5)	0.291	27.8 (2, 23)	**0.000**	0.709
Three months	212.7 (± 129)				
Six months	319.9 (± 74.1)				

Wilks' Lambda: Multivariate test; F (df): The ratio of two mean square values (hypothesis and error degree of freedom); n^2: partial Eta squared (a measure of effect size for group mean difference), p: significance value, n varied from 30-33 for each group.

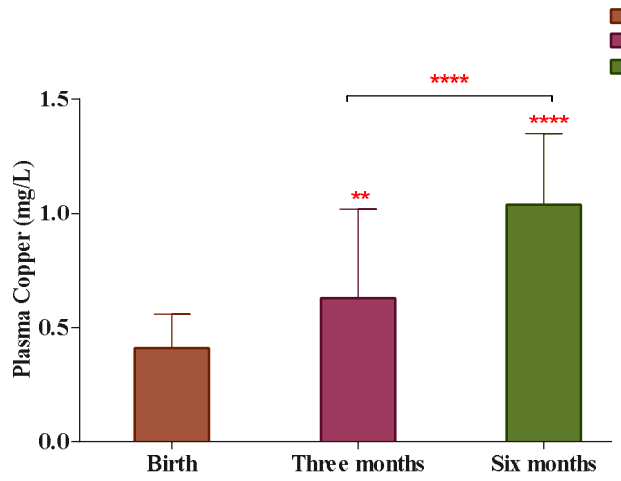
The subsequent post hoc tests for multiple comparisons and linear trend showed that there were differences among micronutrient concentrations at the three time points (**Figure 7.3**). There was a decrease in iron at six months compared with the mean value at birth (p = 0.007). Mean

plasma iron at three and six months was less than at birth ($p = 0.002$, $p = 0.008$ respectively). A significant decline was observed in concentrations of potassium ($p < 0.0001$) and red cell folate ($p < 0.001$), and in the calcium to magnesium ratio ($p < 0.0001$) from birth to six months while there was a linear trend towards increase for copper, magnesium, sodium, phosphorus ($p < 0.0001$) and sulphur ($p < 0.05$) from birth to six months. Zinc at birth was less than in infants at six months ($p = 0.04$). Calcium was greater at three months than at birth or at six months ($p = 0.02$). However, no linear trend was observed for either zinc or calcium from birth to six months (**Figure 7.3**).



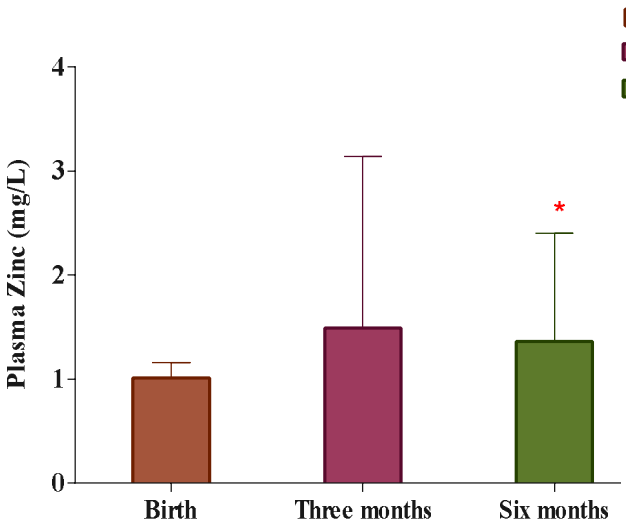
The pairwise comparison between three time points showed differences between mean (± SD) **plasma iron** in infants at birth and at three months (p=0.002, 95% CI: 1.2, 4.8), and between birth and at six months (p=0.008, 95% CI: 0.75, 4.6); however, no difference was observed between three months and six months. Post-test for linear trend was significant (slope= -1.3, p= 0.007).

**: p< 0.01



The pairwise comparison between three time points showed difference between mean (± SD) **plasma copper** measured in infants at birth and at three months (p = 0.005, 95% CI: -0.36 -0.07), and between birth and at six months (p < 0.0001, 95% CI: -0.74, -0.51) and between three and six months (p < 0.0001, 95% CI: -0.54, -0.27). Post-test for linear trend was significant (slope = 0.31, p< 0.0001)

****: p< 0.0001, **: p< 0.01



The pairwise comparison between three time points showed a difference between mean (± SD) **plasma zinc** measured in infants at birth and at six months (p = 0.047, 95% CI: -0.70, -0.005) but not between birth and three months or between three months and six months. Post-test for linear trend was not significant.

*: p< 0.05

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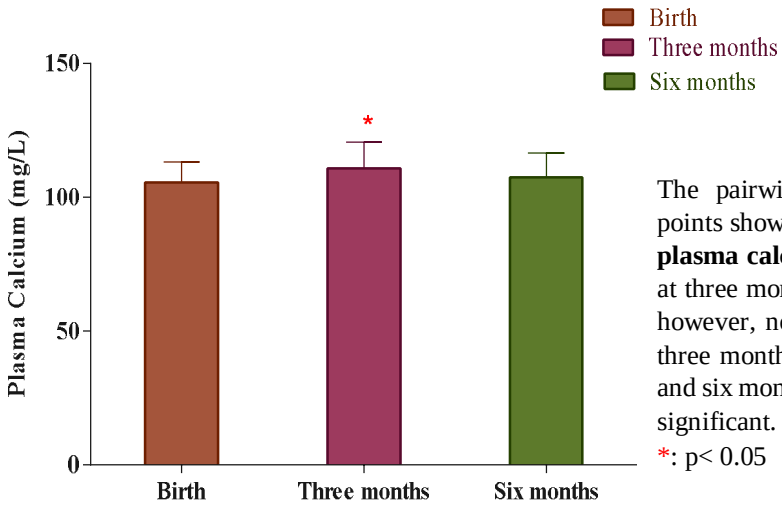
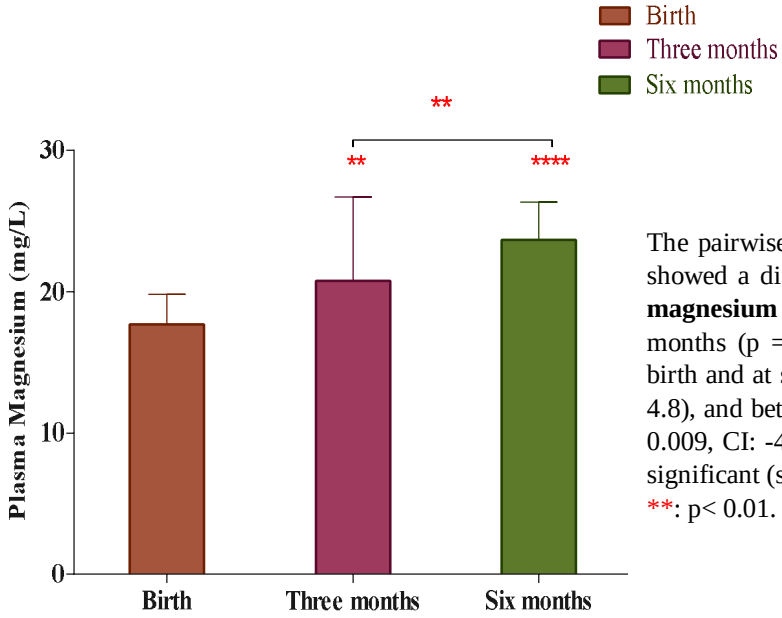


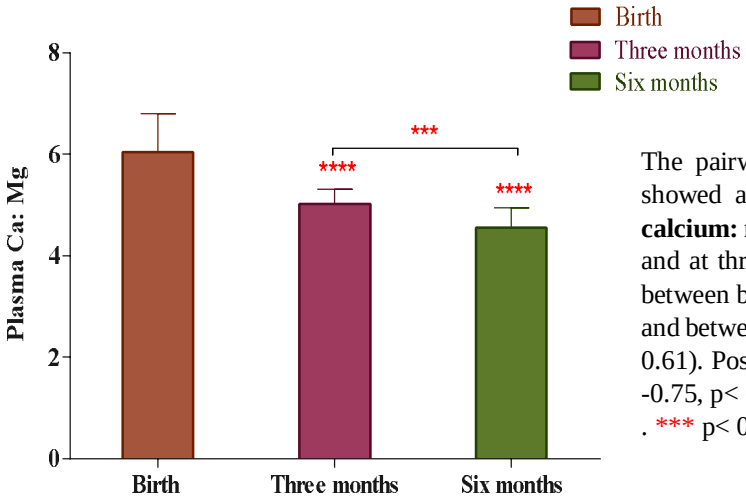
The pairwise comparison between three time points showed a difference between mean (± SD) **plasma calcium** measured in infants at birth and at three months (p = 0.023, 95% CI: -9.7, -0.75); however, no differences were observed between three months and six months, and between birth and six months. Post-test for linear trend was non-significant.

*: p< 0.05



The pairwise comparison between three time points showed a difference between mean (± SD) **plasma magnesium** measured in infants at birth and at three months (p = 0.006, 95% CI: -5.3, -0.97), between birth and at six months (P < 0.0001, 95% CI: -7.2, -4.8), and between three months and six months (p = 0.009, CI: -4.9, -0.75). Post-test for linear trend was significant (slope=3.0, p < 0.0001).

**: p< 0.01. **** p< 0.0001



The pairwise comparison between three time points showed a difference between mean (± SD) **plasma calcium: magnesium ratio** measured in infants at birth and at three months (p < 0.0001, 95% CI: 0.76, 1.2), between birth and six months (p < 0.0001, CI: 1.1, 1.8), and between three and six months (p = 0.0001, CI: 0.33, 0.61). Post test for linear trend was significant (slope= -0.75, p< 0.0001)

. *** p< 0.001, **** p< 0.0001

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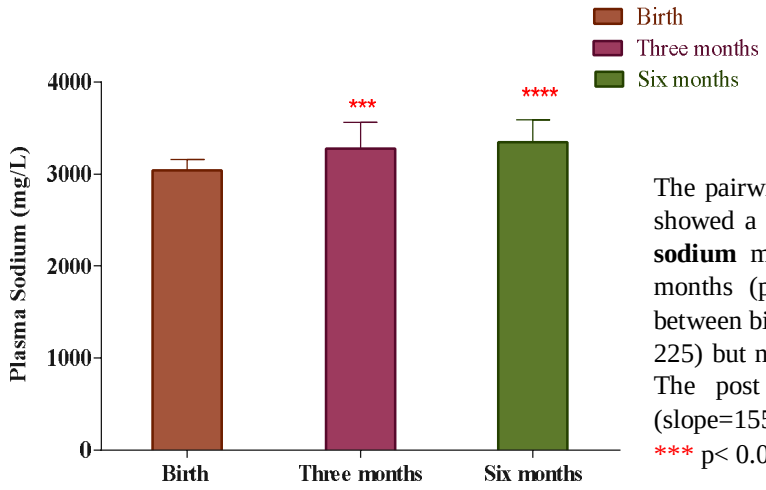
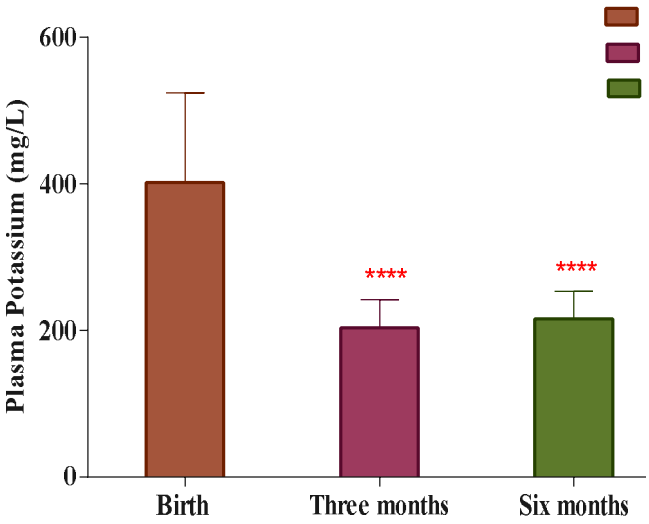


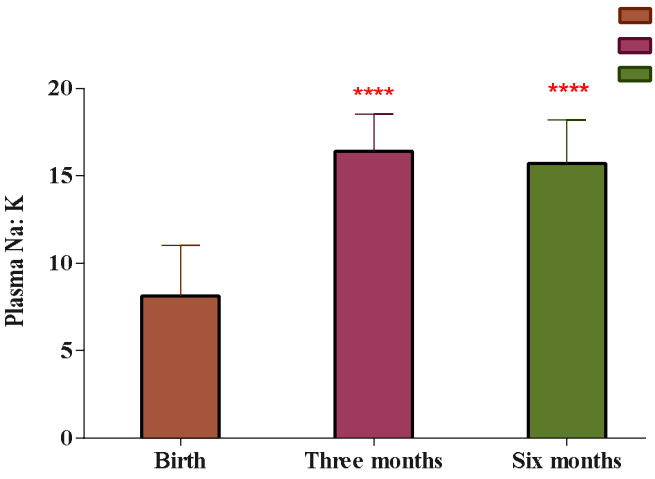
The pairwise comparison between three time points showed a difference between mean (± SD) **plasma sodium** measured in infants at birth and at three months ($p < 0.0001$, 95% CI: -356, -121), and between birth and six months ($p < 0.0001$, CI: -392, -225) but not between three months and six months. The post test for linear trend was significant (slope=155, $p< 0.0001$)

*** $p< 0.001$, **** $p< 0.0001$



The pairwise comparison between three time points showed a difference between mean (± SD) **plasma potassium** measured in infants at birth and at three months ($p < 0.0001$, 95% CI: 149, 246), and between birth and at six months ($p < 0.0001$, CI: 139, 232) but not between three and six months. Post test for linear trend was significant (slope = -93, $p< 0.0001$)

**** $p< 0.0001$



The pairwise comparison between three time points showed a difference between mean (± SD) plasma **sodium: potassium ratio** measured in infants at birth and at three months ($p < 0.0001$, 95% CI: -9.7, -6.7), and between birth and at six months ($p < 0.0001$, CI: - 9.06, -6.09) but not between three and six months. Post test for linear trend was significant (slope = 3.7, $p< 0.0001$)

**** $p< 0.0001$

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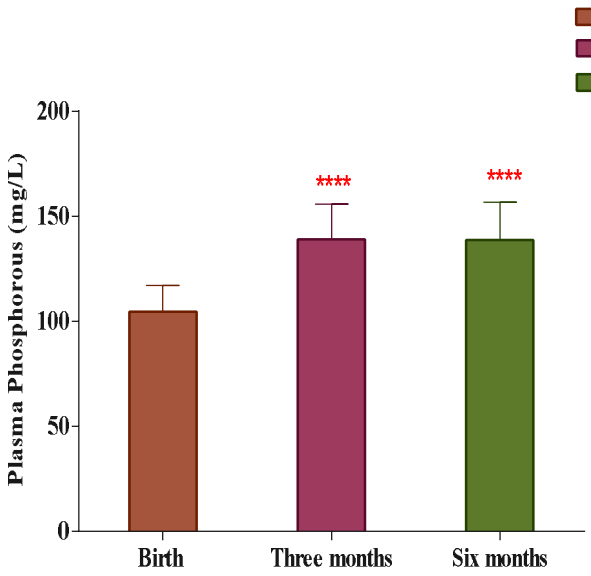
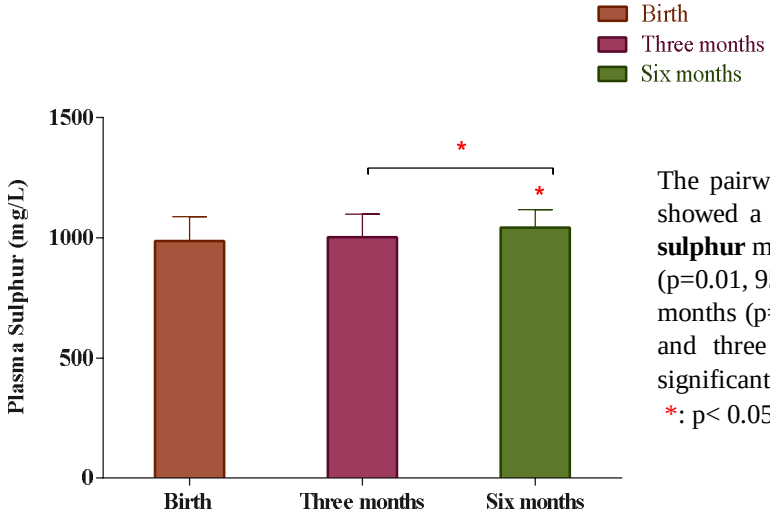


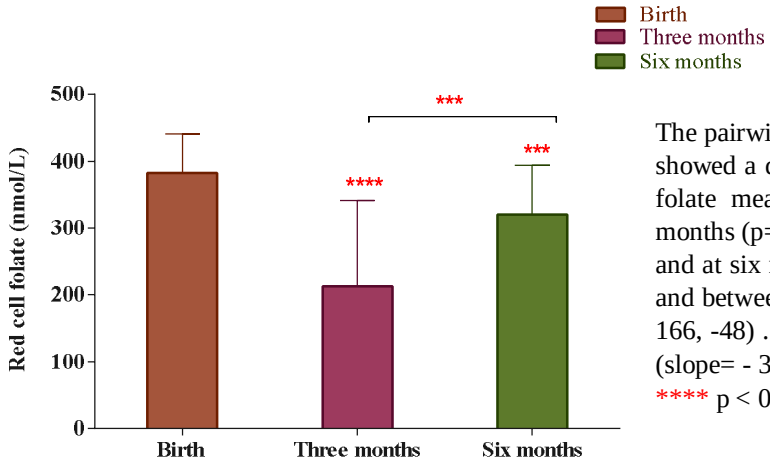
The pairwise comparison between three time points showed a difference between Mean (± SD) **plasma phosphorus** measured in infants at birth and at three months ($p < 0.0001$, 95% CI: -41.5, -27.1), and between birth and at six months ($p < 0.0001$, CI: -42.1, -25.7) but not between three and six months. Post-test for linear trend was significant (slope=16.9, $p < 0.0001$)

*** $p< 0.0001$



The pairwise comparison between three time points showed a difference between mean (± SD**) plasma sulphur** measured in infants at birth and at six months (p=0.01, 95% CI: -97, -13), and between three and six months (p=0.05, CI: -80, 0.69) but not between birth and three months. Post-test for linear trend was significant (slope=27.6, p= 0.01)

*: $p< 0.05$



The pairwise comparison between three time points showed a difference between mean (± SD) red cell folate measured in infants at birth and at three months (p= 0.000, CI: 119, 220), and between birth and at six months ($p = 0.001$, 95% CI: 27.6, -97.7) and between three and six months ($p = 0.001$, CI: -166, -48) . Post-test for linear trend was significant (slope= - 31.3, $p = 0.01$)

**** $p < 0.0001$, ***: $p< 0.001$

Figure7.3: Multiple comparisons of means (±SD) for plasma micronutrients at birth, three and six months

7.6.2 **Association between cord blood micronutrients and maternal anthropometric variables and infant birth outcomes**

The anthropometric variables of mothers [weight, height, body mass index (BMI)] were assessed at recruitment (8-16 week gestation). Infant characteristics of weight, length, head circumference, gestational age (GA) and APGAR score were recorded at birth. The association between cord blood micronutrients and maternal and infant birth outcomes are presented in **Table 7.3**. There was no association of cord blood micronutrients with maternal characteristics: weight, height and BMI.

Plasma copper, ratio of calcium to magnesium, ratio of sodium to potassium and serum vitamin B_{12} were observed to be positively associated with GA ($r = 0.4$, $p = 0.0007$, $r = 0.28$, $p = 0.04$, $r = 0.28$, $p = 0.05$, $r = 0.3$, $p = 0.01$ respectively) while potassium was negatively associated with GA ($r = -0.28$, $p = 0.04$). Calcium was negatively associated with head circumference ($r= -0.3$, $p = 0.01$) and sulphur was inversely associated with APGAR score recorded at 1 minute ($r= -0.3$, $p = 0.04$).

Table 7.3: Correlation analysis between blood micronutrients measured at birth (cord blood) and maternal factors and infant birth outcomes (n =38 to 50)

Cord Blood Micronutrients (mg/L)	Maternal anthropometric variables at recruitment			Infant birth outcomes					
	Weight (kg)	Height (m)	BMI (kg/m^2)	Gestation age (weeks)	Weight (g)	Length (cm)	Head circumference (cm)	Apgar score at 1 min	Apgar score at 5 min
Iron	*r = - 0.1* *p = 0.4*	*r = 0.01* *p = 0.9*	*r = - 0.09* *p = 0.5*	*r = - 0.03* *p = 0.8*	*r = 0.00* *p = 0.9*	*r = - 0.1* *p = 0.3*	*r = 0.12* *p = 0.4*	*r = 0.00* *p=0.9*	*r = - 0.1* *p=0.3*
Copper	*r = 0.00* *p = 0.9*	*r = 0.1* *p = 0.2*	*r = - 0.1* *p = 0.3*	***r = 0.4*** ***p = 0.0007******	*r = 0.02* *p = 0.8*	*r = - 0.05* *p = 0.7*	*r = - 0.04* *p = 0.7*	*r = 0.01* *p=0.9*	*r = -0.2* *p=0.1*
Calcium	*r = 0.00* *p = 0.9*	*r = 0.1* *p = 0.5*	*r =- 0.06* *p = 0.6*	*r = - 0.03* *p = 0.8*	*r = - 0.1* *p = 0.3*	*r = 0.00* *p = 0.9*	***r = - 0.37*** ***p = 0.01****	*r = - 0.2* *p=0.2*	*r = - 0.29* *p=0.07*
Magnesium	*r = 0.02* *p = 0.8*	*r = 0.01* *p = 0.9*	*r = - 0.03* *p = 0.8*	*r = - 0.1* *p = 0.2*	*r = - 0.1* *p = 0.4*	*r = - 0.02* *p = 0.8*	*r = - 0.1* *p = 0.1*	*r = 0.05* *p=0.7*	*r = -0.04* *p=0.7*
Ca: Mg	*r = - 0.04* *p = 0.7*	*r = 0.00* *p = 0.9*	*r = - 0.03* *p = 0.8*	***r = 0.28*** ***p = 0.04****	*r = 0.2* *p = 0.1*	*r = 0.07* *p = 0.6*	*r = 0.08* *p = 0.5*	*r = -0.06* *p=0.7*	*r =0.00* *p=0.9*
Zinc	*r = 0.1* *p = 0.2*	*r = 0.08* *p = 0.5*	*r = 0.1* *p = 0.4*	*r = - 0.1* *p = 0.2*	*r = - 0.1* *p = 0.3*	*r = - 0.1* *p = 0.4*	*r = - 0.1* *p = 0.2*	*r = - 0.1* *p=0.2*	*r = - 0.2* *p=0.1*
Sodium	*r = 0.1* *p = 0.2*	*r = 0.1* *p = 0.3*	*r = 0.1* *p = 0.4*	*r = 0.03* *p=0.7*	*r = - 0.03* *p = 0.7*	*r = - 0.1* *p = 0.3*	*r = 0.06* *p = 0.6*	*r = -0.2* *p=0.2*	*r =- 0.08* *p=0.6*
Potassium	*r = - 0.1* *p = 0.4*	*r = - 0.1* *p = 0.2*	*r = - 0.01* *p = 0.8*	***r = - 0.28*** ***p = 0.04****	*r = - 0.1* *p = 0.4*	*r = 0.09* *p = 0.5*	*r = - 0.1* *p = 0.2*	*r = 0.1* *p=0.4*	*r = 0.03* *p=0.8*
Na: K	*r = 0.1* *p = 0.4*	*r = 0.1* *p = 0.2*	*r = 0.01* *p = 0.9*	***r = 0.28*** ***p = 0.05****	*r = 0.1* *p = 0.4*	*r = - 0.1* *p = 0.4*	*r = 0.1* *p = 0.2*	*r = - 0.1* *p = 0.4*	*r = 0.001* *p = 0.9*
Phosphorus	*r = 0.03* *p = 0.7*	*r = 0.04* *p = 0.7*	*r = - 0.01* *p = 0.9*	*r = - 0.1* *p = 0.2*	*r = - 0.2* *p = 0.1*	*r = - 0.03* *p = 0.8*	*r = - 0.2* *p = 0.1*	*r = - 0.2* *p=0.1*	*r = - 0.3* *p=0.06*
Sulphur	*r = - 0.1* *p=0.3*	*r = - 0.05* *p = 0.7*	*r = - 0.2* *p = 0.1*	*r = 0.2* *p = 0.1*	*r = 0.05* *p = 0.7*	*r = 0.1* *p = 0.4*	*r = - 0.1* *p = 0.3*	***r = - 0.3*** ***p=0.04*** *	*r = - 0.2* *p=0.1*
#Serum B_{12}	*r = 0.1* *p = 0.4*	*r = 0.04* *p = 0.7*	*r = 0.09* *p = 0.5*	***r = 0.3*** ***p = 0.01****	*r = 0.2* *p = 0.09*	*r = 0.1* *p = 0.4*	*r = - 0.01* *p = 0.9*	*r = - 0.1* *p=0.4*	*r = - 0.06* *p=0.6*
•Serum folate	*r = - 0.08* *p = 0.5*	*r = 0.05* *p = 0.7*	*r =- 0.09* *p = 0.5*	*r = 0.03* *p = 0.8*	*r = 0.1* *p = 0.5*	*r = 0.1* *p = 0.1*	*r = 0.1* *p = 0.3*	*r = 0.1* *p=0.5*	*r = 0.08* *p=0.6*
•Red cell folate	*r = 0.1* *p = 0.4*	*r = - 0.02* *p = 0.8*	*r = 0.1* *p = 0.3*	*r = - 0.1* *p = 0.2*	*r = - 0.04* *p = 0.7*	*r = 0.1* *p = 0.2*	*r = - 0.05* *p = 0.6*	*r = - 0.03* *p=0.8*	*r = 0.04* *p=0.7*

#: Lab values in pmol/L; •:Folate lab values in nmol/L, n=number of subjects, Na: sodium, K: potassium, Ca: calcium, Mg: magnesium

7.6.3 **Association between cord blood micronutrients and CBMN-Cyt biomarkers at birth**

The correlation analyses for blood micronutrients and CBMN-Cyt biomarkers at birth are presented in **Table 7.4**. Iron was negatively correlated with NBUD MNC (r= - 0.28, p = 0.001). Magnesium was correlated positively with MN MNC (r = 0.23, p = 0.03). Ratio of calcium to magnesium was significantly correlated with MN BNC (r = 0.28, p = 0.01). Red cell folate was associated positively with necrotic lymphocytes (r = 0.22, p = 0.05). Copper, calcium, sodium, potassium, zinc and sulphur, phosphorous, and serum vitamin B_{12} were not associated with any of the lymphocyte CBMN-Cyt biomarkers measured in cord blood at birth.

Table 7.4: Correlation analysis between cord blood micronutrients and CBMN-Cyt biomarkers at birth

Cord Blood Micronutrients (mg/L)	MNBNC	NPBBNC	NBUDBNC	NDI	Apoptotic cells	Necrotic cells	MNMNC	NBUDMNC
Iron (n = 78)	*r= - 0.06* *p = 0.5*	*r= - 0.1* *p = 0.3*	*r= - 0.16* *p = 0.1*	*r= 0.003* *p = 0.9*	*r= 0.04* *p =0.6*	*r= 0.09* *p = 0.4*	*r =-0.13* *p = 0.2*	***r= - 0.28*** ***p= 0.01****
Copper (n = 78)	*r = - 0.003* *p = 0.9*	*r = 0.05* *p = 0.6*	*r = 0.1* *p = 0.3*	*r = - 0.16* *p = 0.1*	*r = 0.12* *p = 0.2*	*r = -0.05* *p = 0.6*	*r = 0.1* *p = 0.3*	*r = -0.03* *p= 0.7*
Calcium (Nn= 78)	*r = 0.16* *p = 0.1*	*r = 0.1* *p=0.3*	*r = 0.07* *p = 0.4*	*r = - 0.05* *p = 0.6*	*r =- 0.05* *p = 0.6*	*r = 0.0* *p = 0.9*	*r = 0.17* *p = 0.1*	*r = 0.16* *p = 0.1*
Magnesium (n = 78)	*r = - 0.12* *p = 0.2*	*r = 0.15* *p = 0.17*	*r = - 0.04* *p= 0.7*	*r = - 0.16* *p = 0.1*	*r = -0.1* *p= 0.3*	*r = 0.00* *p= 0.9*	***r = 0.23*** ***p= 0.03****	*r = 0.04* *p= 0.6*
Ca: Mg (n = 78)	***r = 0.28*** ***p=0.01****	*r = 0.04* *p = 0.6*	*r = 0.10* *p = 0.08*	*r = 0.13* *p = 0.2*	*r = - 0.07* *p = 0.5*	*r = 0.03* *p = 0.7*	*r = 0.05* *p = 0.6*	*r = 0.15* *p = 0.1*
Zinc (n = 78)	*r = 0.05* *p = 0.6*	*r = 0.14* *p = 0.1*	*r = 0.07* *p = 0.5*	*r = - 0.16* *p = 0.1*	*r = - 0.09* *p = 0.4*	*r = 0.1* *p = 0.3*	*r = - 0.04* *p = 0.6*	*r = - 0.06* *p = 0.5*
Sodium (n = 78)	*r = - 0.05* *p = 0.6*	*r = 0.1* *p = 0.3*	*r = 0.03* *p = 0.7*	*r =- 0.1* *p = 0.3*	*r = - 0.03* *p = 0.7*	*r = 0.00* *p = 0.9*	*r = 0.16* *p = 0.1*	*r = 0.1* *p = 0.3*
Potassium (n = 78)	*r = - 0.01* *p = 0.9*	*r = - 0.01* *p = 0.8*	*r = - 0.15* *p = 0.1*	*r = 0.09* *p = 0.4*	*r = - 0.13* *p = 0.2*	*r =- 0.1* *p = 0.3*	*r = - 0.01* *p = 0.8*	*r = - 0.01* *p = 0.8*
Na: K (n =78)	*r = 0.01* *p = 0.89*	*r = 0.02* *p = 0.85*	*r = 0.14* *p = 0.19*	*r = - 0.10* *p = 0.36*	*r = 0.12* *p = 0.26*	*r = 0.10* *p = 0.34*	*r = 0.04* *p = 0.66*	*r = 0.02* *p = 0.83*
Phosphorus (n = 78)	*r = 0.13* *p = 0.2*	*r = - 0.06* *p = 0.5*	*r = - 0.08* *p = 0.4*	*r = 0.02* *p = 0.8*	*r = - 0.06* *p=0.5*	*r = 0.07* *p = 0.5*	*r =- 0.03* *p = 0.7*	*r = - 0.6* *p = 0.5*
Sulphur (n = 78)	*r = 0.14* *p = 0.2*	*r = 0.2* *p=0.06*	*r = 0.14* *p=0.2*	*r = - 0.19* *p=0.09*	*r = - 0.15* *p = 0.1*	*r = - 0.18* *p = 0.1*	*r = 0.1* *p = 0.3*	*r = 0.05* *p = 0.6*
#:Serum B_{12} (n =81)	*r = 0.18* *p = 0.1*	*r = 0.1* *p = 0.3*	*r = 0.00* *p = 0.9*	*r = - 0.19* *p = 0.07*	*r = - 0.19* *p = 0.07*	*r = - 0.16* *p=0.1*	*r = 0.12* *p = 0.2*	*r = 0.01* *p =0.9*
•Serum Folate (n = 70)	*r = 0.02* *p = 0.8*	*r = 0.00* *p = 0.9*	*r = - 0.08* *p = 0.5*	*r = - 0.13* *p = 0.2*	*r = - 0.09* *p = 0.4*	*r = - 0.11* *p = 0.3*	*r = 0.03* *p =0.7*	*r =- 0.19* *p =0.1*
•Red cell folate (n =76)	*r = 0.16* *p = 0.1*	*r =- 0.08* *p = 0.4*	*r = - 0.14* *p = 0.2*	*r = 0.17* *p = 0.1*	*r = - 0.14* *p = 0.2*	*r = 0.15* *p = 0.19*	*r = 0.18* *p = 0.1*	*r = 0.16* *p = 0.1*

#: Lab values for vitamin B_{12} in pmol/L; •: Folate in nmol/L. Abbreviations: n = number of samples; MN: micronuclei; NPB: nucleoplasmic bridges; NBUD: nuclear buds; BNC: binucleated lymphocyte cells; MNC: mononucleated lymphocyte cells; NDI: nuclear division index; Ca: calcium; Mg magnesium; K: potassium, Na: sodium

7.6.4 Association of blood micronutrients with infant weight, feeding scores and CBMN-Cyt biomarkers at 3 months

Infant weight at three months was negatively associated with plasma concentrations of calcium, sodium and phosphorus (r= - 0.37, p = 0.003; r= - 0.4, p = 0.001; r = - 0.2, p = 0.02 respectively). None of the other plasma nutrients showed any association with infant weight at three months. None of the micronutrients were associated with average feeding scores at three months (**Table 7.5**).

Table 7.5: Association of blood micronutrients with infant weight and feeding scores at 3 months

Infant Blood Micronutrients (mg/L)	Weight at 3 months (g)	Feeding score at 3 months
Iron (n = 58)	*r= -0.07* *p=0.5*	*r= 0.00* *p= 0.99*
Copper (n =45)	*r= -0.0,6* *p= 0.6*	*r= 0.04* *p= 0.7*
Calcium (n= 58)	***r= - 0.37*** ***p=0.003*****	*r= 0.01* *p= 0.8*
Magnesium (n =55)	*r= -0.1* *p= 0.1*	*r= -0.1* *p= 0.1*
Ca: Mg ratio (n=55)	*r= - 0.1* *p= 0.4*	*r= 0.1* *p= 0.3*
Zinc (n =53)	*r= - 0.1* *p= 0.3*	*r= 0.06* *p= 0.6*
Sodium (n= 58)	***r= - 0.4*** ***p= 0.001****	*r= -0.0,2* *p= 0.8*
Potassium (n = 58)	*r= - 0.08* *p= 0.5*	*r= 0.06* *p= 0.6*
Na: K ratio (n = 58)	*r= - 0.06* *p= 0.64*	*r= -0.09* *p= 0.49*
Phosphorus (n = 58)	***r= -0.2*** ***p= 0.02****	*r= 0.09* *p= 0.4*
Sulphur (n= 58)	*r= - 0.2* *p= 0.06*	*r= 0.09* *p= 0.4*
•Red cell folate (n=40)	*r= 0.09,* *p= 0.5*	*r= 0.07* *p= 0.6*

•:Lab values for Folate in nmol/L. Abbreviations: Ca: calcium, Mg: magnesium, K: potassium, Na: sodium

The correlation between micronutrients and CBMN-Cyt biomarkers measured at three months is presented in **Table 7.6**. Iron was inversely associated with apoptotic lymphocytes (r = - 0.32, p = 0.01). While zinc was negatively correlated with NBUD MNC, (r = - 0.27, p = 0.05), ratio of Ca: Mg correlated positively with NBUD MNC (r = 0.3, p = 0.03). Zinc was also positively associated with NPB BNC (r = 0.29, p = 0.03) and apoptotic lymphocytes (r = 0.26, p = 0.05). Phosphorous was negatively correlated with NDI (r = - 0.3, p = 0.02) and red cell folate was associated positively with necrotic lymphocytes (r= 0.3, p = 0.01).

Table 7.6: Correlation analysis between blood micronutrients and CBMN-Cyt biomarkers at three months

Infant Blood Micronutrients (mg/L)	MNBNC	NPBBNC	NBUDBNC	NDI	Apoptotic cells	Necrotic cells	MNMNC	NBUDMNC
Iron (n =55)	*r = 0.02* *p = 0.8*	*r = 0.21* *p = 0.1*	*r = 0.15* *p = 0.2*	*r = - 0.03* *p = 0.8*	***r = - 0.32*** ***p =0.01****	*r = 0.19* *p = 0.1*	*r=0.11* *p= 0.4*	*r= - 0.08* *p= 0.5*
Copper (n =43)	*r = - 0.24* *p = 0.1*	*r = - 0.05* *p = 0.7*	*r = - 0.15* *p = 0.3*	*r = 0.28* *p = 0.06*	*r = 0.1* *p = 0.4*	*r = 0.05* *p = 0.7*	*r = - 0.06* *p = 0.6*	*r = - 0.05* *p = 0.7*
Calcium (n=55)	*r = 0.00* *p = 0.9*	*r = - 0.11* *p = 0.4*	*r = 0.00* *p = 0.9*	*r = -0.21* *p = 0.1*	*r =- 0.04* *p = 0.7*	*r = - 0.17* *p= 0.1*	*r = 0.14* *p= 0.3*	*r = 0.02* *p= 0.8*
Magnesium (n =52)	*r = - 0.02* *p = 0.8*	*r = - 0.02* *p = 0.8*	*r = 0.12* *p = 0.3*	*r = - 0.21* *p = 0.1*	*r = 0.00* *p = 0.9*	*r = - 0.03* *p = 0.8*	*r = 0.01* *p= 0.9*	*r = - 0.23* *p= 0.09*
Ca: Mg ratio(n =52)	*r = - 0.03* *p = 0.7*	*r = - 0.05* *p = 0.7*	*r = - 0.14* *p = 0.2*	*r = 0.08* *p = 0.5*	*r = 0.02* *p = 0.8*	*r = 0.02* *p = 0.8*	*r = 0.07* *p = 0.5*	***r = 0.3*** ***p = 0.03****
Zinc (n =50)	*r = - 0.16* *p = 0.2*	***r = 0.29*** ***p = 0.03****	*r = - 0.03* *p = 0.8*	*r = 0.00* *p = 0.9*	***r = 0.26*** ***p = 0.05****	*r = 0.02* *p = 0.8*	*r = - 0.01* *p = 0.9*	***r = - 0.27*** ***p = 0.05****
Sodium (n =55)	*r = - 0.06* *p = 0.6*	*r = - 0.09* *p = 0.4*	*r = 0.02* *p = 0.8*	*r =- 0.1* *p=0.4*	*r = - 0.03* *p=0.8*	*r = 0.01* *p=0.9*	*r = 0.07* *p=0.5*	*r = 0.03* *p=0.8*
Potassium (n=55)	*r = - 0.03* *p = 0.7*	*r = 0.06* *p = 0.6*	*r = - 0.07* *p = 0.5*	*r =- 0.05* *p=0.6*	*r = - 0.17* *p=0.1*	*r =0.11* *p = 0.4*	*r = 0.00* *p=0.9*	*r = - 0.1* *p=0.4*
Na: K ratio (n =55)	*r = 0.05* *p = 0.69*	*r = - 0.08* *p = 0.53*	*r = 0.09* *p = 0.5*	*r = - 0.02* *p = 0.82*	*r = 0.15* *p = 0.25*	*r = - 0.14* *p = 0.28*	*r = 0.08* *p = 0.55*	*r = 0.16* *p = 0.22*
Phosphorus (n =55)	*r = 0.2* *p = 0.1*	*r = - 0.05* *p=0.6*	*r = 0.14* *p = 0.2*	***r = - 0.3*** ***p = 0.02****	*r = 0.1* *p = 0.4*	*r = - 0.05* *p = 0.6*	*r =0.17* *p=0.2*	*r = 0.25* *p=0.07*
Sulphur (n =55)	*r = - 0.4* *p = 0.7*	*r =- 0.2* *p = 0.07*	*r = 0.04* *p = 0.7*	*r =- 0.13* *p=0.3*	*r = - 0.14* *p = 0.3*	*r = 0.02* *p = 0.8*	*r = 0.09* *p = 0.5*	*r = - 0.09* *p = 0.4*
•Red cell folate (N =37)	*r = 0.14* *p=0.3*	*r =- 0.24* *p=0.1*	*r = 0.11* *p=0.5*	*r = 0.19* *p=0.2*	*r =-0.07* *p=0.6*	***r = 0.3*** ***p=0.01****	*r =- 0.18* *p=0.3*	*r = 0.26* *p=0.1*

•: Lab values for Folate in nmol/L. *Abbreviations*: n = number of samples; MN: micronuclei; NPB: nucleoplasmic bridges; NBUD: nuclear buds; BNC: binucleated lymphocyte cells; MNC: mononucleated lymphocyte cells; NDI: nuclear division index; Ca: calcium; Mg magnesium; K: potassium, Na: sodium

7.6.5 Association of blood micronutrients with infant weight, average feeding scores and CBMN-Cyt biomarkers at 6 months

Infant weight at 6 months was associated with iron (r = 0.31, p = 0.02) and sulphur (r = 0.2, p = 0.05). Plasma calcium and sulphur were positively correlated with average feeding scores at six months (r = 0.2, p = 0.03; r = 0.2, p = 0.05 respectively) (**Table 7.7**).

Table 7.7: Association of blood micronutrients with infant weight and feeding scores at six months

Infant Blood Micronutrients (mg/L)	**Weight at 6 months (g)**	**Feeding score at 6 months**
Iron (n =49)	***r= 0.31*** ***p=0.02****	*r= 0.0* *p= 0.7*
Copper (n =44)	*r= - 0.05* *p= 0.7*	*r= 0.05* *p= 0.7*
Calcium (n =49)	*r=0 18* *p=0.2*	***r= 0.2*** ***p= 0.03****
Magnesium (n = 48)	*r= 0.1* *p= 0.2*	*r= 0.1* *p= 0.4*
Ca: Mg ratio (n = 48)	*r= - 0.03* *p= 0.8*	*r= 0.07* *p= 0.6*
Zinc (n = 48)	*r= 0.00* *p= 0.9*	*r= 0.00* *p= 0.9*
Sodium (n = 49)	*r= 0.14* *p= 0.3*	*r= 0.2* *p= 0.09*
Potassium (n =49)	*r= 0.13* *p= 0.3*	*r= 0.09* *p= 0.5*
Na: K ratio (n=49)	*r= - 0.13* *p= 0.34*	*r= 0.00* *p= 0.99*
Phosphorus (n = 49)	*r= 0.16* *p= 0.2*	*r= 0.11* *p= 0.4*
Sulphur (n= 49)	***r= 0.2*** ***p= 0.05****	***r= 0.2*** ***p= 0.05****
•Red cell folate (n =38)	*r= 0.00* *p= 0.9*	*r= 0.05* *p= 0.7*

•:Lab values for Folate in nmol/L. Abbreviations: Ca: calcium, Mg: magnesium, K: potassium, Na: sodium

Table 7.8 presents correlations between CBMN-Cyt biomarkers and blood micronutrients measured at 6 months. Copper was observed to be positively correlated with MN MNC (r = 0.34, p = 0.02), calcium was positively associated with necrotic lymphocytes (r = 0.3, p = 0.04), and magnesium was negatively associated with NBUD BNC (r = - 0.28, p = 0.05). The ratio of calcium and magnesium was associated positively with NPB BNC (r = 0.31, p = 0.03) and NBUD BNC (r = 0.32, p = 0.02). While red cell folate and sodium: potassium ratio was positively associated with NDI (r = 0.44, p = 0.006, r = 0.27, p = 0.06), magnesium, sodium, potassium, was negatively correlated with NDI (r = - 0.33, p = 0.02, r = - 0.28, p = 0.05, and r = - 0.32, p = 0.02 respectively).

Table 7.8: Correlation analysis between blood micronutrients and CBMN-Cyt biomarkers at six months

Infant Blood Micronutrients (mg/L)	MNBNC	NPBBNC	NBUDBNC	NDI	Apoptotic cells	Necrotic cells	MNMNC	NBUDMNC
Iron (n = 46)	*r = 0.13* *p = 0.3*	*r = - 0.11* *p = 0.4*	*r = 0.11* *p = 0.4*	*r = - 0.11* *p = 0.4*	*r = -0.13* *p = 0.3*	*r = 0.02* *p = 0.8*	*r = 0.19* *p = 0.1*	*r = - 0.07* *p = 0.6*
Copper (n=41)	*r = - 0.14* *p = 0.3*	*r = - 0.12* *p = 0.4*	*r = 0.08* *p= 0.6*	*r = 0.05* *p = 0.7*	*r = 0.04* *p = 0.7*	*r = - 0.02* *p = 0.8*	***r = 0.34*** ***p = 0.02****	*r = 0.15* *p = 0.3*
Calcium (n =46)	*r = 0.23* *p = 0.1*	*r = 0.09* *p = 0.5*	*r = - 0.09* *p = 0.5*	*r = -0.2* *p = 0.1*	*r = - 0.16* *p = 0.2*	***r = 0.3*** ***p= 0.04****	*r = 0.12* *p= 0.4*	*r = - 0.08* *p= 0.5*
Magnesium (n=45)	*r = 0.02* *p = 0.8*	*r = - 0.18* *p = 0.2*	***r = - 0.28*** ***p = 0.05****	***r = - 0.33*** ***p = 0.02****	*r = - 0.1* *p = 0.4*	*r = 0.05* *p = 0.7*	*r = 0.08* *p = 0.5*	*r = - 0.1* *p= 0.5*
Ca: Mg ratio (n =45)	*r = 0.06* *p = 0.6*	***r = 0.31*** ***p = 0.03****	***r = 0.32*** ***p = 0.02****	*r = 0.23* *p = 0.1*	*r = 0.11* *p = 0.4*	*r = 0.18* *p = 0.2*	*r = 0.08* *p = 0.5*	*r = 0.08* *p = 0.5*
Zinc (n =45)	*r = - 0.06* *p = 0.6*	*r = 0.02* *p = 0.8*	*r = 0.05* *p = 0.7*	*r = - 0.21* *p = 0.1*	*r = - 0.16* *p = 0.2*	*r = 0.06* *p = 0.6*	*r = 0.00* *p = 0.9*	*r = - 0.13* *p = 0.3*
Sodium (n = 46)	*r = 0.12* *p = 0.3*	*r = - 0.17* *p = 0.2*	*r =- 0.21* *p = 0.1*	***r = - 0.28*** ***p = 0.05****	*r = - 0.04* *p = 0.7*	*r = 0.03* *p = 0.7*	*r = 0.08* *p = 0.5*	*r = - 0.06* *p=0.6*
Potassium (n = 46)	*r = 0.07* *p = 0.6*	*r = 0.05* *p = 0.7*	*r = 0.11* *p = 0.4*	***r =- 0.32*** ***p = 0.02****	*r = -0.06* *p = 0.6*	*r = - 0.03* *p = 0.7*	*r = 0.23* *p = 0.11*	*r = - 0.11* *p = 0.4*
Na: K (n = 46)	*r = 0.01* *p = 0.89*	*r = - 0.18* *p = 0.22*	*r = - 0.20* *p = 0.16*	***r = 0.27*** ***p = 0.06***	*r = 0.1* *p = 0.5*	*r = 0.08* *p = 0.5*	*r = - 0.22* *p = 0.13*	*r = 0.12* *p = 0.41*
Phosphorus (n = 46)	*r = 0.00* *p = 0.9*	*r = - 0.06* *p = 0.6*	*r = 0.04* *p = 0.7*	*r = - 0.2* *p = 0.1*	*r = 0.02* *p = 0.8*	*r = - 0.05* *p = 0.7*	*r = 0.06* *p = 0.6*	*r = 0.07* *p = 0.6*
Sulphur (n = 46)	*r = - 0.08* *p = 0.5*	*r = - 0.15* *p = 0.3*	*r = - 0.24* *p = 0.1*	*r =- 0.23* *p = 0.1*	*r = - 0.08* *p = 0.5*	*r = 0.00* *p = 0.9*	*r = 0.13* *p = 0.3*	*r = - 0.06* *p = 0.6*
•Red cell folate (n =37)	*r = 0.07* *p = 0.6*	*r = 0.00* *p = 0.9*	*r = - 0.07* *p = 0.6*	***r = 0.44*** ***p = 0.006****	*r =0.00* *p = 0.9*	*r = 0.00* *p = 0.9*	*r =0.04* *p = 0.8*	*r = 0.15* *p = 0.3*

•: Lab values for Folate in nmol/L. *Abbreviations*: n = number of samples; MN: micronuclei; NPB: nucleoplasmic bridges; NBUD: nuclear buds; BNC: binucleated lymphocyte cells; MNC: mononucleated lymphocyte cells; NDI: nuclear division index; Ca: calcium; Mg magnesium; K: potassium, Na: sodium

7.6.6 **Correlation between micronutrients at birth, three and six months**

The micronutrients measured in cord blood were assessed for any correlation with values measured at three and six months, and the results are presented in **Table 7.9**. Zinc at birth was correlated with values at six months ($p = 0.04$). Magnesium measured at birth was correlated with that at three months ($p = 0.003$). The ratio of calcium to magnesium was correlated at birth and at three months ($p = 0.05$). Plasma sulphur also correlated at birth and at three months ($p = 0.04$). None of the other plasma micronutrients were observed to be correlated at any of the three time points.

Table 7.9: Correlation of plasma micronutrient concentrations at birth with those at three and at six months

Micronutrient	Birth and Three months(n = 48)			Birth and Six months (n = 39)		
	r value (95%CI)	p value	n	r value (95%CI)	p value	n
Iron	-0.09 (-0.38 to 0.21)	0.53	44	-0.10 (-0.42 to 0.23)	0.53	37
Copper	0.22 (-0.14 to 0.53)	0.22	32	0.25 (-0.10 to 0.55)	0.14	34
Zinc	-0.19 (-0.48 to 0.12)	0.23	39	0.32 (0.0008 to 0.5)	**0.04**	36
Calcium	-0.09 (-0.38 to 0.21)	0.54	44	0.17 (-0.16 to 0.48)	0.29	37
Magnesium	0.44 (0.15 to 0.66)	**0.003**	41	0.05 (-0.28 to 0.38)	0.74	37
Ca: Mg	0.30 (-0.006 to 0.55)	**0.05**	41	0.10 (-0.23 to 0.41)	0.55	37
Sodium	0.002 (-0.3 to 0.3)	0.98	44	0.26 (-0.07 to 0.55)	0.11	37
Potassium	-0.29 (-0.55 to 0.006)	**0.04**	44	-0.06 (-0.38 to 0.27)	0.70	37
Na: K	- 0.29 (-0.54 to 0.013)	**0.05**	44	- 0.07 (-0.40 to 0.26)	0.64	37
Phosphorus	-0.03 (-0.33 to 0.26)	0.81	44	0.18 (-0.15 to 0.47)	0.28	37
Sulphur	0.14 (-0.16 to 0.43)	0.34	44	0.28 (-0.05 to 0.56)	0.08	37
Red cell folate	0.27 (-0.1 to 0.5)	0.15	48	0.10 (-0.18 to 0.37)	0.48	39

r value: Pearson/spearman coefficient; p: level of significance (two tailed); n: number of subjects; CI: confidence interval (same cohort of infants for whom values were available for each time points were included for correlation analysis)

The correlation matrix for all blood micronutrients measured in cord blood at birth and in heel prick infant blood at three and six months are outlined in **Table 7.10, 7.11** and **7.12** respectively.

At birth, iron was positively associated with copper (r = 0.47, p =0.000), zinc (r = 0.48, p = 0.000) and negatively with calcium (r = - 0.63, p = 0.000). Copper was positively correlated to zinc (r = 0.25, p =0.01) and negatively to calcium (r = - 0.24, p = 0.02). Zinc and calcium were positively correlated with magnesium (r = 0.28, p = 0.009, r = 0.023, p = 0.03 respectively). Calcium was also related to sulphur (r = 0.27, p = 0.04). Magnesium was positively correlated to potassium (r = 0.22, p = 0.04) but negatively with sodium (r = - 0.34, p = 0.01). Sodium was also correlated with sulphur (r = 0.32, p = 0.003). Phosphorous showed positive association to sulphur (r = 0.31, p = 0.004).

Table 7. 10: Correlation Matrix of micronutrients measured in cord blood at birth

		Iron	Copper	Zinc	Calcium	Magnesium	Sodium	Potassium	Phosphorous	Sulphur	Red folate
Iron	r	1	.472**	.484**	-.630**	-.069	-.075	.173	-.020	-.236	-.060
	p		.000	.000	.000	.534	.588	.118	.885	.086	.602
Copper	r	.472**	1	.257*	-.241*	-.085	-.103	-.027	.087	.064	-.078
	p	.000		.019	.028	.443	.457	.808	.533	.646	.497
Zinc	r	.484**	.257*	1	.018	.284**	-.261	.190	-.126	-.265	-.029
	p	.000	.019		.875	.009	.057	.086	.362	.053	.802
Calcium	r	-.630**	-.241*	.018	1	.233*	-.093	-.166	.198	.273*	.186
	p	.000	.028	.875		.034	.504	.134	.151	.046	.104
Magnesium	r	-.069	-.085	.284**	.233*	1	-.344*	.225*	.033	-.137	-.023
	p	.534	.443	.009	.034		.011	.041	.813	.323	.839
Sodium	r	-.075	-.103	-.261	-.093	-.344*	1	-.047	-.037	.323**	.000
	p	.588	.457	.057	.504	.011		.736	.737	.003	1.000
Potassium	r	.173	-.027	.190	-.166	.225*	-.047	1	-.165	-.042	.146
	p	.118	.808	.086	.134	.041	.736		.232	.763	.203
Phosphorous	r	-.020	.087	-.126	.198	.033	-.037	-.165	1	.312**	.071
	p	.885	.533	.362	.151	.813	.737	.232		.004	.611
Sulphur	r	-.236	.064	-.265	.273*	-.137	.323**	-.042	.312**	1	.162
	p	.086	.646	.053	.046	.323	.003	.763	.004		.241
Red cell folate	r	-.060	-.078	-.029	.186	-.023	.000	.146	.071	.162	1
	p	.602	.497	.802	.104	.839	1.000	.203	.611	.241	

At three months iron showed positive association with zinc (r = 0.35, p = 0.006), sodium (r = 0.32, p = 0.01), potassium (r = 0.86, p = 0.000), sulphur (r = 0.41, p = 0.001) but negative with copper (r = - 0.44, p = 0.001) and magnesium (r = - 0.36, p = 0.005). Copper was associated positively with magnesium (r = 0.26, p = 0.04), phosphorus (r = 0.32, p = 0.01) and red cell folate (r = 0.43, p = 0.006). Zinc was positively correlated with sodium (r = 0.34, p = 0.009), potassium (r = 0.38, p = 0.003), sulphur (r = 0.33, p = 0.01) and negatively with magnesium (r = - 0.34, p = 0.009). Calcium showed positive associations with magnesium (r = 0.49, p = 0.000), sodium (r = 0.89, p = 0.000), potassium (r = 0.53, p = 0.000), phosphorous (r = 0.63, p = 0.000) and sulphur (r = 0.87, p = 0.000). Magnesium was associated positively with sodium (r = 0.41, p = 0.001), phosphorous (r = 0.50, p = 0.000) and sulphur (r = 0.34, p = 0.007). Sodium was associated positively with potassium (r = 0.58, p = 0.000), phosphorous (r = 0.59, p = 0.000) and sulphur (r = 0.89, p = 0.000). Potassium and phosphorous were also correlated with sulphur (r = 0.65, p = 0.000 and r = 0.63, p = 0.000 respectively) (**Table 7.11**).

Table7. 11: Correlation Matrix of micronutrients measure in heel prick infant blood at three months

		Iron	Copper	Zinc	Calcium	Magnesium	Sodium	Potassium	Phosphorous	Sulphur	Redfolate
Iron	r	1	-.441**	.357**	.238	-.364**	.323*	.864**	-.069	.415**	-.144
	p		.001	.006	.072	.005	.013	.000	.604	.001	.381
Copper	r	-.441**	1	-.187	.067	.264*	-.013	-.242	.323*	.047	.432**
	p	.001		.159	.617	.045	.925	.067	.013	.725	.006
Zinc	r	.357**	-.187	1	.140	-.341**	.342**	.385**	.016	.337**	-.030
	p	.006	.159		.294	.009	.009	.003	.906	.010	.857
Calcium	r	.238	.067	.140	1	.490**	.893**	.537**	.631**	.875**	-.016
	p	.072	.617	.294		.000	.000	.000	.000	.000	.922
Magnesium	r	-.364**	.264*	-.341**	.490**	1	.410**	-.078	.501**	.348**	.070
	p	.005	.045	.009	.000		.001	.562	.000	.007	.671
Sodium	r	.323*	-.013	.342**	.893**	.410**	1	.587**	.591**	.894**	.039
	p	.013	.925	.009	.000	.001		.000	.000	.000	.815
Potassium	r	.864**	-.242	.385**	.537**	-.078	.587**	1	.162	.658**	.021
	p	.000	.067	.003	.000	.562	.000		.225	.000	.897
Phosphorous	r	-.069	.323*	.016	.631**	.501**	.591**	.162	1	.635**	.223
	p	.604	.013	.906	.000	.000	.000	.225		.000	.173
Sulphur	r	.415**	.047	.337**	.875**	.348**	.894**	.658**	.635**	1	.147
	p	.001	.725	.010	.000	.007	.000	.000	.000		.372
Red folate	r	-.144	.432**	-.030	-.016	.070	.039	.021	.223	.147	1
	p	.381	.006	.857	.922	.671	.815	.897	.173	.372	

Number of samples for micronutrients ranged from 39-58, r: Correlation coefficient, p: significance (two way), *: p<0.05, **: p< 0.01.

At six months, iron was positively associated with calcium (r = 0.45, p = 0.001), magnesium (r = 0.32, p = 0.02), sodium (r = 0.59, p = 0.000), potassium (r = 0.90, p = 0.000), phosphorus (r = 0.67, p = 0.000) and sulphur (r = 0.72, p = 0.000). While copper was not associated with any micronutrient, zinc showed negative association with magnesium (r = - 0.39, p = 0.005). Calcium was positively correlated with magnesium (r = 0.58, p = 0.000), sodium (r = 0.77, p = 0.000), potassium (r = 0.52, p = 0.000), phosphorous (r = 0.58, p = 0.000) and sulphur (r = 0.74, p = 0.000). Magnesium was positively correlated with sodium (r = 0.44, p = 0.001), potassium (r = 0.35, p = 0.01), phosphorous (r = 0.39, p = 0.006) and sulphur (r = 0.41, p = 0.003). Sodium was positively associated with potassium (r = 0.72, p = 0.000), phosphorous (r = 0.74, p = 0.000) and sulphur (r = 0.91, p = 0.000). Potassium was positively correlated with phosphorous (r = 0.77, p = 0.000) and sulphur (r = 0.79, p = 0.000). Phosphorus showed positive association with sulphur (r = 0.78, p = 0.000) (**Table 7.12**).

Table 7. 12: Correlation Matrix of micronutrients measured in heel prick infant blood at six months

		Iron	Copper	Zinc	Calcium	Magnesium	Sodium	Potassium	Phosphorous	Sulphur	Red folate
Iron	r	1	-.013	.167	.454**	.326*	.597**	.902**	.677**	.729**	-.027
	p		.931	.250	.001	.022	.000	.000	.000	.000	.877
Copper	r	-.013	1	-.144	.190	.150	-.140	-.149	-.111	-.084	.016
	p	.931		.324	.192	.304	.337	.306	.447	.568	.929
Zinc	r	.167	-.144	1	.109	-.392**	.205	.227	.232	.252	-.006
	p	.250	.324		.455	.005	.157	.116	.109	.080	.973
Calcium	r	.454**	.190	.109	1	.587**	.774**	.523**	.589**	.741**	-.099
	p	.001	.192	.455		.000	.000	.000	.000	.000	.570
Magnesium	r	.326*	.150	-.392**	.587**	1	.446**	.351*	.390**	.415**	-.198
	p	.022	.304	.005	.000		.001	.013	.006	.003	.254
Sodium	r	.597**	-.140	.205	.774**	.446**	1	.727**	.748**	.914**	-.233
	p	.000	.337	.157	.000	.001		.000	.000	.000	.179
Potassium	r	.902**	-.149	.227	.523**	.351*	.727**	1	.773**	.793**	-.068
	p	.000	.306	.116	.000	.013	.000		.000	.000	.699
Phosphorous	r	.677**	-.111	.232	.589**	.390**	.748**	.773**	1	.781**	-.201
	p	.000	.447	.109	.000	.006	.000	.000		.000	.247
Sulphur	r	.729**	-.084	.252	.741**	.415**	.914**	.793**	.781**	1	-.170
	p	.000	.568	.080	.000	.003	.000	.000	.000		.329
Red cell folate	r	-.027	.016	-.006	-.099	-.198	-.233	-.068	-.201	-.170	1
	p	.877	.929	.973	.570	.254	.179	.699	.247	.329	

7.6.7 **Effect of mode of feeding on genome damage biomarkers at three months**

To test the hypothesis that mode of feeding adopted for infants at three and six months may influence frequency of CBMN biomarkers assessed in lymphocytes collected from infants, correlation analysis was performed. We did not observe significant correlation between CBMN-Cyt biomarkers and feeding scores for either male or female or combined infants in the cohort at three months (**Table 7.13).**

Table 7.13: Correlation analysis of CBMN biomarkers and average feeding scores at 3 months

	Total (n=64)		**Female (n=32)**		**Male (n=31)**	
	'r'	p-value	'r'	p-value	'r'	p-value
MN BNC	-0.01	0.91	- 0.05	0.7	0.11	0.5
NPB BNC	0.07	0.62	0.17	0.3	- 0.28	0.1
NBUD BNC	0.16	0.25	- 0.02	0.8	0.24	0.1
NDI	-0.06	0.67	- 0.21	0.2	0.11	0.5
Apoptotic lymphocyte	0.06	0.65	- 0.22	0.2	0.12	0.4
Necrotic lymphocyte	-0.001	0.99	- 0.16	0.3	0.02	0.8
MN MNC	-0.03	0.84	0.04	0.8	-0.09	0.6
NBUD MNC	-0.15	0.32	-0.20	0.2	-0.17	0.3

Each DNA damage biomarker was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution);
Abbreviations:MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNCs, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD are presented per 100 MNCs]

7.6.8 **Effect of mode of feeding on genome instability biomarkers at six months**

At six months, combined cohort was not observed to have any association between average feeding scores and CBMN-Cyt biomarkers. The female cohort was observed to have significant association of NPB BNC with average feeding scores (r = 0.41, p = 0.05, 95% CI: - 0.01 to 0.7). In the male cohort NBUD BNC measured in was negatively correlated with average feeding scores (r = - 0.39, p = 0.03, 95% CI: -0.67 to-0.02 (**Table 7.14**).

Table 7. 14: Correlation analysis of CBMN-Cyt biomarkers and average feeding scores at 6 months

	Total (n=53)		**Female (n=23)**		**Male (n=29)**	
	'r'	p-value	'r'	p-value	'r'	p-value
MN BNC	-0.13	0.41	-0.03	0.8	-0.25	0.1
NPB BNC	-0.03	0.83	0.41#	0.05*	-0.02	0.8
NBUD BNC	-0.23	0.14	- 0.02	0.9	-0.39# #	0.03*
NDI	0.04	0.80	0.00	0.9	0.08	0.6
Apoptotic lymphocyte	0.09	0.55	0.13	0.5	0.03	0.8
Necrotic lymphocyte	-0.03	0.82	- 0.11	0.5	-0.12	0.5
MN MNC	0.25	0.12	0.21	0.3	0.04	0.8
NBUD MNC	0.05	0.72	0.05	0.8	0.07	0.7

Each DNA damage biomarker was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution);
Significance: *p ≤ 0.05; # 95%CI:-0.01 to 0.7; # # 95% CI: -0.67 to-0.02
Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNCs, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD are presented per 100 MNC

7.6.9 **Gender differences in micronutrients measured at birth, three and six months**

The differences in mean (± SEM) blood micronutrients were assessed between male and female infants at birth, three and six months, and are represented in Tables 7.15, 7.16 and 7.17 respectively.

At birth, there was significant difference observed in concentration of phosphorous (p = 0.03) (**Table 7.15**). At three months, there were significant gender differences observed for plasma calcium, sodium and sulphur concentrations (p = 0.01, p = 0.03, p = 0.03 respectively) and

results are shown in **Table 7.16**. At six months after birth, there were no significant gender differences for any of the micronutrient measured and the results are shown in **Table 7.17.**

Table 7. 15: Gender differences in blood micronutrients at birth, (Mean ± SEM)

Blood Micronutrients (mg/L)	Mean (± SEM) at Birth		Student t test (two tailed)	Significance
	Male (n = 38)	Female (n = 39)		
Iron	6.56 (± 0.93)	9.8 (±2.0)	p = 0.22	NS
Copper	0.42 (± 0.02)	0.40 (± 0.03)	p = 0.23	NS
Zinc	1.00 (± 0.02)	0.99 (± 0.02)	p = 0.8	NS
Calcium	104.4 (± 1.45)	104.7 (± 1.88)	p = 0.60	NS
Magnesium	17.37 (± 0.28)	18.0 (± 0.36)	p = 0.17	NS
Ca: Mg ratio	6.06 (± 0.13)	5.8 (± 0.14)	p = 0.5	NS
Sodium	3019 (± 19.3)	2999 (± 37.1)	p = 0.9	NS
Potassium	392 (± 18.9)	430 (21.4)	p = 0.19	NS
Phosphorus	101 (± 1.8)	107 (± 2.3)	p = 0.03	*
Sulphur	956 (± 20.2)	991 (± 16.8)	p = 0.18	NS
Vitamin B_{12} (pmol/L)	440 (± 38.6)	462 (± 50.0)	p = 0.9	NS
Serum Folate (nmol/L)	62.2 (± 3.0)	64.5 (± 2.7)	p = 0.6	NS
Red cell Folate (nmol/L)	381 (± 10.2)	388 (± 12.1)	p = 0.6	NS

Table 7.16: Gender differences in blood micronutrients at three months (Mean ± SEM) after birth

Blood Micronutrients (mg/L)	Mean (± SEM) at three months		Student t test (two tailed)	Significance
	Male (n =31)	Female (n = 27)		
Iron	3.05 (± 0.40)	3.17 (0.37)	p=0.70	NS
Copper	0.86 (± 0.05)	0.89 (± 0.06)	p=0.76	NS
Zinc	1.26 (± 0.11)	2.38 (± 0.80)	p= 0.33	NS
Calcium	108.7 (± 1.33)	116.2 (± 2.46)	**p=0.01**	*
Magnesium	21.7 (± 0.37)	23.1 (± 0.58)	p=0.17	NS
Ca: Mg ratio	5.05 (± 0.06)	5.06 (± 0.07)	p=0.94	NS
Sodium	3239 (± 30.8)	3401 (± 67.2)	**p=0.03**	*
Potassium	201 (± 6.02)	211 (8.31)	p=0.65	NS
Phosphorus	137.2(± 2.91)	147.5 (± 5.15)	p=0.21	NS
Sulphur	988 (± 10.9)	1061 (± 26.9)	**p= 0.03**	*
Red cell Folate (nmol/L)	290 (± 37.14)	241 (28.94)	p= 0.55	NS

Table 7.17: Gender differences in blood micronutrients at six months (Mean ± SEM) after birth

Blood Micronutrients (mg/L)	Mean (± SEM) at six months		Student t test (two tailed)	Significance
	Male (n = 26)	Female (n =22)		
Iron	3.75 (± 0.49)	7.69 (4.2)	p=0.62	NS
Copper	1.04 (± 0.04)	1.11 (± 0.07)	p=0.40	NS
Zinc	1.78 (± 0.27)	1.38 (± 0.17)	p= 0.41	NS
Calcium	108.5 (± 1.73)	107.5 (± 2.23)	p=0.51	NS
Magnesium	23.6 (± 0.52)	24.4 (± 0.78)	p=0.35	NS
Ca: Mg ratio	4.64 (± 0.06)	4.44 (± 0.08)	p=0.07	NS
Sodium	3373 (± 50.2)	3380 (± 69.6)	p=0.90	NS
Potassium	217 (± 7.42)	233 (20.9)	p=0.98	NS
Phosphorus	141 (± 3.36)	141 (± 5.61)	p=0.62	NS
Sulphur	1048 (± 15.5)	1058 (± 29.5)	p= 0.81	NS
Red cell Folate (nmol/L)	330 (± 10.9)	323 (15.1)	p= 0.54	NS

7.7 Discussion

Approximately 40 micronutrients have been identified including Vitamin B_{12}, folate, iron, zinc, calcium, magnesium and sulphur which are essential in optimal amounts from the human diet to maintain normal health (409,410,429,435,438,451,456,468,470,490,499,763,781). Many of these micronutrients, alone or concomitantly, are substrates and/or cofactors in the metabolic pathways regulating DNA synthesis and/or repair and gene expression (517,763,782,783). An infant is dependent on optimal intakes of these micronutrients through either breast milk or complementary feeds (784,785). There is increasing evidence that deficiency of these micronutrients may cause DNA replication errors and DNA repair defects as well as inducing a pro-inflammatory status in humans that may translate into genome instability (145,254,255,298,299,371,517,521,653,703,786,787). Hence, this prospective study was designed to assess correlations between concentrations of a subset of blood plasma micronutrients (iron, copper, zinc, calcium, magnesium, sodium, potassium, phosphorous, sulphur, vitamin B_{12} and red cell folate) and CBMN-Cyt assay biomarkers, infant birth outcomes and feeding scores during the first six months of life. The study consisted of infants born to South Australian mothers who were of low risk of complications during pregnancy.

7.7.1 Blood micronutrients and maternal anthropometric data and infant birth outcomes

In the cohort of DADHI study, mother's weight, height and BMI, recorded at recruitment (8-16 weeks gestation), were not associated with any micronutrient assessed in cord blood. Maternal anthropometric parameters are indicators of nutritional status. Few studies that have investigated effect of maternal anthropometry on cord micronutrient status have reported association of overweight and obesity with low iron (788), folate (789), vitamin D (790) and zinc (791) in cord blood. In the NewGeneris cohort, maternal serum vitamin D (<50 nmol/L recorded at 14-18 weeks of gestation) was associated with increased MN BNC frequency in cord blood [incidence rate ration (IRR= 1.32 (95%CI: 1.00, 1.72)]. This increase was higher for newborns with birth weight above the third quartile [≥ 3.5 kg; IRR = 2.21 (1.26, 3.89)] (310) indicating epigenetic influence of maternal factors on infants' metabolic profile.

A prospective cohort study on 15 obese (BMI > 30 kg/ m^2) and 15 lean (BMI <18–25 kg/ m^2) women reported significantly lower levels of vitamin B_6, vitamin C, vitamin E, RBC folate and higher CRP and IL-6 levels along with higher ratio of oxidized to reduced glutathione among obese women compared to lean counterparts. Though, the study did not find any differences in cord micronutrient concentrations between infants born to either group of women, but folate, vitamin B_6 and zinc levels correlated strongly between mother and infant (789). A Spanish cohort study investigated effect of maternal weight on iron status that was determined by measuring serum transferrin receptor and ferritin levels at 24 and 34 weeks and at delivery in cord blood. There was no significant effect of maternal BMI on any of the haematological parameters. However, transferrin saturation in cord blood was found to be negatively correlated with maternal BMI ($r = -0.2$, $P = 0.032$ $n = 97$) (788). The reason for non-association observed between maternal weight or BMI with cord micronutrients in our cohort may be attributed to normalcy status of mothers with respect to their mean BMI [25.3 (±

3.7) kg/m^2], mean weight [67.3 (± 11.9) kg] and low risk of medical complication during pregnancy.

In our cohort, iron was not associated with infant weight at birth and at three months. The reason for this non-association may be that iron deficiency is often observed in low birth weight neonates and occurs mainly in underdeveloped and developing countries (792). At birth, infants can be classified either by gestational age (GA) or by weight. Neonates born < 37 weeks are classified as preterm, < 28 weeks as very preterm and < 26 weeks as extremely preterm infants. Our cohort with mean (± SD) GA of 39.7 (±1.1) weeks were all considered to be of normal term (mean birth weight=3463 g, mean GA=39.7 weeks) (Appendix 5-12) from healthy mothers living in Australian population. A Spanish cross-sectional study on paired healthy pregnant mothers and their infants (n=54) investigated the association between 10 trace minerals including iron in cord blood plasma and anthropometric measurements at birth. They also did not find any association between cord iron concentration and birth weight of infants who were categorized according to their weight as small, normal or large for gestational age (793). We observed that infant weight at six months was positively associated with plasma iron ($r = 0.3$, $p = 0.02$). We also found that a significant decline in plasma iron concentration occurred from birth to 6 months. A previous population study of 800 in- and outpatients (0-18 years) that aimed to define paediatric reference intervals (2.5th – 97.5th percentiles) in Washington, DC, also observed a decrease in plasma iron from 0.72-2.35 mg/L (n=76 aged 0-90 days) to 0.23-1.92 mg/L (n=92, aged 91 days to 12 months) (794). In a healthy, normal birth weight infant at term, most of the body iron is found in haemoglobin, while a quarter of total body iron is localized as iron stores in liver (795). However, a fall in iron concentrations as measured by haemoglobin concentrations has been observed during the first few weeks of life, and has been attributed to a change in infant's environment from a relatively hypoxic uterus to the oxygen-rich atmosphere (796). An infant of normal birth weight expands its blood volume while it doubles its birth weight, which occurs at about 6 months of age (796) which may help

to explain the positive association between plasma iron and infant weight that we observed at 6 months.

In the current study a positive association of cord blood copper concentrations was observed with GA ($r = 0.4$, $p < 0.0007$). Similar findings were reported in a French study that aimed to establish reference serum micronutrient values for GA ($r = 0.5$, $p < 0.001$, n=245 term infants) (797). A decreasing trend (though not significant) was observed for cord plasma copper concentration and GA in a small uncomplicated mother-infant cohort study (n=35) (798). Also the increase in plasma copper concentrations in infants from birth to six months that we observed was similar to data from a longitudinal study (n=105 healthy breast fed infants, aged 2, 6 and 12 months) in Turkey (799). Although breast milk is low in copper, deficiency appears to be rare in premature infants fed breast milk, which may be a result of the higher bioavailability of copper from human milk than from cow milk or formula (442). Furthermore, the rapid increase in serum copper concentrations after birth may indicate sufficient copper stores and minimal losses through gastrointestinal tract in infants (800).

The concentration of **calcium** was found to be significantly different at three months compared to birth values but remained unchanged between three and six months. Nevertheless, over the period of six months, no significant increase or decrease was observed. This result is different from a non-blinded study of 132 breast fed infants that reported a decrease in plasma calcium ($p < 0.05$) from birth to twelve months but the infants were administered vitamin D3 supplements (400-600 IU) (801) which may have influenced calcium concentration as vitamin D is known to modulate calcium homeostasis (451). Our observation of a negative association of plasma calcium and head circumference of infants at birth ($r = - 0.37$, $p=0.01$) and infant weight at three months ($r = -0.3$, $p = 0.003$) is contrasted with a prospective cohort study in Turkey on 70 neonates, which reported a positive correlation of plasma calcium with birth weight, birth length and head circumference ($r = 0.308$, $p = 0.009$, $r = 0.324$, $p = 0.006$, $r = 0.296$, $p = 0.013$ respectively) (802). The reasons for this correlation could not be explain. Head

circumference is a measurement of a child's head around its largest area. A measure above the normal percentile may be a sign of hydrocephalus. A very small head size (microcephaly), on the other hand, indicates very slow growth rate and improper brain development (341). When compared with reference WHO growth charts, the mean head circumference for male and female infants in our cohort was at the 50th percentile (**Appendix 5 and 6**) which is usually regarded as normal within a population (336,345). Whether *in utero* environment could have influenced (803,804) need to be further investigated along with measures of urinary excretion of calcium and other hormones involved in calcium regulation, such as parathyroid hormone and vitamin D to understand the association of calcium on infant head circumference.

A significantly increasing trend in plasma **magnesium** concentration was evident from birth to three and six months as observed in other studies (794,805,806) and may be attributed to an increase in infant weight, height and bone growth of infants, given that more than 99% of the body's magnesium is located intracellularly, in bone and skeletal muscle (807). Mean plasma magnesium values at birth (17.7 mg/L or 0.73 mMol/L) (Appendix 13) are close to mean serum magnesium (0.76 mMol/L) reported in cord blood by Fenton et al in a cross sectional study in Canada for healthy term infants (n=53, GA >36 weeks) (808). They also found a negative association of serum magnesium with GA (multiple regression coefficient = - 0.007, p = 0.006) (808), although no such association was evident in our study.

In our cohort, plasma **zinc** concentrations did not show any linear trend from birth to six months among. No significant correlation was found between zinc measured in the cord blood and infant's weight at birth, three and six months. A number of other studies also confirm our observations that there was no association between birth weight and cord zinc (809-811).

A significant increase in mean plasma **sodium** (Na^+) and a concomitant decrease in mean plasma potassium (K^+) were observed in the infants in our study between birth and 6 months although the concentrations were within the normal acceptable physiological range (Appendix 13 and 14). The transition from foetal to newborn life is associated with major changes in water

and electrolyte homeostatic control (477,812). Newborns must rapidly assume fluid and electrolyte homeostasis in an environment in which fluid and electrolyte availability and loss fluctuate much more widely than in utero (813,814). Na^+ and K^+ are the most abundant cations in biological systems. Na^+ ions are mainly present at high concentrations extracellularly, (along with $Chloride^-$ ion) whereas K^+ ions are present at high concentrations intracellularly (along with Mg^{++}) (814). The ionic concentrations in the intracellular and extracellular compartments are inversely proportional to each other. The shift in plasma Na^+ and K^+ concentrations, as observed in our cohort, is commonly observed in normal weight infants during first year of life as the total water content decreases while their weight increases (477). This could explain the negative association that was found between infant mean weight and mean plasma Na^+ at three months ($r = -0.4$, $p = 0.001$). An inverse association of GA with plasma K^+ has previously been observed in a retrospective study of 95 premature infants in Taiwan ($p < 0.05$) (815) confirming our observations. This may represent the body's attempt to maintain homeostasis in intracellular K^+ concentrations, along with other extracellular cations, such as calcium and Na^+, in order to prevent excess or deficiency of either (812).

Plasma **phosphorus** concentrations increased from birth to six months in our cohort and were within the normal physiological range (**Appendix 13**). Phosphorus is a critical element for skeletal development, bone mineralization, membrane composition, nucleotide structure, and cellular signalling (816). The predominant form of phosphorus as it exists in the body is the phosphate ion $(PO_4)^{3-}$. About 85% of phosphate is found in bone and teeth that are being formed during the first six months of life (816). A complex interplay of intestinal absorption, exchange with intracellular and skeletal storage pools, and renal tubular reabsorption aids in the maintenance of normal blood phosphate concentrations (479). Phosphate balance is regulated by vitamin D and parathyroid hormone (817), as well as by fibroblast growth factor 23 derived from the skeleton (818,819). It may be noted that the trend to an increase in plasma calcium matched the reverse trend of plasma phosphorus from birth to six months, and may indicate

normal homeostasis for these ions in our cohort (820). However, further investigations in a larger cohort, with added measures of urinary phosphorus, total body mineral accretion, serum alkaline phosphatase activity, formula/breast milk contents are required to understand critical role of phosphorus homeostasis in infants (821).

Sulphur is an essential nutrient and plays an important role in cellular energy production and regulation of DNA replication and transcription (484,486). Sulphur mainly circulates in combination with other elements in complex molecules, such as sulphates (487). In the current study it was found that GA was not correlated with plasma sulphur as was previously reported in a study that measured plasma sulphate in cord blood (n=80 healthy term infants) (822). The mean plasma sulphur concentrations increased from birth to six months and were negatively associated with APGAR score at birth. The primary source of sulphur in the human body is in the sulphur-containing amino acids: methionine, cysteine and derivatives, such as taurine. Sulphur may also circulate as other complexes, such as inorganic sulphate: extracellular inorganic sulphate is an important pool for intracellular sulphation (485). Whether the increase in plasma sulphur observed during the first six months of life may be attributed to an infant's growing capacity to absorb sulphur from amino acids obtained through breast milk or complementary feeds requires further investigations (487).

Cord **vitamin B_{12}** concentrations were associated with GA in this cohort. The relation between cord vitamin B_{12} and GA has been the subject of few studies but majority of them have investigated cord vitamin B_{12} concentration in infants born to women who have either been supplemented with folic acid or iron and/folic acid and/or vitamin B_{12} (823,824). Hence further investigations are required in a larger cohort of neonates to understand possible explanation for this finding (510) along with other cofactors in carbon metabolism (riboflavin and vitamin B_6) and methyl malonic acid (MMA: a well-recognised marker of B_{12} status) and Hcy (510,825,826).

Folate is required for normal growth and development for a human infant owing to its indispensable role in cellular proliferation, gene expression, DNA synthesis and repair (78,408,498,499,827,828). We observed a 16% decrease in red cell folate (RCF) concentration in the infants from birth to six months ($p < 0.0001$). A similar decline in folate was also observed in an older study (829) where infant folate status was assessed in serum as well as whole blood at 3-6 days, 3-4 months, 6-8 months and at 12 months (n=24, normal full term infants). In the current study we also found that infant weight was positively associated with RCF at three months ($r = 0.2$, $p = 0.05$), indicating folate's role in growth and development (78).

7.7.2 Association of blood micronutrients and CBMN-Cyt biomarkers profiles in infants

Iron

It was found that iron at birth was negatively associated with NBUD MNC ($r = -0.28$, $p = 0.001$) and with apoptotic lymphocytes at three months ($r = -0.32$, $p = 0.01$). A previous cohort study comprising of young children (n=30, mean age 11.5 yrs) of poor economic status in Brazil, also found a negative association between the presence of both MN and NPB with red cell iron status ($r = -0.9$, $p = 0.002$; $r = 0.9$, $p = 0.01$ respectively) (434). Iron deficiency may impair enzymes involved in antioxidant function (e.g. catalase) and nucleic acid metabolism (e.g. DNA glycosylases) (479) leading to increased oxidative stress, decreased antioxidant defences respectively in iron deficient subjects (435), immune system dysfunction and possibly an increased risk of cancer (436). In addition to pathologies associated with iron deficiency, an excess of iron has been shown to be highly toxic. Iron-mediated reactive oxygen species generated via the Fenton reaction may lead to point mutations in DNA, DNA adducts such as the modified guanosine base 8-hydroxydeoxyguanosine (8-OHdG), cell apoptosis and necrosis (439,830,831). Excess iron deposition within the liver has been proposed as a cause of necro-inflammation and fibrosis and production of pro-inflammatory cytokines (436). Iron overload also induces DNA hypermethylation and can reduce telomere length (435). Additional research

is therefore required in order to understand the role of iron homeostasis in neonates and infants. A further consideration is that the optimal intake of iron required to prevent genomic damage may not necessarily be the same as that for anaemia prevention (407).

Copper

A significant positive association was observed between plasma copper with MN MNC at six months ($r = 0.34$, $p = 0.02$). Though copper deficiency is rare in humans, an adequate intake of 0.20 mg/d is recommended in Australia for infants (0-6 months), based on the copper content of breast milk (483). Copper homeostasis is integral to human cell growth and cell protection as it is a functional component of human the endogenous antioxidant superoxide dismutase (441). Many enzymes harness the changes in the bound copper oxidation state, in the presence of oxygen, to catalyse redox chemistry for both cell proliferation and signalling (444). Similar to copper deficiency, excess copper may also result in oxidative stress through the ability of free copper to catalyse the reaction between superoxide anion and hydrogen peroxide producing the hydroxyl radical (441). This observation may explain the association of copper with MN MNC because MN can be generated from acentric chromosomes fragmentation induced by oxidative stress. However further investigations into copper homeostasis, role of copper and copper mediated proteins in cell signalling, gene expression is required in a larger cohort to understand this association.

Calcium

In the current study a positive association was observed between calcium and necrotic cells at six months ($r = 0.3$, $p= 0.04$). A previous cross sectional study in South Australia comprising of healthy children (3, 6 and 9 years, n=462) also reported positive associations of plasma calcium with both MN ($p = 0.01$) and necrosis ($p = 0.05$) (529). Though the mean calcium concentration was normal in our cohort (Appendix 5 and 6), dysfunction in the homeostasis of calcium ions in the cells may elicit mitochondrial dysfunction and generation of reactive oxygen

species and DNA damage (448) that in turn may influence propensity to necrosis and cytotoxicity. However, further investigation, is needed especially in view of the possible role of calcium ions in modulating oxidative stress via the mitochondrial aspartate/glutamate carrier in the brains of autistic children (447). This may perhaps also relate to the finding that intranuclear calcium mediates the regulation of DNA structure and various nuclear functions, particularly during cellular differentiation or regeneration (445,446).

Magnesium

At birth, there was a positive association of magnesium with MN MNC and at six months, a negative association was observed between magnesium and NBUD BNC and NDI, suggesting that magnesium concentration is inversely associated with chromosomal instability and mitogen response respectively. We also find positive association between Ca: Mg ratio and MN BNC at birth NBUD MNC at three months NBUD and NPB in BNC at six months suggesting that low magnesium status relative to calcium may be a risk factor for increased DNA damage and chromosomal instability. To our knowledge, this is the first time that such an association has been observed in humans. Magnesium as a a cofactor for DNA polymerase and DNA repair enzymes (N-Methylpurine-DNA glycosylase, apurinic/apyrimidinic endonuclease, DNA polymerase beta, and ligases) (832) is crucial for the regulation of the cell cycle, as well as for cell proliferation, apoptosis, and differentiation (833). The role of magnesium in DNA stabilization is concentration dependent: at high cellular concentrations of magnesium, there is an accumulation of magnesium binding, which can induce conformational changes in DNA, while at low concentrations, there is destabilization of DNA (834) that may cause initiation of diseases, such as cancer (111,453). Deficiency of magnesium may prove to be carcinogenic under conditions that lead to dysregulation of amino-acid metabolism and the immune system function that may increase free radical species in the cell (410,453,456,835). Interestingly, it has also been shown that concentrations of free intracellular magnesium increase in cells

undergoing apoptosis, indicating that intracellular pools of magnesium (that are dependent on various physiological hormonal factors, as well as on calcium and phosphate ion concentrations) regulate cell cycle control and apoptosis (410).

Zinc

A positive association observed between zinc and NPB BNC, apoptotic cells and negative association with NBUD at three months suggests importance of zinc homeostasis in DNA maintainence (469) and has been reported earlier (470,530). Higher than normal cellular concentrations of zinc (32 - 100 µM) reduced cell viability and increased DNA damage in an *in vitro* study utilizing cultured human oral keratinocytes and lymphocytes. (787). At the cellular level, 30–40% of zinc is localized in the nucleus, 50% is found in the cytosol and the remaining part is associated with membranes (469). Cellular zinc concentrations are minutely controlled through an efficient homeostatic mechanism that avoids accumulation of excess zinc under the regulation of various transporter and imported proteins (469), because dysregulation (either excess or deficiency) may cause oxidative stress and subsequent DNA damage (414,463,464,479). In a study of 462 children aged 3 to 9 years, negative association of plasma zinc status and telomere length was observed (529). Telomere shortening is associated with NPB formation and may explain the positive association between plasma zinc and NPB in this cohort although that was not observed in the study with children.

Sodium and Potassium

A significant negative correlation was apparent between plasma sodium and NDI at 6 months. A negative association between plasma potassium and NDI was also evident at six months. Gradients for these ions across the cell membrane provide the energy source for action potentials generated by opening of Na^+ and K^+ channels, as well as for transporting solutes and other ions across the cell membrane via coupled transporters (476). Even transient changes in the electrolyte balance influences membrane permeability and eventually cell growth (475,836). K^+ channels are expressed differently in various lymphocyte subsets, such as naïve

and regulatory human T cells (475,837,838), and have been shown to potentiate calcium mediated cellular proliferation and migration (478). Na^+ ions contribute to the stabilization of large helix nucleic acids structure (839), along with other ions such as K^+ and Mg^{2+}, thereby influencing the stability and the folding kinetics of nucleic acids during replication (839). Interestingly, a change in intracellular sodium has been detected as part of the programmed cell death process in *in vitro* studies, as apoptotic and necrotic cells exhibit cell shrinkage and volume decrease (481). This could explain the negative association that was observed of both plasma sodium and plasma potassium and the NDI which is a marker of cellular proliferation.

Vitamin B_{12}

No association was found between serum vitamin B_{12} concentrations with any of the DNA damage biomarkers at birth. Vitamin B_{12} plays an important role in DNA metabolism by acting as cofactor in the folate-methionine cycle (408). Another cross sectional study in South Australia conducted on young children (462 healthy children 3, 6, and 9 years of age) also reported no association between vitamin B_{12} with DNA damage biomarkers (529). Both in younger (20-40 years) and older adults (50-70 years), serum vitamin B_{12} concentrations below 150 pmol/L were negatively associated with MN frequency (171,531), however, the mean serum vitamin B_{12} concentration in our cohort at birth (**Appendix 13**) was above that considered to be detrimental to genome health (<300 pmol/L) (242) which may explain non association of Vitamin B_{12} with DNA damage biomarkers in our study.

Sulphur

Plasma sulphur showed a weak positive association with NPB (r= 0.2, p=0.06) at birth. Apart from its presence in the essential amino acids, methionine and cysteine, sulphur is also a component of inflammation-enhancing compounds, such as homocysteine, in human body, as well as being found in various environmental pollutants, such as mustard sulphur and sulphur dioxide (491). Experiments in rats have shown these compounds to be genotoxic (491,840). In

order to understand any effect of sulphur on infant DNA, various other sulphur metabolites, such as sulphate, homocysteine and urinary sulphur metabolites, need to be investigated in order to understand sulphur kinetics in infants.

Phosphorous

At three months, plasma phosphorus was observed to be negatively associated with NDI (r= -0.3, p= 0.02). As phosphorus is a component of cell membranes (as part of phospholipids), a contributor to cell regulation and signalling, and a structural component of, DNA and RNA and energy transfer molecule such as adenosine triphosphate (841), this observation need to be investigated in a large cohort along with other factors such as vitamin D and parathyroid status that may also influence its concentration.

Red cell folate

At six months, red cell folate was associated positively with NDI. A similar association has been previously reported in an *in vitro* study on human lymphocytes (842). The demand for folate is greatly enhanced throughout the time of rapid growth among humans, such as during pregnancy and the neonatal years (204,843). The role of folate in DNA synthesis, repair and in the maintenance of genome integrity has been extensively reviewed (145,409,513,844,845). Hence, the normal concentration of RCF (319.9 nmol/L at six months age, **Appendix 13**) observed in our cohort to be associated with NDI validates the indispensable role of folate in cell proliferation. We also found that RCF was positively associated with necrosis at three months. This is in contradiction to previous data that demonstrated increase in necrotic cells *in vitro* under folate deprivation (846). However, it may be noted that it was a small sample size and this association was observed at three months when a fall in mean plasma concentration of folate (42%) was evident in the cohort. The reasons for this decline and subsequent increase at six months may be attributed to increase frequency of formula feed in this cohort between 3 and 6 months.

7.7.3 **Blood micronutrients, mode of feeding and gender differences**

In current study, it was shown that plasma calcium and sulphur concentrations were positively correlated with feeding scores at 6 months. The majority of Australian infants have been reported to have commenced either formula or solid feeds before 6 months (406,847) as observed in our cohort also. When we compared exclusively breast fed (n =19) and exclusively formula fed infants (n =9) at 6 months in our cohort, formula fed infants were heavier (p = 0.03) but had significantly lower calcium concentrations at 6 months (p = 0.01) while there was no difference in the sulphur concentrations. Whether this finding may be due to concentration of calcium in breast milk (264 mg/L) (483) or calcium content of formula milks [which is usually kept higher (12 mg/100 kJ) to compensate for the low bioavailability of calcium from formulas] (483,848) requires further investigation in a larger cohort. The differences in plasma phosphorous, calcium, sodium and sulphur concentrations among male and female infants (with no difference observed in the average feeding scores at three and six months) may be because of increased demand of the infant who usually doubles his/her weight during the first six months (821) or changes in muscle mass/bone turnover/cartilage among the two genders during early period of growth (487,801,849).

7.8 **Limitations**

Generally, the observed significant associations between plasma micronutrients and DNA damage biomarkers were not strong (r=0.2-0.4) and it is therefore possible that some of the associations occurred by chance alone. Nonetheless, some of the associations (e.g. positive association of RBC folate with NDI) appears to be biologically plausible. Another limitation of this longitudinal study was that we did not measure intracellular concentrations of micronutrients or the intake of micronutrients in the infants, so we cannot be certain that plasma concentration reflects intake and cellular concentrations of micronutrients. Therefore the observed associations with DNA damage biomarkers cannot be considered causative.

Determining causation will be challenging and will require intervention with supplementation and/or depletion of micronutrients.

7.9 **Conclusion**

The main cause of any detected human genome damage in an environment relatively low in genotoxic agents may be cellular deficiency or excess of micronutrients that are required for genome maintenance, for instance, the activation/detoxification of chemicals preventing DNA oxidation, promoting DNA repair, being involved in apoptosis, and contributing to DNA synthesis (108,110,145,430). During the first six months, our cohort was observed to have an increase in the plasma concentrations of some minerals, such as copper, sodium, sulphur and phosphorus and a decrease in plasma concentrations of iron and potassium, and in red cell folate, indicating an infants' adaptation to environment and growth. Significant associations were observed for folate with NDI, indicating its indispensable role in proper cell growth in infants. However, the plasma concentrations of some minerals, such as sodium, potassium, magnesium and phosphorus, were correlated negatively with NDI at six months. Furthermore, the associations of calcium, zinc and magnesium with DNA damage biomarkers (MN, NPB and NBUD) suggest that even oversufficiency of some minerals may be detrimental for cell growth and repair.

We also found that mode of feeding (mother's milk or complementary feeds) could affect plasma micronutrient concentrations. It may thus be suggested that, in formulating recommendations for an infant dietary requirements of micronutrients, the concentrations of such nutrients required for genome protection should also be considered.

8 DNA damage in infants born to women at risk of pre-eclampsia during pregnancy

8.1 **Abstract**

Pre-eclampsia (PE) affects 5-7% of pregnancies all over the world and carries an increased risk of stillbirth (one in five stillbirths in otherwise viable babies), intrauterine growth restriction (IUGR) and preterm delivery. Associated with high oxidative stress and inflammation, PE may also be associated with increased DNA damage among infants born to women affected by PE. Currently, however, there are no studies that have investigated DNA damage in the cord blood of such infants.

A pilot case control study was therefore conducted in a South Australian cohort of the 'Investigations in the Folic Acid Clinical Trial' (INFACT study). The main aim was to collect DNA damage data, utilizing the cytokinesis block micronucleus cytome assay (CBMN-Cyt) in lymphocytes collected from cord blood of infants born to women previously identified as at high risk of PE (n =14) and compare them with gender and birth weight matched control group of infants from the 'Diet and DNA damage in infants' (DADHI) study, a subset of infants born to healthy women at low risk of PE (n =19) (hence indicated as DADHI control in this chapter). The secondary aim was to study the correlation of CBMN-Cyt biomarkers with infant birth outcomes and maternal anthropometric variables.

DNA damage biomarkers were measured *ex vivo* in binucleated lymphocyte cells (BNC) and included: micronuclei (MN), nucleoplasmic bridges (NPB) and nuclear buds (NBUD). Apoptotic and necrotic lymphocytes were also scored and nuclear division index (NDI) was measured using the frequency of mono-, bi- and multinucleated lymphocyte cells. In addition, MN and NBUD were also scored in mononucleated lymphocyte cells (MNC) to assess DNA damage that had already been induced *in vivo*.

Three women of the INFACT cohort were primigravidae. Four reported a family history of PE. Four women were subsequently diagnosed with PE [based on measurements of blood pressure (BP) and proteinuria]. The mean (± SD) highest BP reading recorded for the cohort was 147 (± 14.3)/93.7 (± 11.1) mm Hg.

Within the INFACT cohort, the mean (± SD) frequency for MN, NPB and NBUD per 1000 BNC was 3.6 (± 2.8), 4.0 (± 3.0), and 9.6 (± 5.8) respectively. The mean (± SD) NDI was 1.8 (± 0.08). The mean (± SD) for measures of cytotoxicity: apoptotic and necrotic lymphocyte cells measured per 500 viable cells was 5.8 ± (2.1) and 45.6 (± 16.1) respectively. The mean (± SD) frequency for MN and NBUD per 100 MNC was 0.36 (± 0.24) and 1.3 ± (0.67) respectively.

In the INFACT cohort, mother's age recorded at 8-16 week gestation was associated with NPB BNC (r = 0.61, p= 0.05). Mother's weight and height were associated with NBUD BNC (r = 0.62, p= 0.05 and r = 0.61, p= 0.05). Gestational age at birth was negatively correlated with the frequency of apoptotic lymphocytes (r = -0.56, p= 0.08). Head circumference, a marker of foetal growth, was negatively correlated with the frequency of MN in both BNC (r = -0.61, p =0.05) and MNC (r= -0.55, p = 0.09). APGAR score at 1 minute was negatively associated with the frequency of NPB BNC (r = -0.61, p= 0.05) and at 5 minutes was negatively associated with the frequency of MN in both BNC (r = -0.64, p= 0.04) and MNC (r = -0.65, p = 0.03).

Furthermore, DNA damage biomarkers measured in the cord lymphocytes showed differences between the INFACT cases and the DADHI control group. The frequency of both MN in BNC and MNC was 60% and 58% higher respectively in the INFACT group (p = 0.02, p = 0.0001 respectively). NDI was 17% higher in the INFACT group compared with the controls (p = 0.001). DNA damage biomarkers measured in NBUD MNC was 58% higher among the INFACT cohort compared to the control group (p = 0.0004).

To our knowledge, this is the first time that comprehensive measures of DNA damage, cytostasis and cytotoxicity have been collected from cord blood of infants born to women at high risk of developing PE in Australia, utilizing a reliable and well-validated assay. The data indicate that these infants have higher DNA damage and higher nuclear division rate when compared with infants of healthy low-risk mothers. The results also show that maternal weight and gestational age at birth may modulate DNA damage biomarkers in infants. However, the

results of this pilot case control study need to be interpreted with caution given the small number of subjects studied and as some participants were receiving high dose of folic acid supplementation in the INFACT group. The 95% CI were large for most of the differences, indicating that results could be attributed to chance. Further, some associations were weak (p =0.05 to 0.1). This small but novel dataset may now be used a larger better powered study to confirm the observations and provide robust evidence to support the recommendations that DNA damage in human tissues is detected and monitored at the earliest phase of life to identify those at risk of DNA damage induced accelerated ageing and degenerative diseases requiring preventive diet and lifestyle interventions.

8.2 **Introduction:**

Pre-eclampsia (PE) has been defined as a "multi-system disorder characterized by hypertension (HT) and the involvement of one or more other organ systems and/or the foetus" (1). *De novo* hypertension (≥140/90 mmHg after 20 weeks gestation) is commonly (but not always) the first manifestation of PE. Evidence of multisystem dysfunction observed among women affected by PE, may include proteinuria, abnormal liver and/or renal function tests, thrombocytopenia and/or evidence of placental insufficiency (1,11). PE is classified as early-onset if diagnosed prior to 34 weeks gestation and late-onset if diagnosed after 34 weeks gestation (12). Although the exact cause of PE is still unknown, genetic and epigenetic features are being explored to explain the pathogenesis (13). Two pathological stages have been identified in the development of the disease. The first asymptomatic stage is marked by defective trophoblast invasion during early implantation (14,15), followed by placental ischemia and local oxidative stress (2) and the associated inadequate remodelling of the uterine spiral arteries (18), leading to defective uteroplacental blood circulation. This poor placentation leads to a second stage of systemic inflammatory responses and maternal endothelial dysfunction leading to the manifestation of clinical symptoms (15).

8.2.1 **Pre-eclampsia: a state of increased possibility of stress induced DNA damage?**

The main factor involved in the pathophysiology of PE is considered to be oxidative stress, where excess free radicals produce harmful cellular damage, including damage to macromolecules, such as lipids, proteins and DNA (850-855). During PE, oxidative stress may manifest in the placenta as well as in maternal circulation (856). There is also evidence of decreased expression of antioxidant defence enzymes (superoxide dismutase, catalase and glutathione) and increased free radical formation in the placentas of women with PE (857). Numerous studies have reported increased plasma or serum concentrations of homocysteine (Hcy) in women with PE, suggesting that Hcy induced oxidative stress may be an independent risk factor for this disorder (20-29). Hcy promotes the generation of hydrogen peroxide and

oxygen-derived free radicals through the oxidation of its sulfhydryl component (30,31). This results in abnormal changes to the vascular endothelial cell cytoskeleton, acceleration of LDL oxidation and blood vessel thickening (32). Hcy may also induce apoptosis in human umbilical vein endothelial cells and smooth muscle cells by accumulation of unfolded proteins in the lumen of the endoplasmic reticulum (33). It may also increase thromboxane formation, increase leucocytes adhesion to endothelial cells and increase the concentration of pro-inflammatory cytokines within blood vessels (34). Hcy down-regulates intracellular glutathione peroxidase, leading to a decrease in bioactive nitric oxide which is the body's primary vasodilator as observed in aortic endothelial cell cultures (35). Thus, Hcy may either cause maternal endothelial dysfunction directly through oxidative stress (36) or may interfere with nitric oxide function, leading to secondary placental vasoconstriction and ischemia in PE (37). Further, increased Hcy could induce cellular DNA damage and DNA hypomethylation through increased lipid peroxidation, as has been observed in murine hepatic and neuronal cells (858). A recent *in vitro* study demonstrated that human umbilical vein endothelial cells, when exposed to plasma from women with pregnancies complicated by PE resulted in an increase in superoxide free radical generation in mitochondria compared with cells exposed to plasma from women with uncomplicated pregnancies. Real-time PCR analysis showed increased expression of inflammatory markers tumour necrosis factor- α (TNF-α), toll like receptor-9 (TLR-9) and intercellular adhesion molecule-1 (ICAM-1) in endothelial cells treated with plasma collected from women diagnosed with PE (859). Further, *in vivo* and *in vitro* experiments have shown excessive oxidative DNA damage at the foetal-maternal interface of human placenta coupled with DNA damage/repair response activation, as demonstrated by increased expression of γH2AX (a sensitive marker of DNA damage) in the maternal decidua of placental tissues collected from women with PE (860).

Elevated Hcy has been associated with increased DNA damage in a cross-sectional study coupled with a randomized double-blind placebo-controlled dietary intervention study with

folic acid (FA) and vitamin B_{12} in young Australian adults aged 18-32 years. The study reported DNA damage [as measured by Micronuclei frequency (MN) in lymphocytes] associated with the intervention to be positively correlated with the reduction of plasma Hcy ($r = 0.39$, $p < 0.006$) and negatively correlated with serum vitamin B_{12} ($r = -0.49$, $p < 0.0005$). Noticeably, the greatest decrease in plasma Hcy and MN frequency was observed in the subjects with initial plasma Hcy and MN frequency in the high 50^{th} percentile supporting the hypothesis that hyperhomocysteinemia may increase DNA damage (87).

Hypermethylation and reduced expression of genes encoding various proteins involved in placental implantation, including trophoblast invasive functions, have been discovered in placentae from women with PE. Examples of affected genes include ASTN1 (cell adhesion), ABC 6, MOVI0 (ribonucleotide binding), (147) NR3C1 (glucocorticoid receptors), CRHBP (corticotrophin releasing hormone binding), (148) H-19 (trophoblast invasion),(149) syncytin-1 (cell fusion and trophoblast invasion), (150-152) and also genes involved in transcription, lipid metabolism, membrane transport and the immune system (153). Additionally, significant over-expression of certain genes has been attributed to decreased methylation in the placental tissue of patients with PE in genes such as VEGF, (154) EPAS1 and FLT1 (155) (angiogenic factors), TIMP3 (matrix metalloproteinase inhibitor), (156,157) LAIR-2 (gene encoding for a trophoblast protein), DNAJC5G (gene coding a neuroprotective protein), LAMA3 (gene encoding laminins that are important for endothelial repair), (158) LEP (encoding for protein for regulatory function in reproductive maturity), (159,160) placental matrix metalloproteinase 9 (MMP9; a member of family of zinc-dependent proteases that may interfere extra villous trophoblast invasion) (161) and SERPIN3A (homeostasis in inflammation and coagulation pathway) (134,162). Thus, PE may also be caused by altered gene methylation and gene expression promoting inflammation and oxidative stress, which may induce greater DNA damage in the maternal tissues and body fluids of women with PE compared with women with normal pregnancy.

8.2.2 **Assessing oxidative stress induced DNA damage in Pre-eclampsia**

There are a number of assays that can be used to measure oxidative stress, DNA damage and cellular response to DNA damage and oxidative stress during pregnancy including 8-hydroxy-2′- deoxyguanosine (8-OHdG): an oxidized form of guanine (101), 8-isoprostane (a marker of lipid peroxidation and excessive systemic oxidative stress) (102), activin A: a member of the transforming growth factor β family of cytokines (102), thioredoxin expression: a reductive enzyme involved in repair of oxidatively damaged proteins in various tissues including placenta (103), apurinic/redox factor-1 (ref-1): an essential enzyme in DNA base excision repair possessing both DNA repair and redox regulatory activities (104), the terminal deoxynucleotidyl transferase-mediated assay: direct method for the assessment of DNA fragmentation (105), the Comet assay (106,861,862) and phosphorylated H2AX (107,863): both measure double strand breaks. DNA damage induced by oxidative stress and micronutrient deficiency can also result in chromosome aberrations (deletions, rearrangements) which manifest themselves as nuclear anomalies such as micronuclei, nucleoplasmic bridges and nuclear buds (108,109).

The lymphocyte cytokinesis block micronucleus cytome (CBMN-Cyt) assay is one of the most comprehensive and best validated methods to measure chromosomal DNA damage in lymphocytes (108). The CBMN-Cyt assay has evolved into a robust, sensitive and comprehensive assay of DNA damage, cell death and cytostasis (108). The ‘‘cytome’’ concept in the CBMN-Cyt assay implies that every cell in the system studied is scored cytologically for its DNA damage, proliferation and viability status (108). In this assay, genome damage is measured by scoring: micronuclei (MN): a biomarker of both chromosome breakage and/or loss; nucleoplasmic bridges (NPB): a biomarker of DNA mis-repair and/or telomere end-fusions, nuclear buds (NBUD): a biomarker of gene amplification and /or the removal of unresolved DNA repair complexes (109,110). DNA damage biomarkers expressed *ex vivo* (MN, NPB and NBUD) in short term lymphocyte cultures are measured in binucleated

lymphocyte cells (BNC), because only cells that complete nuclear division can express molecular lesions in DNA and in the mitotic machinery as chromosome breakage or chromosome loss events, respectively, that lead to MN formation. Genome damage already expressed *in vivo* as MN and NBUD is measured in mononucleated lymphocyte cells (MNC) that fail to divide *in vitro* in the CBMN-Cyt assay (325,326). MN frequency in lymphocytes has been associated with anaemia (111), cancer (112,113), cardiovascular diseases (114), neurodegenerative diseases (115), reproductive and pregnancy complications, including pregnancy loss (116), infertility (117) and PE (118).

8.2.3 **DNA damage in infants born to women with Pre-eclampsia**

PE affects approximately 5-7% of pregnancies all over the world (2) and is responsible for stillbirth (one in five stillbirths in otherwise viable babies), intrauterine growth restriction (IUGR) (864,865) and preterm delivery, (866) with a 3- to 25-fold increased risk of abruptio placentae, thrombocytopenia, disseminated intravascular coagulation and pulmonary oedema (867). Maternal exposures (environmental pollutants and diet) are now known to alter pregnancy outcomes and methylation of key genes regulating placental cortisol metabolism (868). Maternal systemic inflammation is be associated with impaired foetal growth (869) that may lead to infants born to mothers with PE developing learning disabilities and low IQ later in life (870). Low birth weight babies (LBW) have also been shown to develop insulin resistance and adiposity in childhood (871). The LBW infants are susceptible to higher DNA damage and oxidative stress when compared with normal weight infants. Pregnancy is considered a highly inflammatory condition owing to (or associated with) increased Hcy concentrations (515,872), as well as being associated with increased angiogenesis and increased immune responses especially at the site of implantation (317). The birthing process creates a hypoxic condition, which is known to increase oxidative stress for both mother and infant (100) and which may modulate expression of placental endothelial growth factors that control cellular growth, differentiation, proliferation and apoptosis (143,318-320). It is

therefore reasonable to hypothesize that infants born to mothers with inflammatory conditions, such as PE may be susceptible to more cellular DNA damage as schematically presented in **Figure 8.1**.

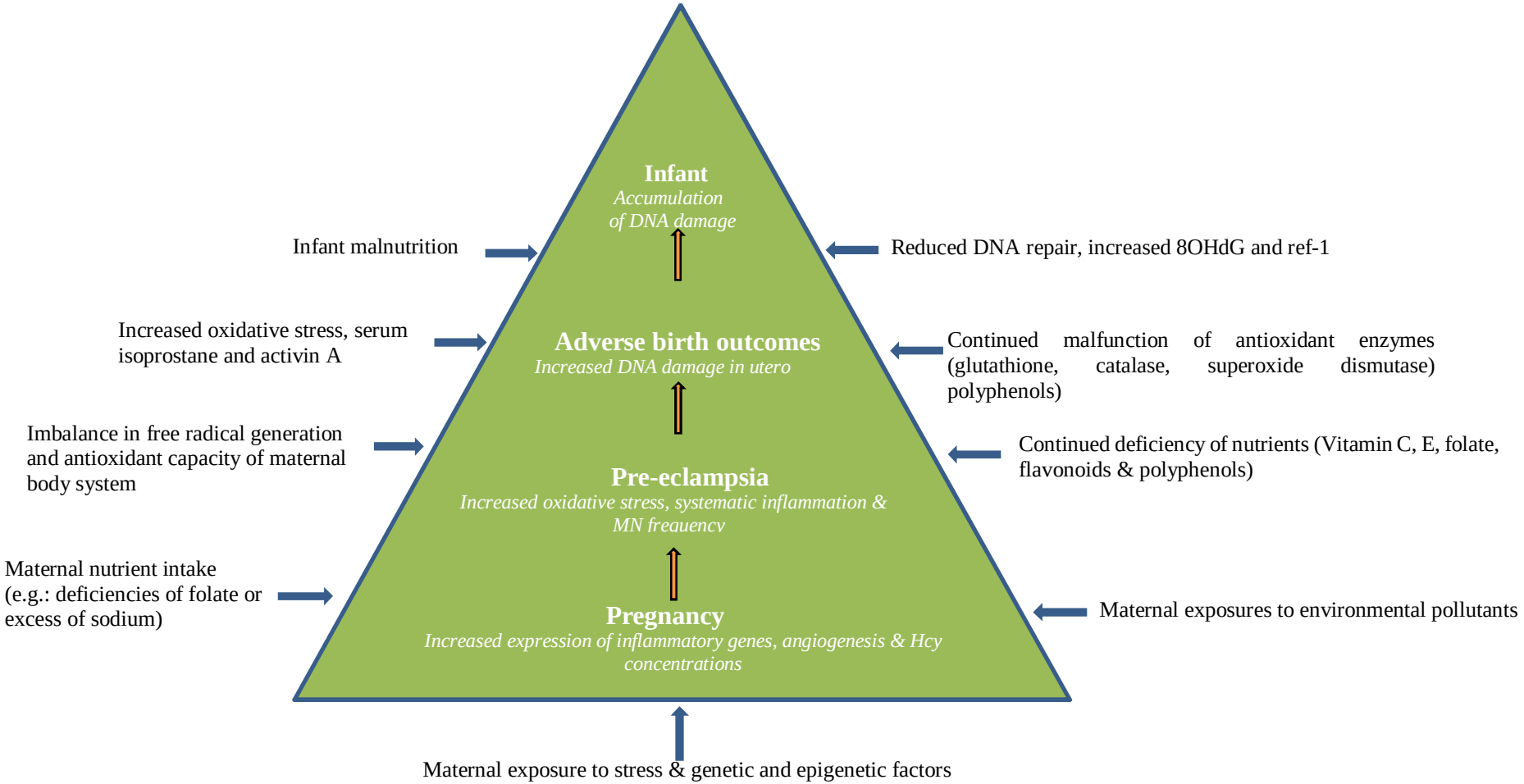


Figure 8.1: A schematic representation of factors associated with increased DNA damage in infants born to women with Pre-eclampsia**.**

(Based on the data of studies summarized in Table 8.1and 8.2)
Abbreviations: Hcy: homocysteine; OHdG: 8-Hydroxy-deoxy-guanine; ref-1: redox factor; MN: micronuclei

There have been few studies that have investigated DNA damage biomarkers in blood of women with/or at risk of PE and their placenta that have been summarised in **Table 8.1**. The first prospective cohort study to investigate the association between genome integrity and PE was conducted on women at both low risk (no previous history of adverse pregnancy outcomes, such as PE) and high risk of adverse pregnancy outcomes (women with pre-existing condition of PE/HT/diabetes) in Australia (118). Increased MN frequency, as measured by the CBMN-Cyt assay, in maternal peripheral lymphocytes at 20 weeks gestation was associated prospectively with PE and IUGR. The odds ratio (OR) for PE and/or IUGR in the cohort of only high risk pregnancies (n=91) was 17.85 (P =0.007) if the MN frequency was greater than 39 per 1000 cells (118). The study suggests that the frequency of MN is increased in lymphocytes of women who later develop PE and/or IUGR compared with women with normal pregnancy outcomes. The same case control study in Australia reported genome instability (frequency of MN and NBUD) to be positively associated with Hcy concentrations in peripheral maternal blood of women at increased risk of PE (r = 0.179, P = 0.038 and r = 0.171, P = 0.047, respectively) (142). A recent case-control study in Japan, demonstrated that oxidative DNA damage, as measured by 8-OHdG, was greater in the placentas of women with early onset PE (143). A further case control study in Australia reported a positive relation (r^2=0.72, p < 0.001) between circulating concentrations of 8-isoprostane and activin A in women with PE (n = 21) compared with normal pregnant women (n = 20) (102). A case control study conducted in Japan observed a higher concentration of 8-OHdG among women with PE and IUGR (n=11) (p = 0.0021), greater thioredoxin expression in PE (n=13) (P=0.045), and increased expression of redox factor-1 in PE (P = 0.017) as well as in PE and IUGR (P = 0.0038) compared with normal pregnant women (n = 23) (144). Interestingly, increased cellular 8-OHdG is correlated with formation of MN in lymphocytes (109), while increased MN frequency has been consistently associated with low

folate and vitamin B_{12} status (145,146) and high Hcy: the metabolic biomarker of deficiency in folate and vitamin B_{12} (242). Further research in a cohort of women at risk of PE may help in explaining the significance of observed genome instability in relation to the folate deficiency and prognosis of PE (523) and confirming the utility of the CBMN-Cyt assay, together with biomarkers of oxidative damage, as potential diagnostic markers of risk of pregnancy complications including PE.

Table 8. 1: Summary of studies of DNA damage in placenta or blood collected from women at risk/or with Pre-eclampsia

Reference	Participants or type of tissue samples	Methods	Results
Kimura *et al* (2013)	Women with uncomplicated pregnancies (n = 10), early-onset PE (n = 13), and late-onset PE (n = 12)	Immunohistochemical analysis conducted to measure the proportion of placental trophoblast cell nuclei staining positive for 8-OHdG and redox factor-1	The proportion of nuclei that stained positive for 8-OHdG was higher in both PE groups compared with the control group, with a higher proportion in the early-onset PE group ($p < 0.001$) than in the late-onset PE group ($p < 0.05$)
Furness *et al* (2013)	Women (<20 weeks gestation) grouped as high (n = 91) or low risk (n = 46) of adverse pregnancy outcomes	Demographic, clinical, and dietary data along with fasting blood samples collected at 18–20 weeks gestation. Detailed information collected on type and dose of multimicronutrient supplement consumption	Maternal folate and plasma Hcy were not increased at 18–20 weeks gestation in those who developed PE. MN frequency and NBUD in lymphocytes were positively correlated with Hcy ($r = 0.179$, $p = 0.038$, and $r = 0.171$, $p = 0.047$, respectively). Multivariate regression analysis showed that reduction of RBC folate was a strong predictor of IUGR ($P = 0.006$)
Shaker *et al* (2013)	Venous blood and placentas from women with PE (n = 25) and age- and parity-matched normal pregnant women (n = 25) during delivery.	Lipid peroxidation was estimated by measuring thiobarbituric acid reactive substances, mainly malondialdehyde (MDA), in placental tissues and serum (by method of Esterbauer and Cheeseman). caspase-8 and -9 activity in placental tissues (determined using Apo Targe colorimetric assay kits), and the percentage of DNA fragmentation in placental tissues was measured by diphenylamine assay and confirmed by agarose gel electrophoresis.	With the exception of caspase-8 activity, the expression of apoptotic markers caspase-9, the percentage of DNA fragmentation (each $p < 0.001$) and the lipid peroxidation product ($p < 0.001$) and placental MDA ($p < 0.05$), and the serum uric acid concentration ($p < 0.05$) were higher in the PE group than the control group.

Reference	**Participants or type of tissue samples**	**Methods**	**Results**
Mert *et al* (2012)	Pregnant women with PE (n = 24), pregnant women with PE and IUGR (n = 20) and healthy pregnant normotensive women (n = 37).	The TOS and TAS of plasma were measured using a novel automated colorimetric measurement method developed by Erel. Sister chromatid exchange (SCE) and micronuclei analysis were performed on peripheral blood lymphocytes of cases and controls.	Increased TOS and TAS in PE + IUGR group compared with healthy pregnant women ($p = 0.001$, $p < 0.001$, respectively). The frequencies of SCE were increased in women with PE + IUGR compared with healthy pregnant women ($p = 0.02$).
Sharma *et al* (2012)	Placental tissue from normotensive nonproteinuric pregnant women (n = 20) and PE women (n = 20) with gestational ages of 30–42 weeks.	Hematoxylin eosin staining, TUNEL assay and M30 immunostaining techniques were used for studying apoptosis in trophoblastic cells of placentas.	The TUNEL apoptotic indices were higher in all the zones of placentas of women with PE when compared with those in the control group but the results were not significant. M30 immunostaining also gave higher apoptotic indices in all the zones of placentas of PE women when compared with the normal group but the result of apoptotic index of basal plate was not significant
Fujumaki *et al* (2011)	Blood and placental tissue samples were collected at delivery from three small groups: women with PE & IUGR (n = 13), women with PE without IUGR (n = 10) and healthy pregnant women without complications (n = 10).	Data were collected on maternal and umbilical concentrations of serum derivatives of reactive oxygen metabolites (d-ROMs: a marker of oxygen free radicals) with the Free Radical Analytical System, and placental localization of 8-OHdG (an indicator of oxidative DNA damage) and redox factor-1(ref-1: indicative of the repair function towards oxidative DNA damage) by standard immunohistochemical procedures.	The study found increased d-ROMs in the maternal blood of women with PE (with IUGR: $p < 0.01$; without IUGR: $p < 0.001$) compared with controls. Umbilical artery of women with PE and IUGR showed higher concentrations of d-ROM ($p < 0.01$), compared with preeclamptic women without IUGR. The 8-OHdG and ref-1 was also higher in women with PE and IUGR ($p < 0.001$) than in the control group.

Reference	**Participants or type of tissue samples**	**Methods**	**Results**
Furness *et al.* (2010)	136 pregnant women: high-risk (n = 91) and low-risk (n = 41)	CBMN-Cyt assay in lymphocytes collected at 20 weeks gestation	Increased DNA damage in maternal peripheral lymphocytes at 20 weeks gestation associated prospectively with PE and IUGR. When genome damage increased to a frequency of 36.7 MN per 1000 BNC , the OR of developing PE and/or IUGR was 15.97
Mandang *et al* (2007)	Women (26–40 weeks gestation) with established PE (n = 21) and gestationally matched healthy pregnant women (n = 20). Placental tissue (n = 11), umbilical cords (n = 6), and maternal peripheral blood (n = 6) from women with a healthy, singleton pregnancy undergoing an elective caesarean section at term (37–40 weeks gestation).	Serum isoprostane and activin A measured in the 2 groups of women. Trophoblast explants, human umbilical vein endothelial cells, and peripheral blood monocytes exposed to oxidative xanthine/xanthine oxidase *in vitro*.	Maternal plasma 8-isoprostane and activin A were higher in women with PE than in controls (333.8 ± 70 vs176.3 ± 26.2 pg/ml, $p = 0.04$, and 49.5 ± 7 vs 13.1 ± 1.2 ng/ml, $p < 0.001$, respectively). Serum 8-isoprostane and activin A were positively correlated ($r^2 = 0.72$, $p < 0.001$) in women with PE vs women with normal pregnancy.
Wiktor *et al* (2004)	Placental tissue samples from chorionic plate of normal pregnancy cases (n=18), pregnancies complicated by severe PE without IUGR (n=17) and thosecomplicated by severe PE with IUGR (n =18).	Cellular DNA was isolated, hydrolysed and analysed using high-performance liquid chromatography. Native nucleosides were monitored at 254 nm and 8-OHdG was measured.	Mean concentration of 8OHdG was higher in placentas collected from women with PE 8OHdG concentrations were higher in PE-IUGR placentas compared with control ($p = 0.008$).
Takagi *et al.* (2004)	Placental tissues from 42 healthy women (6–40 weeks gestation) and women with PE (n = 24). For Western blotting, placental tissue was collected from 8 women with a normal pregnancy (9–39 wk), 5 with PE (28–39 wk), 3 with IUGR (28–36 wk), and 1 with PE + IUGR (36 wk).	Immunohistochemistry and western blotting for 8-OHdG, 4-hydroxynonenal, thioredoxin, and redox factor-1 in the placentas of women with PE, IUGR, PE+IUGR, or normal pregnancy.	8-OHdG lwas increased in IUGR or PE+IUGR group compared with normal pregnancy; thioredoxin expression and redox factor -1 expression were increased in PE ($p = 0.017$), IUGR ($p = 0.016$), and PE + IUGR ($p = 0.0038$)

Abbreviations: PE: preeclampsia, IUGR: intrauterine growth restriction; p: significant value; TAS: total antioxidant status; TOS: and total oxidant status, OSI: oxidative stress index OR: odd ratio; 8-OHdG: 8-hydroxy deoxyguanosine.CBMN-Cyt: cytokinesis block micronucleus assay, MN: micronuclei; NBUD: nuclear bud; SSE: sister chromatin exchange; MDA: malondialdehyde, BNC: binucleated lymphocyte cell; d-ROMs : derivatives of reactive oxygen metabolites ; ref-1: redox factor-1.

Some studies that investigated DNA damage in cord blood are summarized in **Table 8.2**. A cross-sectional study in Turkey measured DNA damage using the alkaline Comet assay in mononuclear leucocytes collected from both the mothers and the cord blood of hypertensive pregnant women (mildly PE, n = 25) and normotensive pregnant women (n = 20) just after delivery. The study reported increased DNA damage ($p < 0.001$), decreased total oxidant status ($P < 0.001$), and increased oxidative stress index ($p < 0.001$) in pre-eclamptic cord blood compared with controls (873).

Table 8. 2: Summary of studies of DNA damage in cord blood samples of women with Pre-eclampsia

Reference	Participants or type of tissue samples	Methods	Results
Negi *et al* (2014)	Umbilical cord blood from neonates born to women with PE (n =19), women with eclampsia (n = 14) normotensive uncomplicated pregnancy (n =18 as control).	8-OHdG [by competitive *in vitro* enzyme-linked immunosorbent assay (ELISA)] kit), protein carbonyl (spectrophotometric DNPH method), nitrite (colorimetric detection of nitrite as an azo dye product of the Griess reaction) catalase (standard method of Aeibi), non-enzymatic antioxidants (vitamin A, E, C), total antioxidant status (using Randox assay kit) and iron status (Ferrozine method) were determined	The study showed a difference between PE group in the concentrations of protein carbonyl ($p < 0.001$), 8-OHdG ($p < 0.001$) and nitrite ($p < 0.001$) compared with controls; as well as a difference between groups in catalase ($p < 0.005$), vitamin E ($p < 0.01$) and TAS ($p < 0.001$) compared with controls. The positive association of risk of pre-eclampsia/eclampsia was observed with protein carbonyl (OR = 1.783, $P < 0.05$), 8-OHdG (OR = 1.088, $p < 0.005$) and nitrite (OR = 1.172, $p < 0.005$).
Hillali *et al* (2013)	Maternal and umbilical cord blood samples from women with PE (n =25), and healthy controls (n =20).	Mononuclear leukocyte DNA damage using the alkaline Comet assay, total antioxidant status (TAS) and total oxidant status (TOS) (using a novel automated method developed by Erel), and the oxidative stress index (OSI) calculated by TOS-to-TAS ratio.	DNA damage, and TOS and OSI concentrations were increased (for all $p <0.001$) in maternal and cord samples, while TAS concentrations decreased in maternal ($p < 0.001$) and cord blood ($p < 0.02$) samples of the PE group.
Mandang *et al* (2007)	Women (26–40 weeks gestation) with established PE (n = 21) and gestationally matched healthy pregnant women (n = 20). Placental tissue (n = 11), umbilical cords (n = 6), and maternal peripheral blood (n = 6) from women with a healthy, singleton pregnancy undergoing an elective caesarean section at term (37–40 weeks gestation).	Serum isoprostane and activin A measured in the 2 groups of women. Trophoblast explants, human umbilical vein endothelial cells, and peripheral blood monocytes exposed to oxidative xanthine/xanthine oxidase *in vitro*.	Maternal plasma 8-isoprostane and activin A were higher in women with PE than in controls (333.8 ± 70 vs 176.3 ± 26.2 pg/ml, $p = 0.04$, and 49.5 ± 7 vs 13.1 ± 1.2 ng/ml, $p < 0.001$, respectively). Serum 8-isoprostane and activin A were positively correlated ($r^2 = 0.72$, $p < 0.001$) in women with PE vs women with normal pregnancy.

Abbreviations: PE: preeclampsia, IUGR: intrauterine growth restriction; p: significant value; TAS: total antioxidant status; TOS: and total oxidant status, OSI: oxidative stress index ; OR: odd ratio; 8-OHdG: 8-hydroxy deoxyguanosine; ELISA : enzyme-linked immunosorbent assay

Though increased expression of inflammatory genes and higher concentrations of oxidative stress biomarkers have been demonstrated in placentas, cord and blood samples collected at delivery from women with PE compared with those seen in normotensive healthy women, but we do not have information on comprehensive DNA damage and cytotoxicity measures. We also do not know whether infant birth outcomes and maternal anthropometric indicators could modulate DNA damage biomarkers in infants born to women at risk of developing PE.

Numerous studies have shown a correlation between the frequency of DNA damage in lymphocytes of mothers/fathers and their offspring, suggesting a common environmental, nutritional or lifestyle insult (304,326-330), utilizing the comprehensive CBMN-Cyt assay. Further, correlation has been observed between the frequency of DNA damage measured as MN frequency in mothers and that seen in their infants (328,330,661,662). Infants born to women with diabetes and epilepsy have been reported to have increased MN frequency when compared with infants born to healthy women (334,554). Women at risk of developing PE have increased DNA damage as measured by frequency of MN at 20 week gestation compared with healthy women (control) at low risk of complications during pregnancy indicating that (118), infants born to women with at high risk of PE will be susceptible to increased genome damage (**Figure 8.1)**. However, prior to the present study we did not have data on DNA damage in infants born to women at risk of PE during pregnancy in Australia.

A pilot case control study was therefore initiated, comparing offspring from a cohort of pregnant women at high risk of PE taking part in the Folic Acid Clinical Trial (FACT), with a control group recruited from a subset of cohort of gender and birth weight matched infants from mother-infant pairs of a concurrent longitudinal prospective study of women at low risk of complications during pregnancy: the Diet and DNA damage in Infants (DADHI) study (hence indicated as DADHI control in this chapter). Investigations of the FACT group (known as the INFACT study) were conducted to collect comprehensive DNA damage data utilizing

the CBMN-Cyt assay from the infants at birth, just as had been collected from the DADHI infants.

8.3 Hypotheses

1. Birth outcomes of infants born to women at high risk of developing pre-eclampsia during pregnancy (INFACT cohort) are associated with DNA damage biomarkers as measured in cord blood by CBMN-Cyt assay.
2. Maternal anthropometric parameters measured at 8-16 week gestation (INFACT cohort) are correlated with DNA damage biomarkers as measured in cord blood by CBMN-Cyt assay.
3. Maternal anthropometric parameters of women at high risk of PE (INFACT cohort) are different when compared to women at low risk of PE (DADHI control).
4. Birth outcomes of infants in the INFACT cohort are different from infants in the DADHI control.
5. The frequency of DNA damage in cord blood as measured by CBMN-Cyt assay is greater among INFACT cases compared with that of DADHI control.
6. Infants in the INFACT cohort have higher red cell folate status when compared with that of the infants in the DADHI control.

8.4 Aims

1. To study the association of infant birth outcomes in the INFACT cohort with DNA damage biomarkers as measured in cord blood lymphocytes by CBMN-Cyt assay.
2. To study the correlation of maternal anthropometric parameters in the INFACT cohort with DNA damage biomarkers as measured in cord blood by CBMN-Cyt assay.
3. To study the differences in maternal anthropometric parameters between the INFACT and the DADHI control.
4. To study whether the birth outcomes of infants in the INFACT cohort are different compared with those of the DADHI control.

5. To determine whether the frequency of CBMN-Cyt biomarkers measured in cord blood lymphocytes are higher in the INFACT cases compared with that seen in the DADHI control at birth.
6. To determine whether red cell folate status is increased in infants in the INFACT cohort compared with the DADHI control at birth.

8.5 **Methods**

A small group of women at high risk of complications during pregnancy were recruited from the Folic Acid Clinical Trial (FACT) study for a pilot study (the Investigations in the Folic Acid Clinical Trial [INFACT] study). The Folic Acid Clinical Trial (FACT) is a randomised, double-blind, placebo-controlled, Phase III, international multi-centre clinical study of 4.0 mg of Folic Acid supplementation in pregnancy (started between 8-16 weeks gestation) for the prevention of pre-eclampsia (PE), funded through the Canadian Institutes of Health Research (286). Women were recruited for the FACT study on the basis of an increased risk of PE (previous pre-eclampsia, twin pregnancy, chronic hypertension, pre-existing diabetes, obesity), and those in the Adelaide cohort were approached for participation in the INFACT study. The INFACT study was designed to evaluate the effect of high dose FA on maternal and infant folate status, on DNA damage markers in mother, neonate and the infant, on neonatal and infant adiposity, and on the development of an allergic cytokine profile in the offspring. The study was approved by the Human Research Ethics Committee of WCHN, Adelaide. All the women were informed about the INFACT study aim and requirements through a detailed Information sheet before giving their informed consent. The schematic representation of the study design is given in **Figure 8.2.**

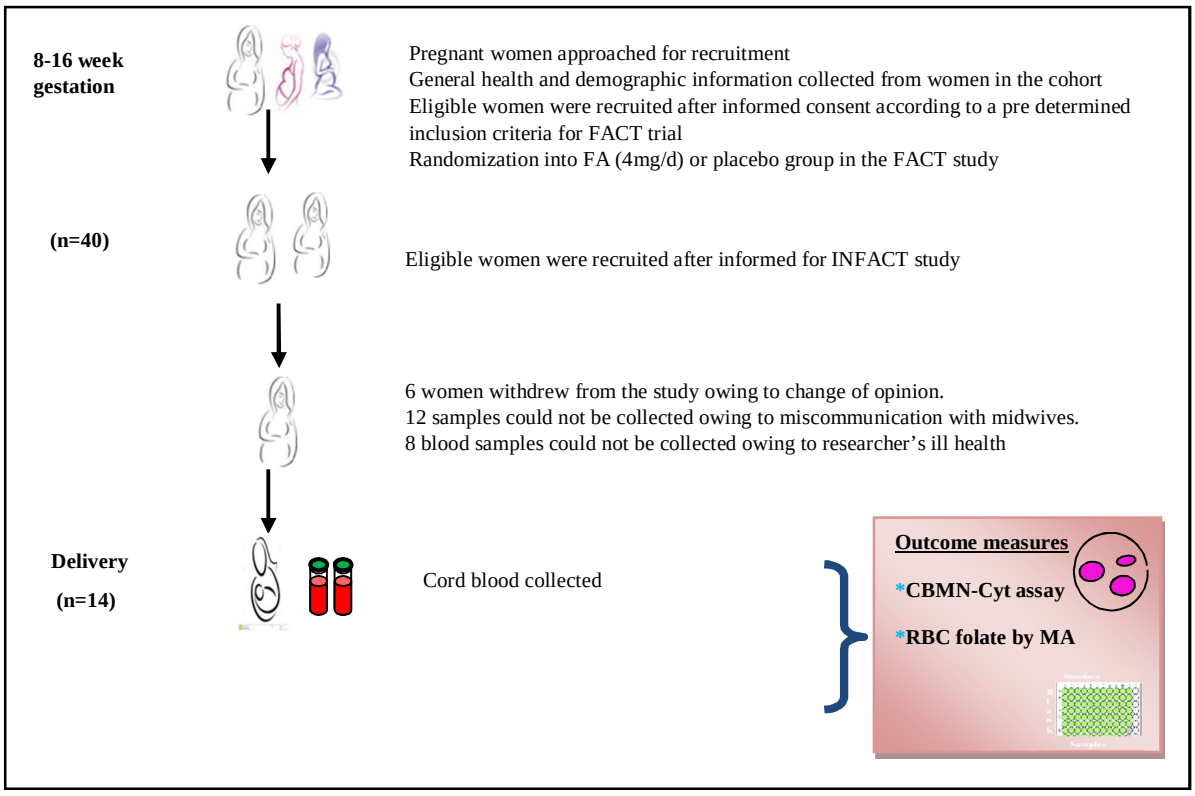


Figure 8.2: Schematic representation of the pilot project in the INFACT study

Abbreviations: (CBMN-Cyt: cytokinesis block micronucleus assay, RBC: red blood cell, MA: microbiological assay for folate, FACT: folic acid clinical trial)

8.5.1 Inclusion criteria

- ≥18 years of age at the time of consent
- Taking ≤1.1 mg of FA supplementation daily at the time of randomization.
- Live foetus
- Gestation age (GA) between $8^{0/7}$ and $16^{6/7}$ weeks of pregnancy (GA is based on the first day of the last menstrual period or ultrasound performed before $12^{6/7}$).
- At least one of the identified risk factors for PE:

- Pre existing hypertension (documented evidence of diastolic blood pressure ≥90 mm Hg or use of hypertensive medication during this pregnancy specifically for the treatment of hypertension prior to randomisation)
- Pre pregnancy diabetes (documented evidence of Type I or Type II diabetes mellitus)
- Twin pregnancy

- Documented evidence of history of PE in a previous pregnancy
- Body mass index (BMI) ≥35kg/m^2

8.5.2 **Exclusion criteria**

- Known history or presence of any clinically significant disease which would be a contraindication to FA supplementation
- Known foetal anomaly/demise
- History of medical complications including renal disease, epilepsy, cancer or use of FA antagonists
- Current enrolment in other clinical trials or who have received an investigational drug within 3 months of randomisation
- Higher order (>2) multiple pregnancy
- Known hypersensitivity to FA
- Known current alcohol abuse (≥2 drinks per day)

8.5.3 **Sample size**

In total, 124 women enrolled in the FACT study were approached to participate in the INFACT study up to March 2015. 40 women consented to be part of the sub study of INFACT project. 6 women withdrew from the study owing to change of opinion. 12 samples could not be collected owing to miscommunication with midwives. 8 blood samples could not be collected owing to the researcher's ill health. Thus, at delivery, cord blood was collected from 14 women enrolled in INFACT to be part of this pilot study. The control group comprised infants (n=19) born to women with low risk of pregnancy complications (subset from the DADHI study) that has been discussed in detail in chapter 6 and 7, and were matched for gender and birth weight (± 150g) at birth (indicated as DADHI control in this chapter).

8.5.4 **General health questionnaire and Anthropometric data collection**

A general health questionnaire was administered to participating women at between 8 and 16 weeks gestation to collect detailed information about the mother's demographics, medical and family history, lifestyle habits (such as smoking), dose and duration of FA supplementation, and other supplements and medicines consumed during the pregnancy period. Mother's weight at recruitment was recorded using a digital balance accurate to within 100 g, and height was determined using a stadiometer accurate to within 1 cm of overall height. BMI was then calculated using the formula weight (kg)/ height (m) ^{2}. Maternal blood pressure (BP): systolic/diastolic was measured using a manual sphygmomanometer by a trained nurse on three occasions (10 minutes apart) during every study visit before the delivery. The BP readings were then averaged and the highest reading among all the three measurements was noted. Dipstick urinalysis was done to assess proteinuria during every maternal visit to WCH: any positive finding (>=1+ protein) was confirmed with a measurement of urinary protein/creatinine ratio (mg/mmol). At delivery, type of labour and delivery (normal/spontaneous/induced/no labour and elective/emergency Caesarean section) and any complications during labour were also recorded. Details regarding the infant's birth weight, birth length, head circumference, gender and gestation age at birth were also recorded. APGAR scores were assessed for infants at 1 and 5 minutes after birth. APGAR score is a tool that measures comprehensive vitality at birth with respect to breathing effort, heart rate, muscle tone, reflexes and skin colour. A score of 7 and above is considered normal while below 3 is considered critically abnormal (339,731,874).

8.5.5 **Blood collection**

Approximately 3-5 ml of cord blood was collected immediately after birth into a 9 ml sterile Lithium Heparin coated collection containers (green top; Greiner Vacuette 2 mL Cat.No. 454089). The tubes were kept at 4^{o}C before being transported to the CSIRO Nutrigenomics laboratory in a lab top cooler within 4-6 hours of collection. The cord blood was then kept at room temperature (18-22^{o}C) and was prepared for the CBMN-Cyt assay. After removing the blood required for CBMN-Cyt assay (2*100µl) by carefully avoiding the clots, the whole blood

tubes were centrifuged at 3000 rpm for 20 minutes to separate the plasma. The red blood cells (RBC) were stored at -80 ºC until analysis for folate by Microbiological assay was performed.

8.5.6 **CBMN-Cyt assay**

A whole blood CBMN-Cyt assay was conducted in duplicate on all collected samples (108). The detailed protocol of the assay has been explained in chapters 3 and 5. Briefly, duplicate whole blood lymphocyte cultures for each blood sample from a participant were prepared. On day 0, 100 µl aliquots of heparinised whole blood were cultured in 810 µl medium. The mitogenic activity in lymphocytes was initiated by adding 90 µl phytohaemagglutinin (PHA) to give a final concentration of 202.5 µg/ml. The time of PHA addition was recorded. The cells were incubated at 37 ºC with loosened lids in a humidified atmosphere containing 5% carbon dioxide for 44 h.

At 44 hrs, the cell cultures were carefully removed from the incubator and 100 µl of cytochalasin-B stock solution was added and gently mixed to achieve a final concentration of 6 µg/ml. The cells were returned to the incubator for a further 24 hrs.

At 68 hrs, cultures were removed from the incubator, and the cells were resuspended by mixing gently. The cell suspension was underlaid with 400 µl of Ficoll-Paque (Amersham Pharmacia Biotech, Sweden, cat no. 17144002) in a TV10 tube (Techno Plas, S9716VSU, Australia) using a ratio of 1 (Ficoll):3 (cell suspension) without disturbing the interface. The tube containing cell suspensions overlaid on Ficoll was then centrifuged once at 400g for 30 min at 18 - 20ºC to separate the lymphocytes. Using a pipette with a 200 µl clear plugged tip, the 'buffy' lymphocyte layer at the interface of the Ficoll-Paque and culture medium was removed, carefully avoiding uptake of Ficoll. The lymphocyte suspension was washed in three times its volume of Hanks balanced salt solution (Hanks HBSS, Trace Scientific, Melbourne, Australia, Cat no. 111010500-V) by gently pipetting in 1320 µl HBSS solution and then centrifuging at 180g for 10 min at room temperature to remove any residual Ficoll and cell debris. The supernatant was gently removed, leaving approximately 200 µl cell suspension. Subsequently,

15 µl dimethyl sulfoxide (DMSO 7.5% v/v of cell suspension Sigma, Sydney, Australia) was added to prevent cell clumping and to optimize identification of cytoplasmic boundaries. The assay was conducted in duplicate for each blood sample. This was followed by harvesting of cells by cytocentrifugation onto cleaned slides. The slides were air-dried for 10 minutes. Then the slides were transferred directly into Diff Quick stain: 10 dips in the orange stain followed by 5 dips in the blue stain. The extra stain was washed off with tap water and slides were left to air-dry for 10 minutes. Cover-slips were finally applied to the slides, using DePeX mounting medium (BDH laboratory, Poole, UK) in a fume-hood. One slide, each with two stained cytospin spots of cells, was prepared from each of the duplicate cultures. A conventional light microscope (Model Leica DMLB2: Leica Microsystem, Wetzlar, Germany) was used to examine the cells at 1000 x magnification. For each scoring analysis, two scorers (MH and TA) individually determined cytostatic and cytotoxic events by scoring 500 cells including mono-, bi-, multinucleated cells, necrotic and apoptotic cells, according to previously published classification criteria (108). This allowed calculation of the nuclear division index (NDI).(108,540), a measure of the proliferative status of the viable cell fraction which thus indicates mitogenic response in lymphocytes (108).

The formula for calculating NDI is as follows (540).

$$NDI = \frac{(M1 + 2M2 + 3M3 + 4M4)}{N}$$

*where M1–M4 represent the number of cells with 1–4 nuclei

*N is the total number of viable cells scored (excluding necrotic and apoptotic cells).

The CBMN-Cyt assay genome damage biomarkers (MN, NPB, NBUDs) in 1000 binucleated lymphocyte cells (BNC) were counted from each duplicate culture to give an overall total for each biomarker per 2000 BNC scored per sample. The results were then averaged and presented for every 1000 BNC. An average of 500 mononucleated lymphocyte cells (MNC) were also scored for MN and NPBs in each duplicate culture in MNCs, using criteria previously described (539). The results in MNCs were expressed as MN and NBUD per 100 MNCs per subject. The

HUMN scoring criteria recommends that the MN frequency be determined in a minimum of 1000 cells (539) but in 40% of our slides, there were insufficient MNC to score 1000 cells.

8.5.7 **Measure of Red cell folate**

The method outlining the red cell folate measurement (94,629,641) is presented in chapter 5. A brief outlined is included in this section.

Chemicals required

- 0.5% sodium ascorbate solution: 5g sodium ascorbate (Sigma-Aldrich, New South Wales, Australia) dissolved in 1000 ml Milli Q water
- Working standard solution B of 5-methylTHF solution (concentration=1nmol/L)
- Folic acid casei medium (Difco): 9.4g media was added to 100 ml Milli Q water. The solution was boiled for 2-3 minutes and then filtered with a 0.22µm filter
- .The bacteria inolculum was thawed. 50 µl of the inoculum was added to 4950 µl of folic acid casei media and mixed well. This constitute the inoculated media.
- Blood samples (cord and heel prick bloods collected from the infants) of unknown folate concentration.

The Assay

Briefly, in a 96 well flat-bottom plate, 0.5% sodium ascorbate was added in all the wells. In the blank wells, 100 µl of 0.5% sod ascorbate solution and 100 µl inoculated media was added. Lastly, 100 µl of inoculum was added in standard and sample wells. Final volume in each well was 200 µl. Secondly, in the standard wells, 100-0 µl (decreasing concentration from first to last well) of 0.5% sodium solution was added. Then the working standard solution of 5-methyl THF (1nmol/L) was added in the standard well in increasing concentration (0-100 µl) corresponding to the sodium ascorbate solution. Each concentration was achieved in triplicate. In the sample wells, 80 µl of sodium ascorbate solution was added. Then 20 µl of blood sample was added in the sample well. The study ID was used as the label for each sample well to

carefully define each well. Each concentration was achieved in triplicate. Recovery wells were included for each sample to estimate percentage recovery of folate from the sample. Each recovery well had 60 µl 0.5% sodium solution, 20 µl of sample and 20 µl of standard solution. Lastly, 100 µl of inoculum was added in standard and sample wells. Final volume in each well was 200 µl. The plate was sealed and incubated for 18 hours in an incubator at 37°C. After 18 hours, the bacteria were resuspended by shaking the plate which was covered with the seal to avoid cross-contamination. The plate was read at 590 nm on a spectrophotometer (UV MAX 250, multi-mode micro plate reader, Molecular devices, USA). The optical density values in triplicates were recorded for all wells (standard, sample and recovery). The average value was obtained for each well. Standard deviation and coefficient of variation (CV) was calculated for each point. If the CV values were > 10%, the readings were discarded and sample were re tested. A standard concentration response curve or calibrator curve was obtained by plotting average optical density value as ordinate and concentration of 5-methyl-THF standard as abscissa in logarithm scale utilizing MS Excel 2010 (a snap shot of calculation is included as Appendix 4). The regression equation [y = a ln (x) + c] and R-square value of the calibration curve were computed in MS Excel (641). If the R value was below 0.98, the assay was repeated. The optical value of the sample and recovery was put in a regression equation (interpolate) to calculate the folate concentration in the sample well. The value was adjusted for the dilution factor (x100) to obtain the final folate content in nmol/L per sample (641).

8.5.8 **Statistical analysis**

All CBMN-Cyt biomarkers and infant birth outcomes variables (gestational age at birth, birth weight, birth length, head circumference and APGAR score at 1 and 5 minutes) were first analysed for normality utilizing the D'Agostino Pearson omnibus test. Degree of association between continuous variables was evaluated by correlation analysis. Pearson correlation coefficients were calculated for Gaussian distributed data. Correlation analysis for non-Gaussian distributed data was performed using the Spearman rank test. Gender and birth weight matched samples were selected from the DADHI control to compare the differences among

measured biomarkers from the INFACT cohort. Differences in all the variables (CBMN-Cyt biomarkers in cord blood, infant birth outcomes, red cell folate status in cord blood and maternal anthropometric data) for the DADHI and the INFACT cohorts were assessed by Student's paired t-test (two tailed) for Gaussian distributed data. When the sample distribution was not normal, Wilcoxon matched-pairs signed rank test was performed. Each INFACT case was matched for gender and birth weight (± 150g) with atleast one or two DADHI control, however, for few cases, birth weight matched control could not be found [weight 1890 g and 4940 g, (hence values were not included for this analysis); and for three cases only one match could be found]. All values are presented as Mean [± standard error for mean (SEM)]. For all analyses, differences were accepted as significant at a P-value of < 0.1. Graph Pad Prism version 6.04 for Windows (Graph Pad Inc., San Diego, CA, USA) and SPSS 23.0 (IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY, USA: IBM Corp) were used for all statistical analyses.

8.6 Results

8.6.1 General maternal demographic characteristics and infant birth outcomes for INFACT cases and DADHI control

The mean ± (SD) data for general demographic characteristics measurements for mother-infant cohort are presented in Table 8. 3. The maternal anthropometric data were measured at recruitment at 16-24 week gestation. Mean (± SD) age of mothers (N=14) was 33.3 (± 4.7) years, height was 1.63 (± 5.2) m, weight was 93.0 (± 24.7) kg and BMI was 34.4 (± 8.1) Kg/m^2. Mean (± SD) of highest BP readings recorded for the cohort was 147 (± 14.3)/93.7 (± 11.1) mm Hg. The Mean (± SD) of BP readings recorded at first and second visit were: 117 (± 13.1)/69.9 (± 9.8) mm Hg and 119 (± 12.9)/72.2.9 (± 13.3) mm Hg respectively. Four participants reported family history of PE, three women were primigravida and two women had pregnancy with assisted reproductive technology. Two women were diagnosed with thrombophilia and 4 women were diagnosed with PE (based on measurements of blood pressure and proteinuria).Seven women delivered by caesarean. One participant had placental abruption. Two women delivered twin babies. 13 women reported consumption of folic acid (400-800

µg/d) supplement during pregnancy. The infant birth outcomes were recorded after delivery. Mean (± SD) gestation age for INFACT infant cohort (n=14) was 37.5 (± 1.1) weeks, birth weight was 3086 (± 875) gm, birth length 48.1 (± 3.9) cms and head circumference was 34.4 (± 2.2) cms (Table 8.3). Four infants were of low birth weight (<2500 gm).

Table 8. 3: General demographic data for INFACT mother-infant cohort [mean (± SD)]

Mothers (n=14)*		**Combined infants (n=14)**		**Female (n=9)**	**Male (n=5)**
Age (years)	33.3 (± 4.7)	Gestation age (weeks)	37.5 (± 1.1)	37.3 (± 1.3)	37.7 (± 1.0)
BMI (kg/m^2)	34.4 (± 8.1)	Birth weight (gm)	3086 (± 875)	2663 (± 541)	3848 (± 879)
Height (m)	1.63 (± 5.2)	Birth length (cms)	48.1 (± 3.9)	46.4 (± 3.1)	51.2 (± 3.6)
Weight (Kg)	93.0 (± 24.7)	Head circumference (cms)	34.4 (± 2.2)	33.8 (±2.2)	35.6 (± 2.0)
Women who took Folic acid supplement (400 -800 µg)*#	13	APGAR score at 1 minute	7.3 (± 1.5)	7.2 (± 1.8)	7.6 (± 1.1)
Women who smoked during pregnancy *	2	APGAR score at 5 minutes	8.8 (± 0.5)	9.0 (± 0.5)	8.6 (± 0.5)
Women who consumed alcohol during pregnancy *	none				

*Maternal demographic data was collected at recruitment (8-16 weeks of gestation), 5 women had previous history of PE, 4 women were diagnosed with PE in this pregnancy, 4 women had family history of PE. n=number of women/infants, BMI: body mass index

Approximately half of the women in the INFACT group were consuming high dose (4mg/d) of folic acid (after 8-16 week gestation) and the information cannot be unblinded till the completion of FACT trial in year 2018.

Each INFACT case was matched for gender and birth weight with at least one or two DADHI control, however, for few cases, birth weight matched control could not be found [weight 1890 g and 4940 g];and for three cases only one match could be found]. The mean ± (SD) data for general demographic characteristics measurements for subset of mother-infant pairs from DADHI cohort (n=19) that were gender and birth weight matched with INFACT cases are presented in Table 8. 4. The maternal anthropometric data were measured at recruitment at 8016 week gestation. Mean (± SD) age of mothers (n=19) was 29.6 (± 5.2) years, height was 1.6 (± 0.07) m, weight was 67.6 (± 11.2) kg and BMI was 25.4.4 (± 3.7) Kg/m^2. 18 women reported consumption of FA (400-800 µg/d) supplement during pregnancy. The infant birth outcomes were recorded after delivery. Mean (± SD) gestation age for gender and birth weight matched subset of DADHI infant control (n=19) was 39.3 (± 0.99) weeks, birth weight was 3236 (± 585) gm, birth length 48.7 (± 2.1) cms and head circumference was 34.3 (± 1.77) cms (**Table 8.4**).

Table 8. 4: General demographic data for subset of mother-infant pairs of DADHI control [mean (± SD)]

Mothers (n=19)*		**Combined infants (n=19)**		**Female (n=11)**	**Male (n=8)**
Age (years)	29.6 (± 5.2)	Gestation age (weeks)	39.3 (± 0.99)	39.0 (± 0.89)	39.7 (± 1.05)
BMI (kg/m^2)	25.4.4 (± 3.7)	Birth weight (gm)	3236 (± 585)	2923 (± 443)	3666 (± 484)
Height (m)	1.6 (±0.07)	Birth length (cms)	48.7 (± 2.1)	47.7 (± 1.4)	50.1 (± 2.1)
Weight (Kg)	67.6 (± 11.2)	Head circumference (cms)	34.3 (± 1.77)	33.5 (±1.4)	35.5 (± 1.4)
Women who took Folic acid supplement (400 -800 µg)*	18	APGAR score at 1 minute	8.07 (± 1.2)	7.7 (± 1.2)	8.4 (± 1.1)
Women who smoked during pregnancy *	1	APGAR score at 5 minutes	8.78 (± 0.42)	8.7 (± 0.48)	8.8 (± 0.37)
Women who consumed alcohol during pregnancy *	2				

*Maternal demographic data was collected at recruitment (8-16 weeks of gestation), n=number of women/infants. DADHI controls were matched for gender and birth weight with INFACT cohort.

8.6.2 **Correlation analysis of mother's anthropometric measures at recruitment with infant birth outcomes at birth-INFACT cohort**

Infant birth weight was positively associated with mother's weight at recruitment ($r = 0.60$, $p = 0.02$) and similarly infant birth length was positively also associated with mother's weight ($r = 0.45$, $p = 0.09$). No correlation was observed between mother's age and BMI and any of the infant birth outcomes (**Table 8.5**). The GA was correlated positively with infant birth weight ($r= 0.48$, $p = 0.07$) (**Table 8.6**).

Table 8. 5: Correlation analysis of mother's anthropometric characteristics at recruitment and infant birth outcomes at birth-INFACT cohort

Mother's characteristics (n = 14)	**Infant birth outcomes (N = 14)**				
	Weight (gms)	Length (cms)	Head circumference (cms)	APGAR score at 1min	APGAR score at 5 min
Age (yrs)	r = 0.41 p = 0.14	r = 0.31 p = 0.26	r = 0.04 p = 0.86	r = - 0.32 p = 0.26	r = - 0.24 p = 0.39
Weight (kg)	r = 0.41 p = 0.13	**r = 0.45** **p = 0.09**	r = 0.25 P = 0.38	r = 0.32 P = 0.26	r = 0.01 p = 0.96
Height (m)	**r = 0.60** **p = 0.02****	r = 0.44 p = 0.10	r = 0.21 P = 0.46	r = 0.00 P = 0.98	r = - 0.14 p = 0.62
BMI (kg/m^2)	r = 0.30 p = 0.29	r = 0.39 p = 0.16	r = 0.20 p = 0.48	r = 0.36 p = 0.19	r = 0.03 p = 0.91

Table 8. 6: Correlation analysis of gestation age and infant's birth outcomes-INFACT cohort

	Infant birth outcomes				
	Weight (gms)	Length (cms)	Head circumference (cms)	APGAR score at 1 min	APGAR score at 5 min
Gestation age (weeks	**r = 0.48** **p= 0.07***	r = 0.29 p = 0.31	r = - 0.17 p = 0.54	r = - 0.32 p = 0.25	r = - 0.23 p = 0.40

Each infant birth outcome was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution);
**: significant at $p \leq 0.05$, * ≤ 0.1 (All P value are two tailed)

8.6.3 **DNA damage biomarkers and red cell folate measures at birth -INFACT cohort**

The CBMN-Cyt biomarkers for DNA damage as assessed in lymphocytes collected from cord blood of infants born to women at high risk of pre-eclampsia is summarised in **Table 8.6.** The mean (± SD) frequency for MN, NPB and NBUD per 1000 BNC was 3.6 (± 2.8), 4.0 (± 3.0), and 9.6 (± 5.8) respectively. The mean (± SD) NDI was 1.8 (± 0.08). The mean (± SD) for measures of cytotoxicity: apoptotic and necrotic lymphocytes measured per 500 viable cells were 5.8 ± (2.1) and 45.6 (± 16.1) respectively. The mean (± SD) for MN and NBUD in MNC was 0.36 (± 0.24) and 1.3 ± (0.67) respectively. The red cell folate was 599 (± 140) nmol/L) (**Table 8.7**).

Table 8. 7: Mean (± SD) CBMN-Cyt biomarkers and red cell folate measured at birth -INFACT cohort

CBMN-Cyt biomarker	Combined infants (n=10)	Female cohort (n=5)	Male cohort (n=5)
MN BNC	3.6 (± 2.8)	3.4 (± 2.7)	3.8 (± 3.2)
NPB BNC	4.0 (± 3.0)	3.8 (± 2.9)	4.3 (± 3.4)
NBUD BNC	9.6 (± 5.8)	10.1 (±5.9)	9.0 (± 6.3)
NDI	1.8 (± 0.08)	1.7 (±0.08)	1.8 (± 0.07)
Apoptotic lymphocyte	5.8 (± 2.1)	5.5 (± 2.3)	6.1 (± 2.1)
Necrotic lymphocyte	45.6 (± 16.4)	39.6 (± 14.8)	51.7 (± 17.3)
MN MNC	0.36 **(±** 0.24)	0.29 (± 0.22)	0.44 (± 0.26)
NBUD MNC	1.3 (± 0.67)	1.67 (± 0.70)	1.0 (± 0.5)
Red cell folate (nmol/L)	599 (± 140)	527 (± 114)	684 (± 127)

The four slides had lysed cell and hence CBMN-Cyt biomarkers was not available.
Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD are presented per 100 MNC; n: number of subjects.

8.6.4 **Correlation analysis of maternal anthropometric data and Infant birth outcomes with CBMN-Cyt biomarkers measured in cord blood at birth-INFACT cohort**

Mother's age recorded at the time of recruitment was found to be positively associated with NPB BNC (r = 0.61, p = 0.05). Mother's weight and height were observed to be positively associated with NBUD BNC (r = 0.62, p = 0.05 and r = 0.61, p= 0.05) (**Table 8.8**).

The association between infant birth outcomes and CBMN-Cyt biomarkers measured in lymphocytes collected at birth was assessed. The study observed negative association of GA with apoptotic lymphocytes (r = - 0.56, p = 0.08). Head circumference was negatively correlated with MN in BNC (r = - 0.61, p =0.05) and MNC (r= - 0.55, p = 0.09). APGAR score at 1 minutes was negatively associated with NPB BNC (r = - 0.61, p = 0.05) and at 5 minutes was negatively associated with MN BNC (r = - 0.64, p = 0.04) and MN MNC (r = - 0.65, p = 0.03) (**Table 8.9**).

Table 8. 8: Correlation analysis of maternal anthropometric characteristics at recruitment and CBMN-Cyt biomarkers in cord blood at birth-INFACT cohort

Maternal characteristics	**CBMN-Cyt biomarkers in cord lymphocytes at birth (n=10)**							
	MN BNC	NPB BNC	NBUD BNC	NDI	Apoptotic cells	Necrotic cells	MN MNC	NBUD MNC
Age (yrs)	r = - 0.05 P =0.88	**r = 0.61** **P =0.05***	r = 0.02 P = 0.95	r = 0.00 P = 0.99	r = - 0.31 P = 0.37	r = - 0.08 P = 0.81	r= 0.05 P =0.88	r = - 0.10 P =0.76
Weight (kg)	r = - 0.09 P =0.78	r = 0.28 P =0.42	**r = 0.62** **P =0.05***	r = 0.41 P = 0.22	r = - 0.47 P = 0.16	r = - 0.39 P = 0.25	r= 0.03 P = 0.93	r = 0.22 P = 0.52
Height (m)	r = 0.07 P = 0.84	r = 0.50 P = 0.13	**r = 0.61** **P = 0.05***	r= 0.49 P=0.14	r = - 0.49 P = 0.14	r = - 0.49 P = 0.14	r = 0.12 P = 0.73	r = - 0.31 P =0.38
BMI (kg/m^2)	r = - 0.12 P =0.72	r = 0.16 P =0.65	r = 0.53 P =0.10	r = 0.34 P =0.33	r = - 0.40 P =0.23	r = - 0.31 P =0.37	r = 0.01 P = 0.97	r = 0.38 P = 0.27

Each DNA damage biomarker was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution);
**: significant at $p \leq 0.05$, * $p \leq 0.1$ (All p value are two tailed)
The four slides had lysed cell and hence CBMN-Cyt biomarkers was not available.
Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD are presented per 100 MNC;
n: number of subjects.

Table 8. 9: Correlation analysis of infant birth outcomes and CBMN-Cyt biomarkers measured in cord blood at birth-INFACT cohort (n=10)

Infant Birth outcomes	MN BNC	NPB BNC	NBUD BNC	NDI	Apoptotic cells	Necrotic cells	MN MNC	NBUD MNC
Gestation age (weeks)	r = - 0.23 P = 0.50	r = 0.46 P = 0.17	r = 0.49 P = 0.14	r = 0.16 P = 0.64	**r = - 0.56** **P= 0.08***	r = - 0.54 P = 0.10	r = - 0.07 P = 0.83	r = 0.12 P = 0.72
Birth weight (gm)	r = - 0.12 P = 0.72	r = 0.51 P = 0.12	r = 0.35 P = 0.31	r = 0.17 P = 0.63	r = - 0.33 P = 0.34	r = - 0.04 P = 0.90	r = 0.05 P = 0.87	r = - 0.41 P = 0.22
Birth length (cms)	r = - 0.30 P =0.38	r = 0.35 P = 0.31	r = 0.09 P = 0.79	r = 0.15 P = 0.67	r= - 0.25 P= 0.46	r = 0.06 P = 0.85	r = - 0.04 P = 0.90	r = - 0.27 P = 0.44
Head circumference (cms)	**r = - 0.61** **P =0.05****	r = 0.23 P = 0.51`	r = - 0.03 P = 0.93	r = - 0.37 P = 0.28	r = 0.31 P = 0.38	r = 0.19 P = 0.58	**r= - 0.55** **P = 0.09***	r= 0.10 P= 0.76
APGAR score at 1 minute after birth	r = 0.06 P =0.85	**r = - 0.61** **P =0.05****	r = - 0.16 P = 0.65	r = 0.08 P = 0.82	r = 0.02 P = 0.95	r = 0.51 P = 0.12	r = 0.15 P = 0.67	r = - 0.32 P = 0.36
APGAR score at 5 minutes after birth	**r = - 0.64** **P =0.04****	r = - 0.34 P =0.32	r = - 0.10 P = 0.76	r = - 0.22 P = 0.52	r = 0.25 P = 0.48	r = - 0.07 P = 0.84	**r = - 0.65** **P = 0.03****	r = 0.06 P = 0.86

Each DNA damage biomarker was tested for Gaussian distribution and then Pearson 'r' (parametric test for normal distribution data) and Spearman' 'r was calculated (non-parametric test for non-Gaussian distribution); **: significant at $P \leq 0.05$, * $P \leq 0.1$ (All P value are two tailed)
The four slides had lysed cell and hence CBMN-Cyt biomarkers was not available.
Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD are presented per 100 MNC

8.6.5 **Comparison of maternal and infant characteristics between INFACT and DADHI cohort**

The INFACT infant cases were matched with infants in the subset of DADHI cohort with respect to birth weights and gender. The comparison between the cases and controls is presented in **Table 8.10**.

The mothers in the INFACT group had significantly higher mean weight ($p < 0.0001$) and mean BMI ($p = 0.002$) compared to mothers of the DADHI cohort. No other significant difference was observed in the two cohorts with respect to maternal anthropometric markers. The GA of the infants in INFACT cohort was lower when compared with the infants in the DADHI cohort ($p < 0.0001$). The red cell folate in cord blood was significantly higher for INFACT cases when compared with the DADHI control ($p < 0.0001$).

Table 8. 10: Comparison between infant birth outcomes & RCF between INFACT and birth weight matched DADHI control (n ranged from 14-19)

Maternal anthropometric variables	**INFACT [Mean (± SE)]**	**DADHI [Mean (± SE)]**	**p-value**	**95% CI**
Age (years)	32.92(± 1.3)	29.6(± 1.2)	0.13	-7.17 to 1.06
Weight (Kg)	95.6 (± 7.4)	67.6 (± 2.6)	**<0.0001****	-27.45#
Height (m)	1.64 (± 0.01)	1.6 (± 0.02)	0.51	-0.05 to 0.03
BMI (Kg/m^2)	35.22 (± 2.4)	25.4 (± 1.0)	**0.002****	-16.08 to -4.49
Infant's birth outcomes				
Gestation age (weeks)	37.5 (± 0.31)	39.3 (± 0.23)	**<0.0001****	1.55#
Birth length (cms)	48.1 (± 1.0)	48.7 (± 0.48)	0.9	-1.19 to 1.19
Birth weight (g)	3086 (± 233)	3236 (±134)	0.9	-47.6 to 47.6
Head circumference (cms)	34.4 (± 0.61)	34.3 (± 0.4)	0.06	-0.05 to 3.51
APGAR at 1 min	7.3 (± 0.42)	8.0 (± 0.32)	0.21	1.0#
APGAR at 5 min	8.8 (± 0.14)	8.7 (± 0.11)	0.68	0.0#
Folate (nmol/L)	599 (± 42.3)	364 (± 15.9)	**<0.0001****	-265.5 to -166.6

Paired 't' test for performed for comparison between INFACT cases and DADHI control for Gaussian distribution and Wilcoxon matched-pairs signed rank test was performed for non-Gaussian distribution. Each INFACT case was matched for gender and birth weight with atleast one or two DADHI control, however, for few cases, birth weight matched control could not be found [weight 1890 g and 4940 g, (hence values were not included for this analysis); and for three cases only one match could be found].
All values are presented as Mean (± standard error for mean); p value: level of significance; 95% CI: confidence intervals; #: Median of differences for Wilcoxon test, n=number of samples; All p values are two tailed. *: $p \leq 0.05$; ** $p \leq 0.01$; ***: $p \leq 0.001$, ****: $p \leq 0.0001$].

8.6.6 **Comparison between CBMN-Cyt biomarkers measured in cord blood between INFACT cases and subset of DADHI control**

The DNA damage biomarkers measured in the cord lymphocytes indicated significant differences between INFACT cases and DADHI control groups and are shown in the **Table 8.11**. MN BNC were significantly higher in the INFACT group ($p = 0.02$) compared to the control group. NDI was higher in the INFACT cases when compared with the subset of DADHI control ($p = 0.001$). DNA damage biomarkers measured in MNC (MN and NBUD) were observed to be higher among the INFACT cohort compared to the control group ($p = 0.0001$, $p = 0.0004$ respectively) (**Table 8.11**).

Table 8. 11: Comparison between CBMN-Cyt biomarkers measured in cord blood between INFACT cases and DADHI control

	INFACT (n=10) Mean (± SE)	**DADHI(n=10) Mean (± SE)**	**p-value**	**95% CI**
MN BNC	3.66 (± 0.89)	1.45 (± 0.18)	**0.02***	- 2.25 #
NPB BNC	4.05 (± 0.95)	6.2 (± 1.0)	0.23	-1.48 to 5.55
NBUD BNC	9.6 (± 1.8)	9.4 (± 1.1)	0.60	- 4.58 to 2.78
NDI	1.8 (± 0.02)	1.5 (± 0.05)	**0.001*****	- 0.43 to -0.12
Apoptotic lymphocytes	5.8 (± 0.67)	6.1 (± 0.9)	0.9	- 0.12 #
Necrotic lymphocytes	45.6 (± 5.2)	32.6 (± 3.4)	0.21	- 11.63 #
MN MNC	0.36 (± 0.07)	0.11 (± 0.03)	**0.0001******	-0.22 #
NBUD MNC	1.3 (± 0.21)	0.54 (± 0.12)	**0.0004*****	- 0.8 #

Paired 't' test for performed for comparison between INFACT cases and DADHI control for Gaussian distribution and Wilcoxon matched-pairs signed rank test was performed for non-Gaussian distribution. Each INFACT case was matched for gender and birth weight with atleast one or two DADHI control, however, for few cases, birth weight matched control could not be found [weight 1890 g and 4940 g, (hence values were not included for this analysis); and for three cases only one match could be found].
All values are presented as Mean (± standard error for mean); p value: level of significance; 95% CI: confidence intervals; #: Median of differences for Wilcoxon test, N=number of samples; All p values are two tailed. *$p \leq 0.05$; ** $p \leq 0.01$; ***: $p \leq 0.001$, ****: $p \leq 0.0001$].
Abbreviations: MN: micronuclei; BNC: Binucleated lymphocyte cells; NPB: Nucleoplasmic bridge; NBUD: Nuclear buds; MNC: mononucleated lymphocyte cells; MN, NPB and NBUD are presented per 1000 BNC, NDI, apoptotic and necrotic lymphocyte are presented per 500 cells, MN and NBUD are presented per 100 MNC;

8.7 **Discussions**

Preeclampsia affects approximately 5-7% of pregnancies all over the world (2) and is now understood to be a state of increased oxidative stress and inflammation (850-854,856,872,875). It is speculated that altered expression of inflammatory genes may be contributing to inflammatory response and endothelial dysfunction during placental implantation in women who develop PE (523). The pre-eclamptic placental tissue and maternal blood samples have been shown to have higher concentrations of oxidative damage biomarkers such as 8-OHdG, ref-1, activin A and F2 isoprostane as well as elevated Hcy (102,143,144,860,876,877). Women with elevated MN frequency measured at 20 week gestation have been shown to have a higher risk to develop PE later in pregnancy (118). However it is not known whether infants born to women at risk of developing PE may carry high DNA damage biomarkers at birth. Further, the cord blood of women at risk of PE has not been investigated utilizing a comprehensive DNA damage assay that measure genotoxicity and cytotoxicity and cytogenetic level. Hence, a case control study was designed as a pilot project to collect comprehensive DNA damage data utilizing the CBMN-Cyt assay from cord blood collected at delivery from the women who were enrolled in the Investigations in the Folic acid clinical trial (INFACT study) in South Australia. (286). A small number of women could be enrolled owing to some unavoidable circumstances. Firstly, only 5% of women are at risk of developing PE. Secondly there were some administrative delays in initiating the FACT project in Australia. Also, owing to time constraint of a PhD project, the recruitment could not be continued for more than 2 years. Further, owing to researcher's health issues and miscommunication with midwives, some cord samples could not be collected. The data was thus collected from 14 women and their infants and was compared with birth-weight and gender matched subset of infants born to women at low risk of complication from the DADHI study. (indicated as DADHI control in this chapter).

8.7.1 ***Association of infant birth outcomes with maternal anthropometric characteristics***

The average BMI for our INFACT cohort was high and could be categorized in obese (II) category (562). Maternal weight at recruitment was positively associated infant birth length and maternal height with infant birth weight indicating that maternal anthropometric parameters may influence infant's birth outcomes. Male and female cases were between 50 th and 75th percentile when compared with Australian standard for GA and birth weight (**Appendix 15 and 16**). GA was correlated positively with infant birth weight which is normally expected. PE may cause early delivery and low birth weight infants (878) or IUGR (865) but in our cohort comprising of four women with diagnosed condition of PE, only four infants were born LBW (< 2500 gms) and the mean (± SD) birth weight was 3086 (± 875) suggesting that maternal overweight could be a causal factor for increased infant birth weight (358). A previous population based cohort study in Australia also reported birth weight ≥ 4500 g (Adjusted OR 19.94, 95 % CI: 6.81-58.36) of infants born to super-obese women with a median BMI of 52.8 kg/m^2 (879). An estimate by a meta-analysis showed that maternal obesity increases the risk of infants born large for gestation age and birth weight greater than 4000g i.e: macrosomia, (360). Additionally, studies have consistently shown association of increased maternal BMI and obesity with infant's metabolic profile shift towards that observed in obesity (350,355,358,359,361,362), increased blood pressure (362,363), metabolic syndrome (364) or type 2 diabetes (365) during young adulthood. We also found positive association of maternal weight and height with NBUD BNC and maternal age with NPB suggesting that metabolic stress and ageing in the mother may cause increased chromosomal instability in the foetus which is manifested at birth in the infant. Interestingly, the MN frequency index in the infants was strongly inversely correlated with head circumference suggesting an inhibitory effect of increased DNA damage on brain size. The female INFACT cases were observed to have mean head circumference below 50th percentile when compared with WHO standards (**Appendix 22**). Recently, it was shown that microcephaly is associated with increase MN and NPB in humans

with defects in condensing proteins required for proper segregation of chromosomes (342). Furthermore, MN and NPB were also negatively correlated with APGAR score suggesting an association with poor lung and/or heart function in the infants (731).

8.7.2 Comparison of DNA damage CBMN-Cyt biomarkers between INFACT and DADHI cohorts

The mother-infant cohort's anthropometric and DNA damage data for INFACT group was compared with infant weight and gender matched sample from DADHI control which comprised of healthy infants born to women at low risk of complications during pregnancy. The women participants in the INFACT cohort were significantly heavier in weight and BMI compared to DADHI control. The infants in both cohorts were similar in all birth outcomes except gestation age. The INFACT cohort had significantly shorter gestation age than DADHI control that is usual outcome for infants born to women at risk of PE. The INFACT cases were also observed to have higher red cell folate status which may be owing to folic acid supplementation (4mg/d) in this group. As the investigator was blinded from the detailed information on placebo or supplementation group in FACT trial so the reasons for higher red folate status could not be explored.

The INFACT cases had higher frequency of MN BNC ($p = 0.02$) and MNC ($p = 0.0001$), frequency of NBUD MNC was also higher in INFACT cases ($p = 0.0004$) compared to control. To our knowledge, this is the first time that infants born to women at high risk of PE were assessed for frequency of CBMN-Cyt biomarkers at birth. There have been few studies that have investigated oxidative DNA damage biomarkers in infants born to women with/or at risk of PE (860,865,873,875,876,880-882). A cross-sectional study in Turkey measured DNA damage using the alkaline comet assay in mononuclear leukocytes collected from mothers and cord blood of hypertensive pregnant women (mildly PE, n = 25) and normotensive pregnant women (n=20) just after delivery. The study reported increased DNA damage ($p< 0.001$), decreased total oxidant status ($p < 0.001$), increased oxidative stress index ($p < 0.001$) in pre-

eclampic mothers compared to control (873). Fujimaki *et al* investigated association of placental oxidative stress with IUGR in PE women by measuring placental oxidative DNA damage and its repair in blood and placental tissue collected at delivery from three small groups: women with PE and IUGR (n = 13), women with PE without IUGR (n = 10) and healthy pregnant women without complications (n = 10) (880). The study found increased serum derivatives of reactive oxygen metabolites (ROMs) in the maternal blood of women with PE (with IUGR: $p < 0.01$; without IUGR: $p < 0.001$) compared with controls. The 8-OHdG and ref-1 was also higher in women with PE and IUGR ($P < 0.001$) than in the control group indicating the possibility of transfer of maternal ROMs to infants born to women with PE (880). Furthermore, infants born to women with diabetes (334) and epilepsy have also been observed to have higher MN frequency (554). More than one mechanism can explain the origin of MN, including terminal acentric chromosome fragments, acentric chromatid fragments, whole chromosome malsegragation, misrepair of DNA strand breaks, inappropriate base incorporation (e.g. uracil) or base damage (e.g. 8 -OHdG that leads to transient DNA break (109). Among all CBMN-Cyt biomarkers, MN frequency has been the most investigated among cord blood and mainly in cohorts of healthy mother-infant cohort (326,328,329). A meta-analysis of MN frequency based on 13 field studies in children (n = 440) of varying age groups (0-18 years), residing in different countries and a pooled analysis of individual data (n = 332) reported an overall mean of 4.48 and pooled baseline estimate of 3.27 MN per 1000 BNCs for infants (0-1 year) (555). These values are close to mean MN observed in our INFACT cohort. However, MN frequency is usually reported to increase in response to exposure to pollutants (315,551,571,574,575,664), disease state (331,334,554,556,682), and deficiency of micronutrients especially folate, B_{12}, vitamin E, and iron (145,242,435). Thus it is not possible to compare our values collected from a small number of infants born to women at high risk of PE in Australia with those collected form healthy infants born to normal women residing in a different geographical condition.

Interestingly, the INFACT cases were observed to have significantly higher NDI compared to DADHI control indicating either that this slight increase in DNA damage may not be sufficient to suppress proliferation potential of infant cells or that cell cycle checkpoint were too permissive allowing lymphocytes with DNA damage to survive and replicate. Furthermore, NDI could be affected by various *in utero* conditions, cell culture conditions or replication stress factors (558,687,698) that were not measured in this pilot study. The study did not find significant differences in measures of cytotoxicity: apoptosis and necrosis among cases and controls. Further studies are hence required on a large sample of infants born to women at high risk of PE to investigate biomarkers for various nutritional and environmental factors that are known to modulate CBMN-Cyt DNA damage biomarkers and NDI.

8.8 **Limitation**

However, the results of this pilot case control study need to be interpreted with caution given the small number of subjects studied and some participants were receiving high dose of folic acid supplementation in the INFACT group. The 95% CI were large for most of the differences, indicating that results could be attributed to chance. Further, some associations were weak (p =0.05 to 0.1

8.9 **Conclusions**

To our knowledge, this is the first time that comprehensive DNA damage, cytostasis and cytotoxicity data was collected from cord blood of infants born to women at high risk of developing PE in Australia by utilizing a reliable and well-validated CBMN-Cyt assay. The data indicates that these infants have higher DNA damage and higher cytostasis when compared with healthy control group. The results also show that higher maternal weight, height and gestation age may increase DNA damage biomarkers in infants. This baseline data may now be used to form the design of further investigations on large cohorts to build the evidence so that DNA damage in human tissues can be detected and monitored at the earliest possible phase of

life and to identify preventive strategies for maintenance of genome integrity and supporting healthy development and ageing.

9 Conclusions, knowledge gaps and future directions

This PhD project was conducted in four stages, and the knowledge arising from each stage including knowledge gaps are presented in four subsections of this chapter.

Stage I- A systematic review: The aim of the review was to explore the literature and identify potential knowledge gaps in relation to the role of folate at the genomic level in either the aetiology or the prevention of pre-eclampsia. A systematic search strategy was designed to identify citations in electronic databases. 43 articles were selected according to predefined selection criteria**.** The studies, selected on the basis of the inclusion criteria (n=43), were then grouped into Genome stability in women at risk of pre-eclampsia (n=5), DNA methylation in women at risk of pre-eclampsia (n=25) and 'Folic acid supplementation in pre-eclampsia' (n=13). The diverse subject group and the different type of variables studied across the articles selected prohibited statistical assessment of heterogeneity and meta-analysis. Hence a narrative synthesis was conducted.

One of the ***main findings*** of the review as outlined in chapter 1 is that deficiency of micronutrients, mainly folate, vitamin B_6, vitamin B_{12}, and vitamin $B_{2,}$ together with differences in frequency of polymorphisms of genes required for the function of key enzymes in one carbon metabolism (OCM), and increased homocysteine (Hcy) are observed in women with pre-eclampsia. Also, a higher concentration of numerous oxidative stress biomarkers: activin A, 8-deoxy hydroxyl guanosine (8-OHdG), 8-isoprostane, increased thioredoxin expression in various maternal tissues and fluids (maternal blood, cord blood, omental arteries and placenta), have been observed in pre-eclamptic women when compared with women at low risk of pre-eclampsia. Further, altered DNA methylation is consistently reported in various tissues of women with PE, highlighting possible defects in OCM or inadequate intake of dietary methyl donors. The women with increased DNA damage measured by micronuclei (MN) frequency in lymphocytes collected at 20 weeks gestation may develop PE. The review also highlighted evidence in the literature that some of this dysregulations may be rectified epigenetically with oral intake of methyl donors (e.g.: folate), B_2, B_6 and B_{12}.

Knowledge gaps:

1. Does folic acid reduce blood pressure in women at risk of PE and adverse infant birth outcomes among women at risk of pre-eclampsia?
2. Does vitamin B_2 supplementation reduce BP in those carrying MTHFR C667T polymorphism?
3. Can folic acid (FA) supplementation in the diet reduce plasma Hcy concentrations in humans with an efficacy that may be dependent on genotype (e.g. of methylenetetrahydrofolate reductase - MTHFR) and dose and whether the same can be achieved in women with pre-eclampsia under placebo-controlled randomized conditions?
4. The amount of folic acid required, the time of initiating supplementation and the duration for such an effect to become evident with respect to prevention of pre-eclampsia, are all not known.
5. It is not known if any observed effect on PE following folate prophylaxis is influenced by common polymorphisms in the genes coding for the key folate pathway enzymes.
6. Folate deficiency has been reported to alter lymphocyte DNA methylation in humans. Altered global DNA methylation has also been reported in the placentas of women with PE. It is not known, however, if high dose folic acid therapy alters DNA methylation patterns in placental tissue consistently and in a beneficial manner: intervention studies are required. It is also not known whether DNA methylation in lymphocytes correlates with that of placental and fetal tissue.
7. There is a complex interplay among all methyl donors, including $B_{2,}$ B_6, B_{12}, choline and folate, in maintaining various metabolic functions. It is not known how these factors, severally and together, might improve the prognosis and the prevention of pre-eclampsia.

8. Folate deficiency causes the increased appearance of micronuclei (MN: a biomarker of DNA damage) in human lymphocytes, which has been observed in women at 20 week gestation to predict subsequent development of PE and/or intrauterine growth restriction (IUGR). It is not known whether the appearance of MN in lymphocytes correlates with DNA damage and epigenetic modifications, either in the uterine spiral arteries or in the placental cells or fetal tissues other than blood cells.

Future directions: The international folic acid clinical trial (FACT) study and the Investigations in FACT (INFACT) study in Australia are currently underway, and may help in finding answers to some of these gaps in the literature.

*****Intervention studies in a large cohort of women at risk of pre-eclampsia are required to answer whether the observed changes in MN frequency are a cause or a consequence of PE and also whether there is any change in the MN frequency, alongside changes in plasma Hcy, in women at increased risk of PE following prophylactic treatment with high dose FA and/or other B vitamins such as B_{12}, B_2 or B_6.

******Further, it needs to be tested whether infants born to women at increased risk of pre-eclampsia have increased DNA damage when compared with infants born to women at low risk of PE, utilizing a comprehensive validated assay for measuring genome instability of infants.

Stage II: A longitudinal prospective study on DNA damage in infants at birth, three and six months after birth

The observation of high measures of oxidative stress in placenta and cord blood has led to the hypothesis that infants born to mothers with inflammatory conditions, such as PE, may therefore be born with increased cellular DNA damage compared with infants born to women at low risk of PE. Damage to the genome is recognised as an important pathological event that may lead to developmental defects, increases in inflammatory cytokines, immune system dysfunction and an increase in the risk for early onset of degenerative diseases, including cancer. It is of note that the incidence of various childhood cancers has been observed to be

rising in Australia although mortality is decreasing due to better treatment. DNA damage, identified in the immediate perinatal period and sustained during infancy may reflect the genomic impact of maternal diet, such as deficiency of folate, as well as any life-style and/or genotoxic exposure of the mother One of the modifiable environmental factors that may influence the stability and integrity of the infant genome is choice of nutrition for the baby, whether it be through breast milk, formula or complementary feeds. However, there are no DNA damage data at the time of birth from infants born in Australia, and in particular none that have utilized a well validated assay that measures genome health comprehensively to include DNA damage markers, cytostasis and cytotoxicity markers. Furthermore, there are also no data on whether the mode of feeding may subsequently modulate these biomarkers in infants born in Australia.

Hence, a prospective cohort study has been conducted; '**Diet and DNA Damage in Infants'** (The DADHI study), with the aim of collecting data on lymphocyte genome integrity and DNA damage markers, utilizing the robust and well-validated cytokinesis block micronucleus cytome (CBMN-Cyt) assay, in Australian infants at birth and followed at 3 and 6 months of age) born to mothers at low risk of inflammatory conditions. The subset of these data have then been used for comparison with the degree of DNA damage in infants born to women at high risk of PE during pregnancy in stage IV of this PhD project.

The ***main finding*** from this prospective cohort study was the signidicant association of both infant birth outcomes (Birth weight, head circumference, birth length and APGAR score) and maternal anthropometric variables (weight and body mass index) with CBMN-Cyt biomarkers in cord blood, suggesting the possibility of a genotoxic effect of metabolic processes that promotes excessive growth and high BMI and that larger birth size may be consequential to more chromosomal damage possibly due to failure of cell cycle checkpoints.

Also, the mean frequency of CBMN-Cyt biomarkers in cord blood decreased significantly at three and six months after birth relative to cord blood. The decrease in DNA damage biomarkers

was not associated with type of feeding for the infants suggesting that formula and complementary foods used in South Australia are adequate to meet the nutritional needs of infants for maintenance of genome integrity.

Knowledge gaps:

1. The sample size of the DADHI study was small and needs to be replicated to verify the observed associations. It may now be utilized as a baseline dataset for the frequency of DNA damage biomarkers in Australian infants. These initial baseline data will be useful to form the design of similar but larger prospective studies including testing whether infants born to women at high risk of PE may have greater DNA damage compared with infants born to women at low risk of PE as was suggested by our pilot investigation..
2. DNA damage and repair in the offspring may be influenced by numerous environmental factors both pre- and postnatally, by the diet and lifestyle of their mothers, but it is not known what effects these exposure variables might have on DNA damage in cord blood and infants.
3. Does DNA damage vary substantially between lymphocyte subset and their precursors?
4. Variation in the nutritional profile of breast milk and the actual amount of milk consumed by the infant needs to be quantified and similarly complementary food and formula milk needs more detailed analysis.
5. The possibility that breast milk may also be contaminated with environmental pollutants (e.g.: pesticides) should also be taken into consideration
6. It is not known how environmental factors, mainly breast feeding and maternal diet and lifestyle variables, might modulate telomere length in infants and how these might interact with differing risks of pregnancy complications in the mother.
7. It is not known if and how the status of micronutrients relevant for genome maintenance in cord blood might be subsequently affected by different modes of infant feeding

Future directions: The knowledge on the effect of dietary factors in infants on telomere length should also be investigated. In addition a database of knowledge on environmental genotoxins that contribute to genome damage in infants in Australia should be established.

Stage III: The association of blood micronutrients in South Australian infants with birth outcomes, feeding methods and genome damage during first six months after birth.

An optimal balance of dietary micronutrients is essential for the maintenance of human genome integrity. A range of dietary micronutrients including folate and B vitamins, as well as various minerals and other vitamins, are required as enzymatic cofactors or substrates of reactions involved in DNA synthesis or repair or prevention of oxidative damage to DNA. Hence, dietary deficiency of micronutrients at any stage of human development may induce DNA damage and epigenetic changes and accelerated telomere shortening or dysfunction. Plasma minerals, serum vitamin B_{12}, folate and red cell folate were analysed in order to understand effect of micronutrients on DNA integrity.

The resources of the SA Pathology laboratory were used to measure most of the micronutrients, but folate was also measured in red blood cells by the more robust 'Microbiological assay': the "gold standard" for folate measurement. The assay was set up and optimised at CSIRO laboratory after an initial training period.

The ***main findings*** of this study were that decreases in the concentration of plasma iron and potassium and of red cell folate, and in contrast, there was increase in copper, magnesium, sodium and sulphur in infant blood from the time of birth to 6 months of age.

Blood micronutrient status was associated with infant birth outcomes: copper, the ratio of Ca to Mg, and vitamin B_{12} concentrations were observed to be positively associated with gestational age, while potassium was negatively associated with gestational age. Calcium was negatively associated with head circumference at birth and sulphur was inversely associated with APGAR score at 1 minute after birth. Associations of individual micronutrients with

different CBMN-Cyt biomarkers varied with infant age. Iron, magnesium and zinc were negatively associated with NBUD, ratio of Ca and Mg was negatively associated with NBUD BNC. Magnesium, sodium potassium were negatively associated with NDI while folate was positively associated with NDI. The associations of some minerals (calcium, zinc and magnesium) with DNA damage biomarkers suggest that oversufficiency of such minerals may be detrimental for cell growth and repair.

Knowledge gaps

1. Metabolites indicative of the efficacy of micronutrients (e.g.: Hcy for folate, methylmalonic acid for vitamin B_{12}) should also be measured
2. As the study demonstrates that micronutrient concentrations may modulate cellular proliferation and DNA damage, further investigations are required to know the dosage/plasma concentration of micronutrients required for genome maintenance in infants.
3. It is not known how the bioavailability of nutrient content of breast milk (and other feeds) given to an infant may be affected by environmental pollutants in air, plastic content of bottles used for feeding, and/or lifestyle habits of pregnant women, including smoking and alcohol.

****Future directions***: Further randomized controlled trials are needed to gain knowledge for recommendations on infant dietary requirements of micronutrients (through breast milk/formula feed /complementary feed).

Stage IV: DNA damage in infants born to women at risk of pre-eclampsia during pregnancy

Pre-eclampsia (PE) affects approximately 5-7% of pregnancies all over the world and is a main cause of perinatal morbidity and mortality. It is a state of high oxidative stress and inflammation

and, therefore, might be associated with increased DNA damage in infants born to women either at risk of or affected by clinical PE. There are currently no data that has investigated comprehensive DNA damage at the cytogenetic level in the cord blood from such infants. The opportunity was therefore taken of an intervention trial of folic acid supplementation in the prevention of pre-eclampsia (the FACT study) to perform a pilot case control study in the 'Investigation in FACT'- the 'INFACT study' to collect comprehensive DNA damage data utilizing CBMN-Cyt assay from cord blood collected from participating women at high risk of PE in South Australia.

The ***main findings*** of this small case control study were that maternal anthropometric variables (weight, height) and gestational age at birth may influence infant birth outcomes, mainly increased birth weight. Further, observation of positive association of maternal weight and height with NBUD BNC and the negative association of infant birth outcomes (head circumference, APGAR score) with CBMN-Cyt biomarkers (MN, NPB) in our cohort suggests that a larger infant size may be consequential to relaxation of cell cycle checkpoints to allow greater cell division and tissue growth resulting in tolerance of higher DNA damage rates. When compared with the DADHI controls that were matched for infant birth weight and gender at birth, the INFACT cases had higher frequency of CMMN-Cyt biomarkers. To our knowledge, this is the first time that infants born to women at high risk of pre-eclampsia have been assessed for frequency of DNA damage biomarkers at birth. All the previous studies have measured various oxidative stress biomarkers in cord blood.

Knowledge gaps:

1. The cohort size of this study was small, so these novel findings need to be tested and verified in a larger prospective group.

2. It is also important that the maternal data, such as blood pressure, be measured prospectively in a low risk cohort.
3. Further telomere length should also be studied in placental tissues of women at low/high risk of pre-eclampsia and their infants to obtain a more comprehensive assessment of genome damage.
4. The frequency of CBMN-Cyt biomarkers needs to be investigated in the infants born to women who have been administered a high dose of FA to prevent neural tube defects, to investigate for possible protective or harmful effects of high FA on DNA integrity.
5. It would also be important to measure Hcy and methymalonic acid concentrations in cord blood collected from women at low/or high risk of PE to understand whether these toxic metabolites are associated with DNA damage and whether FA supplementation mitigates their genotoxic effects.
6. It is not known how effects on genome damage of any micronutrient supplementation might interact with different polymorphisms in genes, both maternal and foetal, that code for enzyme function in one carbon metabolic pathways (MTHFR C667T).

Future directions: The INFACT and FACT studies are both still ongoing and tissue samples from these studies could be utilized to investigate some of the knowledge gaps mentioned above.

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11 Appendix

Appendix 1: An example of DADHI – Infant feeding record sheet

Code number :	BABY FEEDING RECORD	Diet and DNA Health in Infants (DADHI)

In the table below, please indicate with a tick, your baby's feeding pattern from birth.

	Period after birth					
	1st month	2nd month	3rd month	4th month	5th month	6th month
Exclusively breast fed						
Mainly breast fed						
Mainly formula fed						
Exclusively formula fed						

If you bottle fed with formula milk which product/s did you usually use?_____.

If you fed solids, at what age (months) was your baby when you introduced solid foods? _____months old.

If you fed other drinks, which other drinks did you usually feed your baby? _____.

Appendix 1a: Calculation of Infant feeding scores

FINAL FEEDING SCORE AND WEIGHT RECORD FOR ALL DADHI INFANTS

4=Exclusively breast fed
3=Mainly breast fed
2=Mainly formula fed
1=Exclusively formula fed

V1=Visit 1 at 3 months after delivery
v2=visit 2 at 6 months after delivery

W=weight in grams

Study co	Age V1(M	W(G)V1	1 month	2 month	3 month	Total V1	Average	Age V2	W(G)V2	4 month	5 month	6 month	Total V2	Average V2	Total V1+V2	Average V1+V2
DA001	12.5	5130	3	3	3	9	3	22.5	7470	2	1	1	4	1.5	13	6.5
DA002	11	6835	3	2	1	6	2	24	8555	1	1	1	3	1	9	4.5
DA004	12	5830	4	4	4	12	4	24	7465	4	4	4	12	4	24	12
DA005	11	5920	1	1	1	3	1	#N/A						WITHDRAWN	3	1.5
DA011	11	6150	4	4	4	12	4	#N/A						WITHDRAWN	12	6
DA012	13	6505	4	4	4	12	4	26	7890	4	4	4	12	4	24	12
DA015	12	5940	3	4	4	11	3.66667	25.5	7740	4	4	4	12	4	23	11.5
DA016	13.5	5345	1	4	4	9	3	24	6685	4	4	4	12	4	21	10.5
DA022	13	6810	4	4	4	12	4	23	8615	4	3	3	10	3.5	22	11
DA023	12	6300	4	4	4	12	4	23	7665	3	3	3	9	3	21	10.5
DA024	11	4950	4	3	3	10	3.33333	23	7410	2	1	1	4	1.5	14	7
DA028	11.5	5970	4	1	1	6	2	24.5	8225	1	1	1	3	1	9	4.5
DA030	12	6030	4	4	4	12	4	25	7890	4	3	3	10	3.5	22	11
DA031	13	6715	1	1	1	3	1	24	8125	1	1	1	3	1	6	3
DA032	12	7350	2	3	3	8	2.66667	22	8755	3	3	3	9	3	17	8.5
DA033	13.5	5980	4	4	4	12	4	#N/A						WITHDRAWN	12	6
DA034	11.5	5685	4	4	4	12	4	23	7370	2	2	2	6	2	18	9
DA037	12.5	5785	4	4	4	12	4	#N/A					0	WITHDRAWN	12	6
DA038	11.1	6330	4	4	4	12	4	24.1	8020	4	4	4	12	4	24	12
DA039	13	8870	4	4	4	12	4	24	1200	4	4	4	12	4	24	12
DA041	11	5965	4	4	3	11	3.66667	22	7120	4	4	4	12	4	23	11.5
DA043	14	6660	3	2	1	6	2	24	8025	1	1	1	3	1	9	4.5
DA044	13	7840	3	3	3	9	3	24	9305	3	2	1	6	2.25	15	7.5
DA045	13	6425	4	4	4	12	4	24	8500	4	4	3	11	3.75	23	11.5
DA049	12	6650	3	4	4	11	3.66667	24	8890	4	3	3	10	3.5	21	10.5
DA050	13	5465	4	4	4	12	4	#N/A						WITHDRAWN	12	6
DA052	13	5595	3	3	4	10	3.33333	26	7550	2	1	1	4	1.5	14	7
DA054	12	5965	4	4	4	12	4	24	7375	4	4	4	12	4	24	12
DA055	14	6935	4	4	4	12	4	24	7800	4	3	3	10	3.5	22	11
DA056	14	6505	4	4	4	12	4	22	8000	2	2	2	6	2	18	9
DA060	12.2	5765	4	4	4	12	4	23	7130	4	4	4	12	4	24	12
DA061	13	7025	1	1	1	3	1	22	8460	1	1	1	3	1	6	3
DA063	12.5	5390	4	4	4	12	4	25	6425	4	3	3	10	3.5	22	11
DA065	12.3	6010	3	4	4	11	3.66667	23.5	7470	4	4	4	12	4	23	11.5
DA067	12	5760	4	4	4	12	4	24	7155	4	4	4	12	4	24	12

Appendix 2: Scoring sheet used for recording CBMN-Cyt biomarkers

Study ID	Slide	Scorer	MNC	BNC	Multi	Apo	Necro	Total BNC	MNBNC	NPBBNC	NBUDBNC	Total MNC	MNMNC	NBUDMNC
DA001	Slide A													
	Slide B													
DA002	Slide A													
	Slide B													
DA003	Slide A													
	Slide B													
DA003	Slide A													
	Slide B													

Abbreviations: MN: micronuclei; NPB: nucleoplasmic bridges; NBUD: nuclear buds, BNC: binucleated lymphocyte cells, MNC: mononucleated lymphocyte cells, Multi: multinucleated cells; Apo: apoptotic cells; Necro: necrotic cells

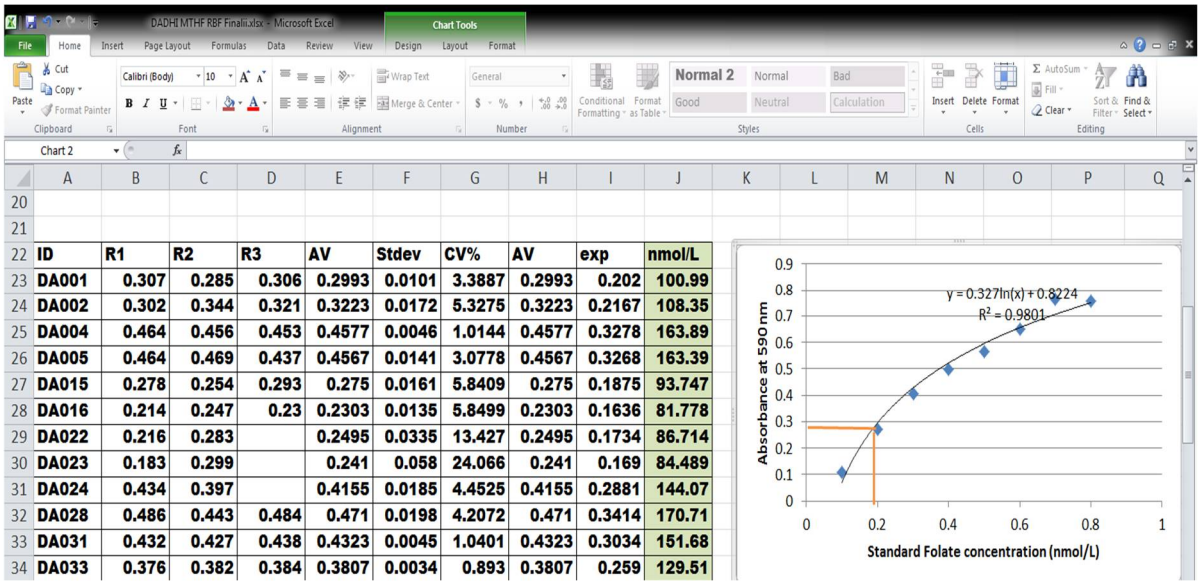
Appendix 3: A snapshot of scoring sheet used for recording CBMN-Cyt biomarkers

CBMN Assay Score Sheet For Micronucleus, Apoptosis,
Necrosis, Nuclear Budding & Nucleoplasmic Bridge Studies
DADHI STUDY cord blood

				Cytostatic Scores*					MN, NPB & NBUD in BNCs**				
				M1	M2	M3					Total	#BNCs	#BNCs
				MONOs	BNCs	MULTIs	APOPT	NECR	#BNCs	#BNCs	#MN in	with	with
	Study ID	Slide #	Scorer	scored	scored	scored	scored	Scored	scored	with MN	BNCs	NPBs	Nbuds
	DA002	Slide A	MH	83	49	1	9	16	1000	1	1	4	4
		Slide B	MH	99	67	10	5	17	1000	0	0	11	11
		Slide C											
		Slide A	TA	89	46	5	1	30	1000	1	1	4	8
		Slide B	TA	128	32	2	0	21	1000	1	1	11	6
		Average					**3.75**	**21**	**1000**	**0.75**	**0.75**	**7.5**	**7.25**
	DA004	Slide A	MH	183	47	3	9	28	1221	1	2	1	3
		Slide B	MH										
		Slide C	MH										
		Slide A	TA	273	46	0	2	33	1794	1	1	41	39
		Slide B											
		Average					**5.5**	**30.5**	1507.5	**1**	**1.5**	**21**	**21**
									1000	**0.66**	**0.99**	**13.9**	**13.9**
	DA005	Slide A	MH	94	131	4	6	48	469	0	0	4	6
		Slide B	MH	69	145	15	22	10	536	1	1	4	2
		Slide C											
		Slide A	TA	66	75	9	2	39	534	0	0	6	0
		Slide B	TA	62	56	10	3	32	472	1	1	1	0
		Average					**8.25**	**32.25**	**502.75**	**0.5**	**0.5**	**3.75**	**2**
	DA011	Slide A	MH	47	166	36	13	14	1000	1	1	8	12

Abbreviations: MN: micronuclei; NPB: nucleoplasmic bridges; NBUD: nuclear buds, BNC: binucleated lymphocyte cells, MNC: mononucleated lymphocyte cells, Multi: multinucleated cells; Apo: apoptotic cells; Necro: necrotic cells; MH: initials for scorer 1; TA: initials for scorer 2

Appendix 4: A snapshot of detailed calculation of folate concentration in a sample



ID	R1	R2	R3	AV	Stdev	CV%	AV	exp	nmol/L
DA001	0.307	0.285	0.306	0.2993	0.0101	3.3887	0.2993	0.202	100.99
DA002	0.302	0.344	0.321	0.3223	0.0172	5.3275	0.3223	0.2167	108.35
DA004	0.464	0.456	0.453	0.4577	0.0046	1.0144	0.4577	0.3278	163.89
DA005	0.464	0.469	0.437	0.4567	0.0141	3.0778	0.4567	0.3268	163.39
DA015	0.278	0.254	0.293	0.275	0.0161	5.8409	0.275	0.1875	93.747
DA016	0.214	0.247	0.23	0.2303	0.0135	5.8499	0.2303	0.1636	81.778
DA022	0.216	0.283		0.2495	0.0335	13.427	0.2495	0.1734	86.714
DA023	0.183	0.299		0.241	0.058	24.066	0.241	0.169	84.489
DA024	0.434	0.397		0.4155	0.0185	4.4525	0.4155	0.2881	144.07
DA028	0.486	0.443	0.484	0.471	0.0198	4.2072	0.471	0.3414	170.71
DA031	0.432	0.427	0.438	0.4323	0.0045	1.0401	0.4323	0.3034	151.68
DA033	0.376	0.382	0.384	0.3807	0.0034	0.893	0.3807	0.259	129.51

Abbreviations: ID: identity number of the sample; R: reading; AV: average of three readings for sample and standard, Stdev: standard deviation; CV: coefficient of variation; exp: exponential value.

Appendix 5: Comparison of DADHI male cohort birth weight with Australian national birthweight percentiles by sex and gestational age

3 Birthweight percentiles for live singleton male infants, Australia, 1998–2007

Gestational age (weeks)	Number of births	Mean (SD) birthweight (g)	Birthweight percentile (g)										
			1st	3rd	5th	10th	25th	50th	75th	90th	95th	97th	99th
20	230	349 (60)	210	248	254	273	310	340	390	430	450	470	500
21	335	418 (66)	270	290	300	335	375	420	460	500	540	542	575
22	401	505 (76)	350	370	390	410	460	500	554	600	630	650	690
23	395	595 (82)	390	450	470	500	540	588	650	700	730	756	800
24	640	681 (105)	426	470	500	550	618	684	750	810	850	875	970
25	715	783 (131)	440	505	530	620	700	785	865	944	995	1030	1100
26	937	894 (152)	500	576	621	680	802	900	996	1078	1130	1155	1210
27	1 069	1016 (194)	510	605	660	752	904	1030	1138	1250	1320	1352	1440
28	1 345	1146 (217)	591	680	735	844	1030	1165	1295	1395	1470	1522	1640
29	1 524	1301 (252)	662	782	860	964	1150	1311	1463	1620	1700	1757	1860
30	2105	1474 (283)	774	900	984	1091	1300	1498	1650	1800	1920	1980	2182
31	2576	1666 (304)	915	1055	1126	1270	1480	1680	1855	2028	2142	2230	2435
32	3895	1867 (331)	1075	1214	1294	1430	1659	1880	2080	2270	2405	2503	2710
33	5599	2106 (371)	1200	1381	1473	1638	1880	2106	2340	2560	2710	2845	3070
34	9 824	2340 (385)	1400	1580	1690	1860	2100	2340	2580	2810	2990	3120	3343
35	16 054	2585 (408)	1600	1795	1920	2080	2330	2578	2835	3095	3275	3410	3665
36	32747	2826 (428)	1805	2015	2120	2295	2550	2820	3095	3360	3550	3690	3930
37	73986	3093 (449)	2050	2265	2372	2540	2800	3080	3378	3670	3865	3990	4235
38	230 003	3344 (439)	2340	2540	2640	2800	3050	3330	3625	3910	4090	4215	4445
39	293 109	3486 (430)	2510	2700	2800	2950	3195	3470	3765	4040	4220	4335	4560
40	409 976	3632 (434)	2650	2840	2940	3090	3340	3620	3915	4195	4370	4490	4708
41	192154	3769 (438)	2780	2970	3070	3220	3470	3755	4060	4340	4515	4630	4850
42	19 804	3832 (462)	2760	2980	3095	3250	3520	3820	4130	4430	4615	4740	4970

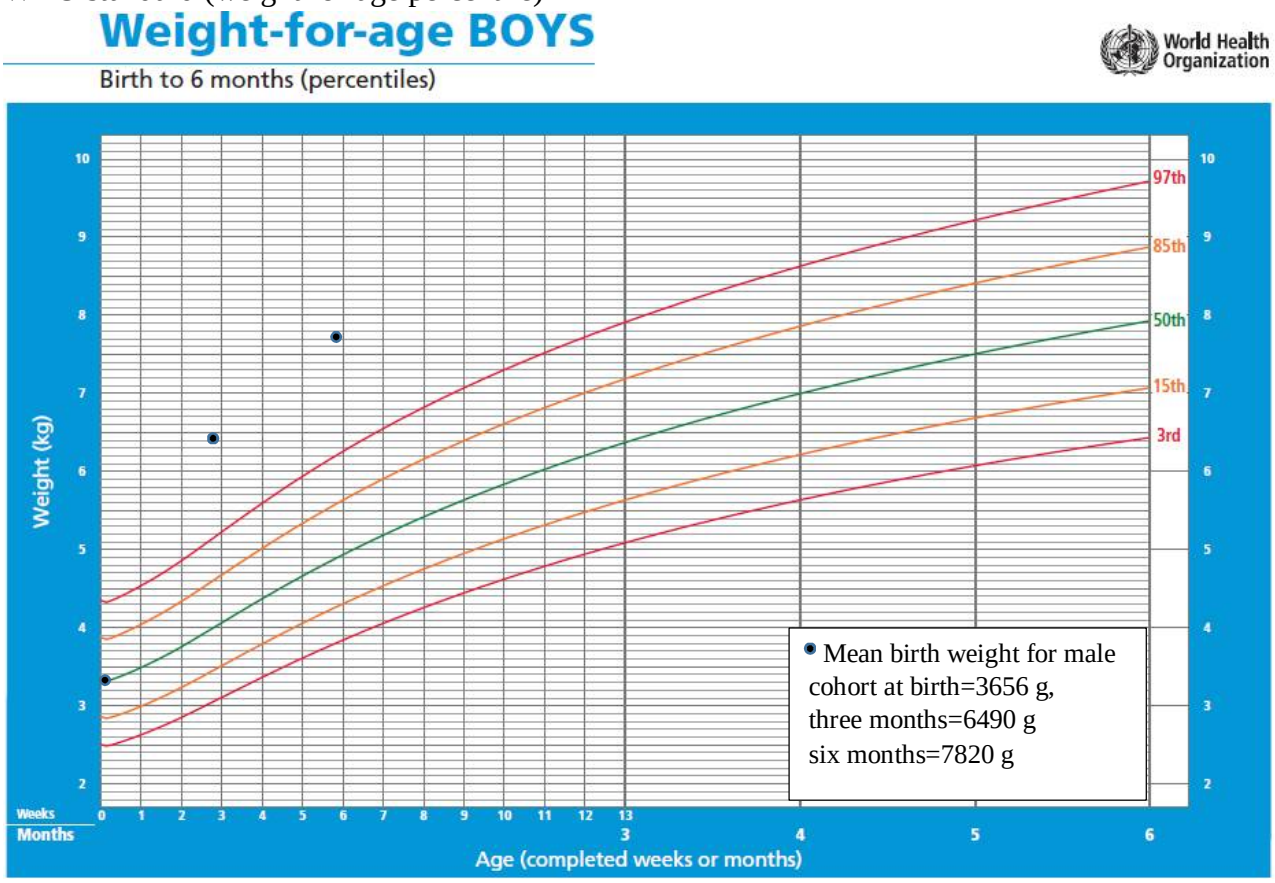
Appendix 6: Comparison of DADHI female cohort birth weight with Australian national birthweight percentiles by sex and gestational age

4 Birthweight percentiles for live singleton female infants, Australia, 1998–2007

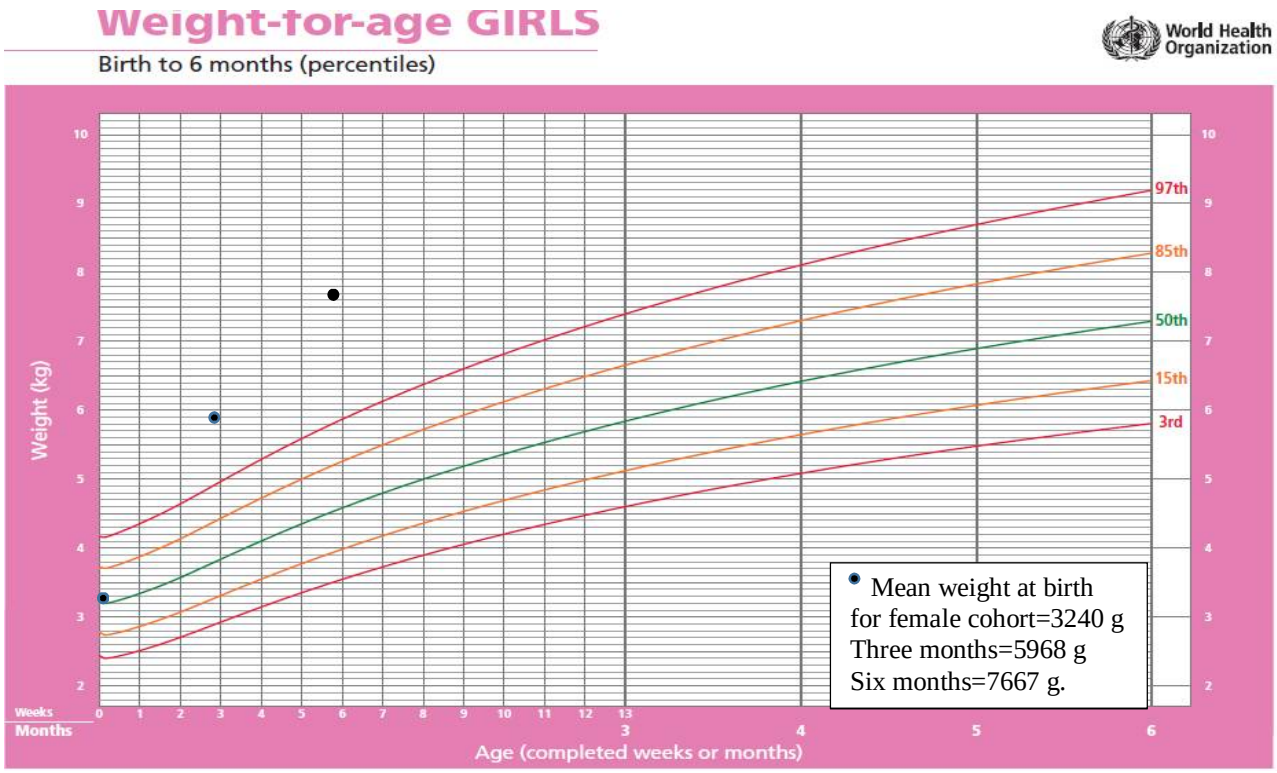
Gestational age (weeks)	Number of births	Mean (SD) birthweight (g)	Birthweight percentile (g)										
			1st	3rd	5th	10th	25th	50th	75th	90th	95th	97th	99th
20	197	333 (65)	190	210	230	265	290	320	374	410	450	490	525
21	256	386 (69)	210	250	270	300	340	390	433	470	510	515	530
22	333	474 (72)	260	325	355	400	425	480	520	560	589	610	620
23	376	558 (89)	320	375	400	445	506	560	615	660	700	725	800
24	528	637 (95)	380	430	480	520	580	641	700	754	793	815	860
25	599	730 (128)	410	470	498	559	645	740	817	884	940	975	992
26	809	825 (166)	428	490	520	594	717	840	940	1026	1072	1106	1186
27	879	949 (188)	500	568	598	675	840	965	1077	1175	1240	1280	1390
28	1136	1073 (230)	495	622	675	764	928	1090	1230	1347	1410	1470	1610
29	1188	1215 (252)	572	712	790	870	1055	1240	1380	1494	1595	1680	1840
30	1656	1394 (277)	725	870	918	1030	1220	1400	1571	1715	1840	1920	2130
31	2052	1582 (302)	880	1000	1060	1190	1385	1590	1780	1948	2065	2146	2338
32	3119	1772 (322)	970	1140	1230	1348	1570	1780	1970	2170	2290	2400	2620
33	4 421	2014 (356)	1180	1330	1424	1560	1790	2011	2235	2450	2616	2746	2970
34	8108	2242 (375)	1331	1525	1615	1764	2005	2240	2470	2705	2870	2995	3220
35	13104	2486 (403)	1525	1710	1820	1980	2230	2480	2735	2995	3175	3300	3516
36	28386	2720 (420)	1750	1940	2040	2198	2445	2710	2980	3250	3450	3575	3810
37	66928	2979 (439)	1970	2175	2275	2430	2690	2965	3255	3545	3735	3865	4100
38	214002	3215 (425)	2256	2440	2540	2690	2930	3200	3490	3770	3945	4062	4290
39	282046	3351 (415)	2420	2600	2690	2830	3070	3340	3620	3890	4060	4175	4390
40	398257	3493 (416)	2566	2740	2830	2975	3210	3480	3765	4030	4200	4316	4525
41	181434	3619 (424)	2680	2855	2945	3090	3330	3605	3900	4170	4340	4455	4670
42	17701	3665 (445)	2670	2850	2950	3110	3360	3650	3955	4240	4420	4545	4760
43	801	3579 (463)	2660	2800	2865	3010	3240	3560	3880	4210	4385	4560	4760

Reference: Dobbins et al 2012, Australian national birthweight percentiles by sex and gestational age, 1998–2007.

Appendix 7: Comparison of DADHI male cohort weight at birth, three and six months with WHO standard (weight for age percentile)

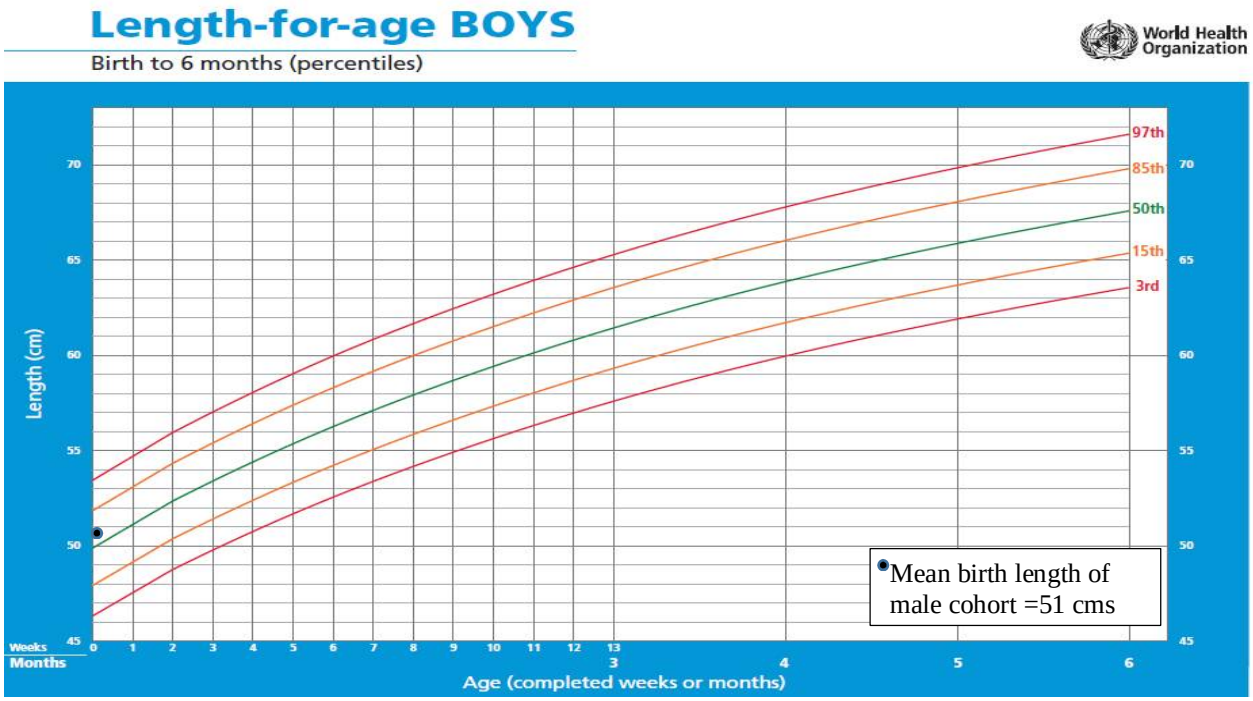


Appendix 8: Comparison of DADHI female cohort weight at birth, three and six months with WHO standard (weight for age percentiles) Reference: (http://www.who.int/childgrowth/en/, 2016)

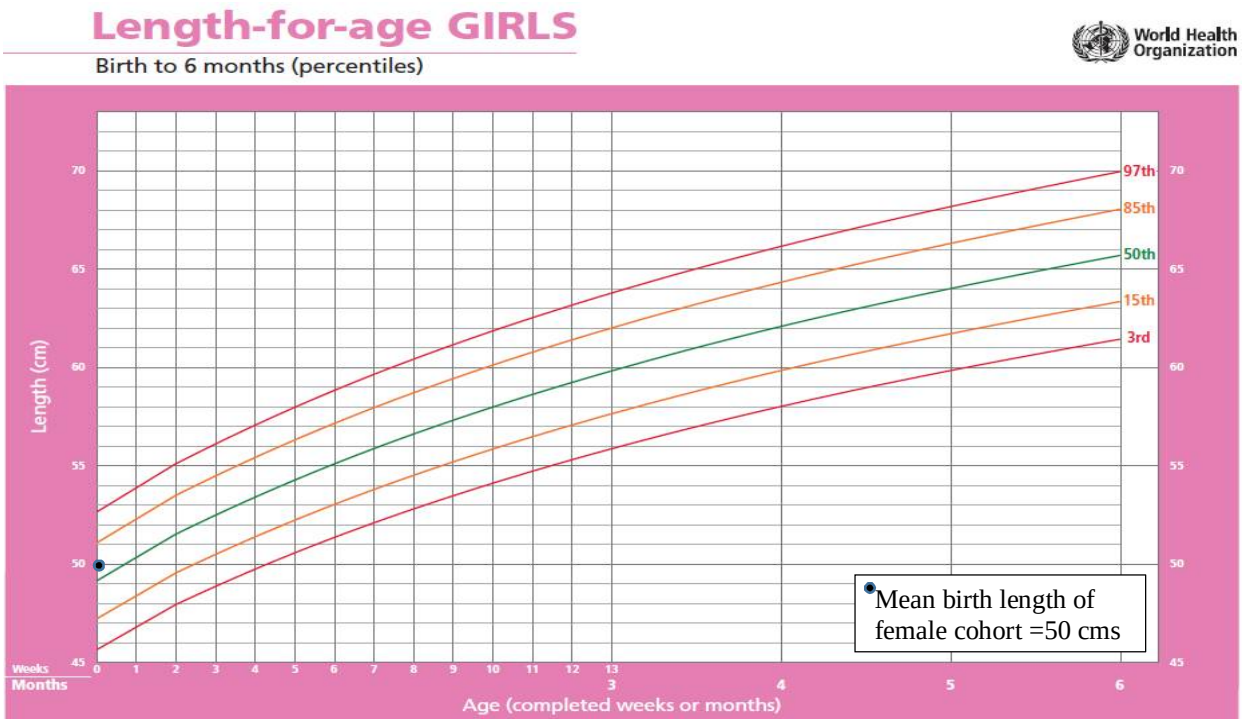


Reference: (http://www.who.int/childgrowth/en/, 2016)

Appendix 9: Comparison of DADHI male cohort birth length with WHO standard (length for age percentiles)

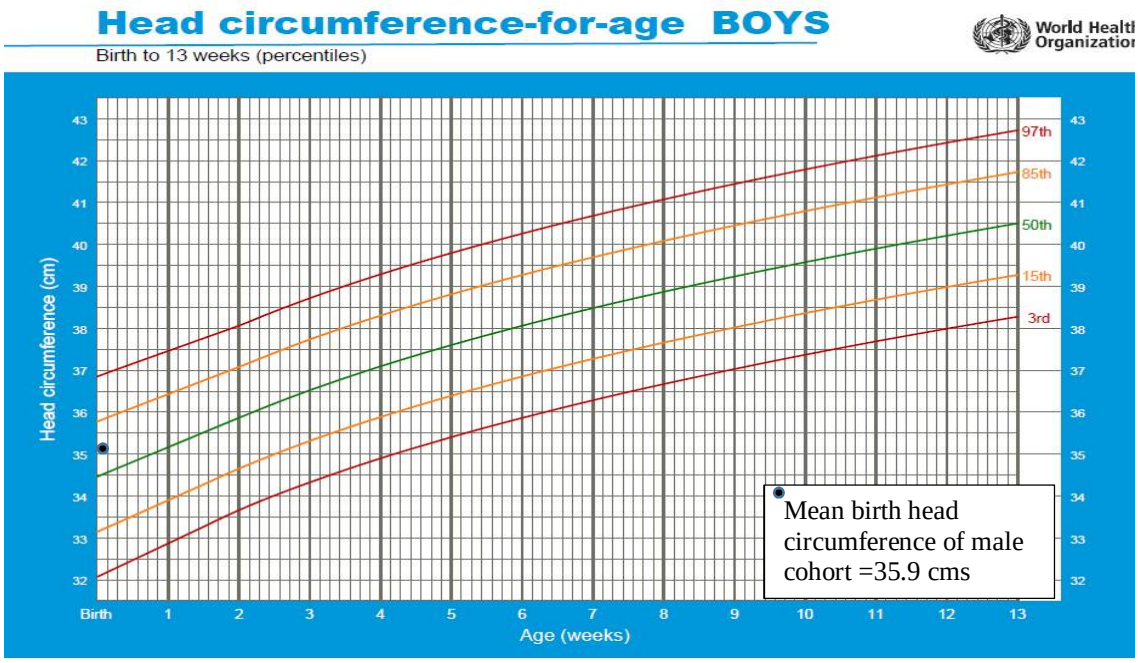


Appendix 10: Comparison of DADHI female cohort birth length with WHO standard (length for age percentiles)

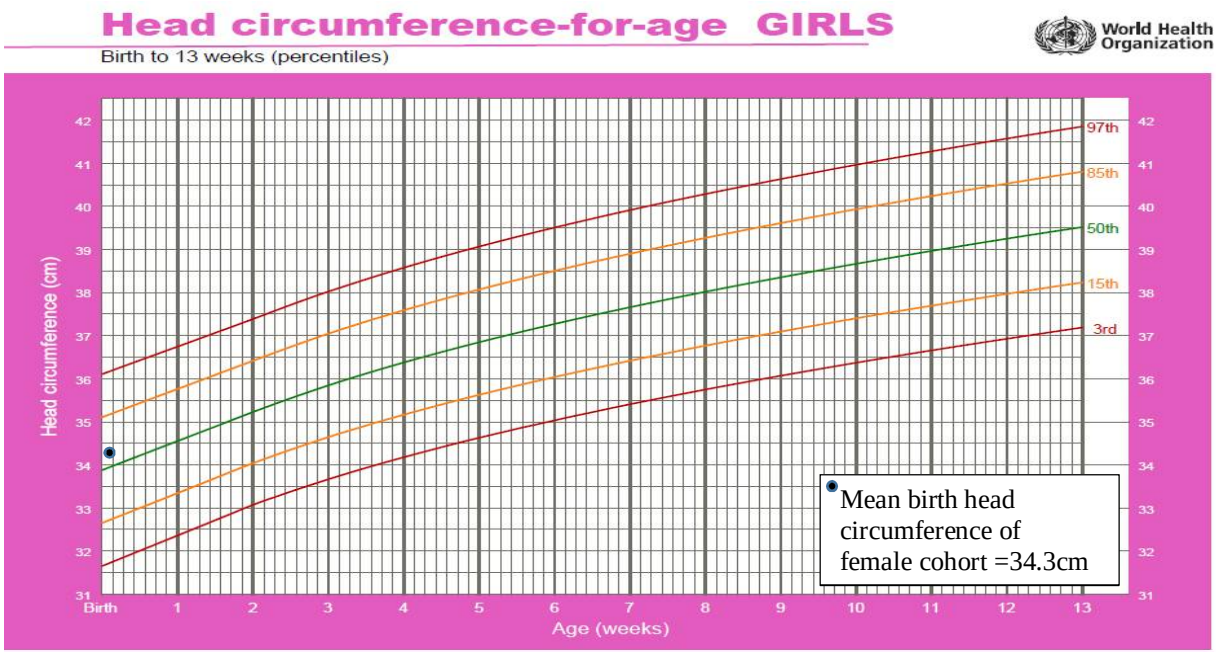


Reference: (http://www.who.int/childgrowth/en/, 2016)

Appendix 11: Comparison of DADHI male cohort birth head circumference with WHO standard (length for age percentiles)



Appendix 12: Comparison of DADHI female cohort birth head circumference with WHO standard (length for age percentiles)



Appendix 13: Recommended dietary intakes and normal plasma minerals values for infant

Micronutrients	AI mg/d *	UL mg/d *	Normal Plasma/serum levels #	DADHI Cohort (SI units)			Comments
				Birth	Three months	Six months	
Iron	0.2 mg/d	20mg/d	Neonates: 17.9–44.8 µmol/l Infants: 7.2–17.9 µmol/l	112.9 µmol/L	57.8 µmol/L	64.4 µmol/L	High
Copper	0.20 mg/d	Not possible to establish	Birth to 6 months: 3.1–4.2 µmol/l	6.4 µmol/L	9.8 µmol/L	16.3 µmol/L	High
Calcium	210 mg/d	Not possible to establish	Serum: Preterm: 1.6–2.8 mmol/l Term to 10 days: 1.9–2.6 mmol/l 10 days to 2 years: 2.3–2.8 mmol/l	2.62 mmol/L	2.75 mmol/L	2.67 mmol/L	Normal
Magnesium	30mg/d	Not possible to establish	0.63–1.05 mmol/l	0.72 mmol/L	0.85 mmol/L	0.97 mmol/L	Normal
Zinc	2 mg/d	4 mg/d	10.7–18.4 µmol/l	15.4 µmol/L	22.7 µmol/L	20.8 µmol/L	Low
Sodium	Sodium: 120	Not possible to establish possible to establish	Sodium: (less than 1 year age) 130–145 mmol/L	132.1 mmol/L	142.6 mmol/L	145.6 mmol/L	Normal
Potassium	400mg/d	Not possible to establish possible to establish	Neonates: 3.7–5.9 mmol/L Infants: 4.1–5.3 mmol/L	10.31 mmol/L	5.22 mmol/L	5.52 mmol/L	Normal
Phosphorous	100 mg/d	Not possible to establish	Neonates: 1.45–2.91 mmol/l 10 days to 2 years: 1.45–2.10 mmol/l	3.38 mmol/l	4.48 mmol/l	4.47 mmol/l	High
Sulphur	As protein component	As protein component	not known	987.7mg/L	1003 mg/L	1043mg/L	could not assess
Vitamin B_{12}	0-6 months: 0.5µg/d 7-12 months: 0.5µg/d	No evidence to determine toxicity	Neonates: 118–959 pmol/l Infants/children: 148–616 pmol/l	443.5 pmol/L	-	-	Normal
Folate	65 ug/d	Not possible to establish	RBC: Newborn: 340–453 nmol/L Infants: 168–2254 nmol/L	382.67 nmol/L	212.7 nmol/L	319.9 nmol/L	Normal

Abbreviations: AI: Adequate intakes; UL: Upper limit; SI: standard units*:AI and UL as per NHMRC nutrient intakes
#:Normal plasma values for infants from 'The Harriet Lane Handbook Mobile Medicine Series - Expert Consult; 20th ed.; 2015'

Appendix 14: Conversion of lab values for the cohort into standard unit

Nutrient	Lab values (mg/L) (IMVS)	Intermediate conversion	Conversion factor *	Standard International unit
Iron	6.29 3.23 3.6	629 (ug/L) 323 360	× 0.179	112.59 µmol/L 57.8 64.4
Copper	0.41 0.63 1.04	41(ug/L) 63 104	× 0.157	6.4 µmol/L 9.89 16.3
Calcium	105.7 110.9 107.6	10.57 mg/dl 11.09 10.76	× 0.25	2.62 mmol/L 2.75 2.65
Magnesium	17.7 20.8 23.7	1.77 mg/dl 2.08 2.37	× 0.411	0.727 mmol/L 0.85 0.97
Zinc	1.01 1.49 1.36	101 µg/dl 149 136	× 0.153	15.4 µmol/L 22.7 20.8
Sodium	3040 3280 3350	304 mg/dl 328 335	mEq/L# mg × valance/atomic weight)**	132.17 mmol/L 142.6 145.65
Potassium	402 204 216	40.2 mg/dl 20.4 21.6	× 0.256	10.31 mmol/L 5.22 5.52
Phosphorous	104.7 139 138.6	10.47 mg/dl 13.9 13.86	× 0.323	3.38 mmol/L 4.48 4.47
Vitamin B12	443.5 pmol/L - -			443.5 pmol/L
Red cell folate	382.67 nmol/L 212.7 319.9			382.67 nmol/L 212.7 319.9

#:mEq conversion for sodium% Pot http://nephron.com/cgi-bin/SI.cgi *: Bloch, A., and Shills, M. (2006) Conversion factors. In Shills, M., Shike, M., Ross, C., Caballero, B. and Cousins, R. (eds.), *Modern Nutrition in Health and Disease*. Lippincott Williams and Wilkins, A Wolters Kluwer Company, Philadelphia, pp. 1840-1846

Appendix 15: Comparison of INFACT male cohort birth weight with Australian national birthweight percentiles by sex and gestational age

3 Birthweight percentiles for live singleton male infants, Australia, 1998–2007

Gestational age (weeks)	Number of births	Mean (SD) birthweight (g)	Birthweight percentile (g)										
			1st	3rd	5th	10th	25th	50th	75th	90th	95th	97th	99th
20	230	349 (60)	210	248	254	273	310	340	390	430	450	470	500
21	335	418 (66)	270	290	300	335	375	420	460	500	540	542	575
22	401	505 (76)	350	370	390	410	460	500	554	600	630	650	690
23	395	595 (82)	390	450	470	500	540	588	650	700	730	756	800
24	640	681 (105)	426	470	500	550	618	684	750	810	850	875	970
25	715	783 (131)	440	505	530	620	700	785	865	944	995	1030	1100
26	937	894 (152)	500	576	621	680	802	900	996	1078	1130	1155	1210
27	1 069	1016 (194)	510	605	660	752	904	1030	1138	1250	1320	1352	1440
28	1 345	1146 (217)	591	680	735	844	1030	1165	1295	1395	1470	1522	1640
29	1 524	1301 (252)	662	782	860	964	1150	1311	1463	1620	1700	1757	1860
30	2105	1474 (283)	774	900	984	1091	1300	1498	1650	1800	1920	1980	2182
31	2576	1666 (304)	915	1055	1126	1270	1480	1680	1855	2028	2142	2230	2435
32	3895	1867 (331)	1075	1214	1294	1430	1659	1880	2080	2270	2405	2503	2710
33	5599	2106 (371)	1200	1381	1473	1638	1880	2106	2340	2560	2710	2845	3070
34	9 824	2340 (385)	1400	1580	1690	1860	2100	2340	2580	2810	2990	3120	3343
35	16 054	2585 (408)	1600	1795	1920	2080	2330	2578	2835	3095	3275	3410	3665
36	32747	2826 (428)	1805	2015	2120	2295	2550	2820	3095	3360	3550	3690	3930
37	73986	3093 (449)	2050	2265	2372	2540	2800	3080	3378	3670	3865	3990	4235
38	230 003	3344 (439)	2340	2540	2640	2800	3050	3330	3625	3910	4090	4215	4445
39	293 109	3486 (430)	2510	2700	2800	2950	3195	3470	3765	4040	4220	4335	4560
40	409 976	3632 (434)	2650	2840	2940	3090	3340	3620	3915	4195	4370	4490	4708
41	192154	3769 (438)	2780	2970	3070	3220	3470	3755	4060	4340	4515	4630	4850
42	19 804	3832 (462)	2760	2980	3095	3250	3520	3820	4130	4430	4615	4740	4970

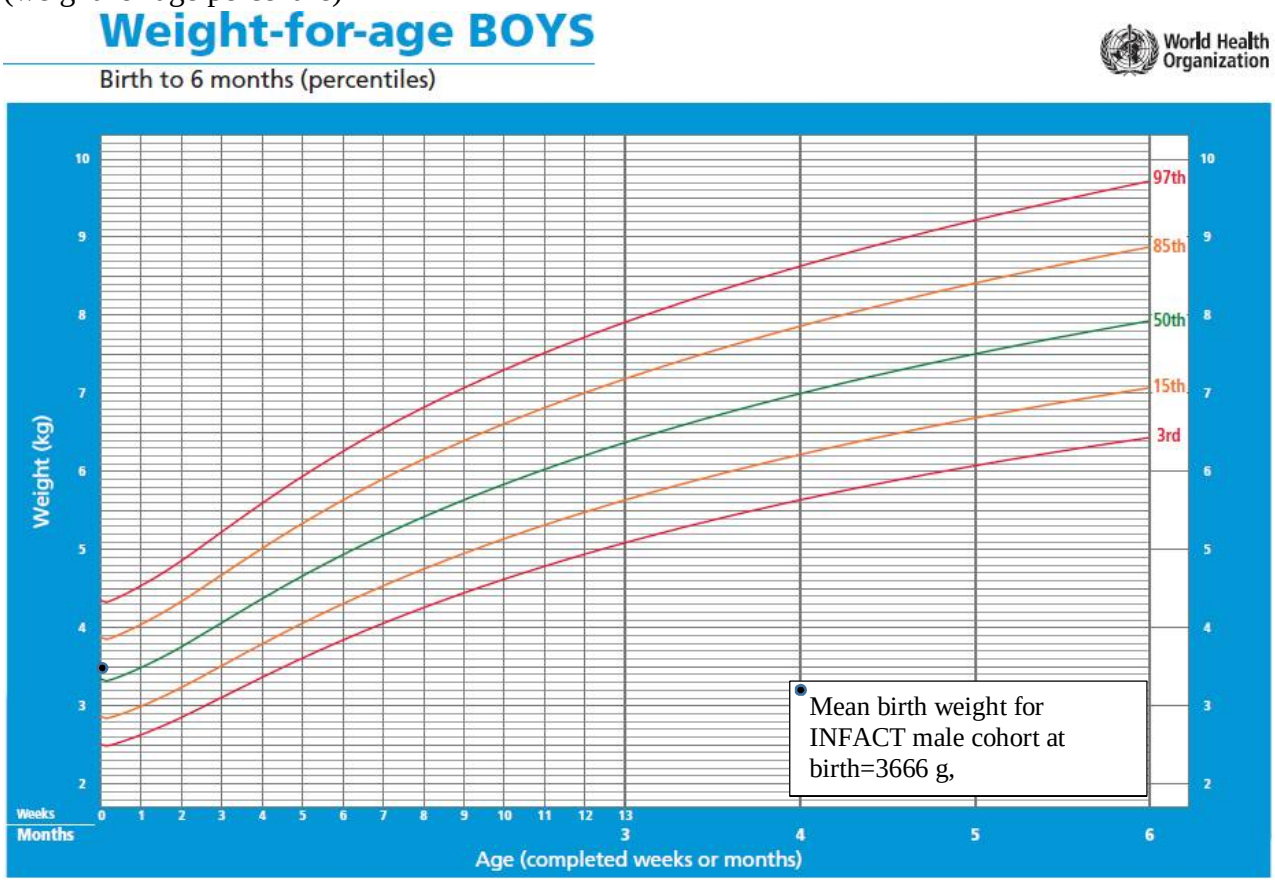
Appendix 16: Comparison of INFACT female cohort birth weight with Australian national birthweight percentiles by sex and gestational age

4 Birthweight percentiles for live singleton female infants, Australia, 1998–2007

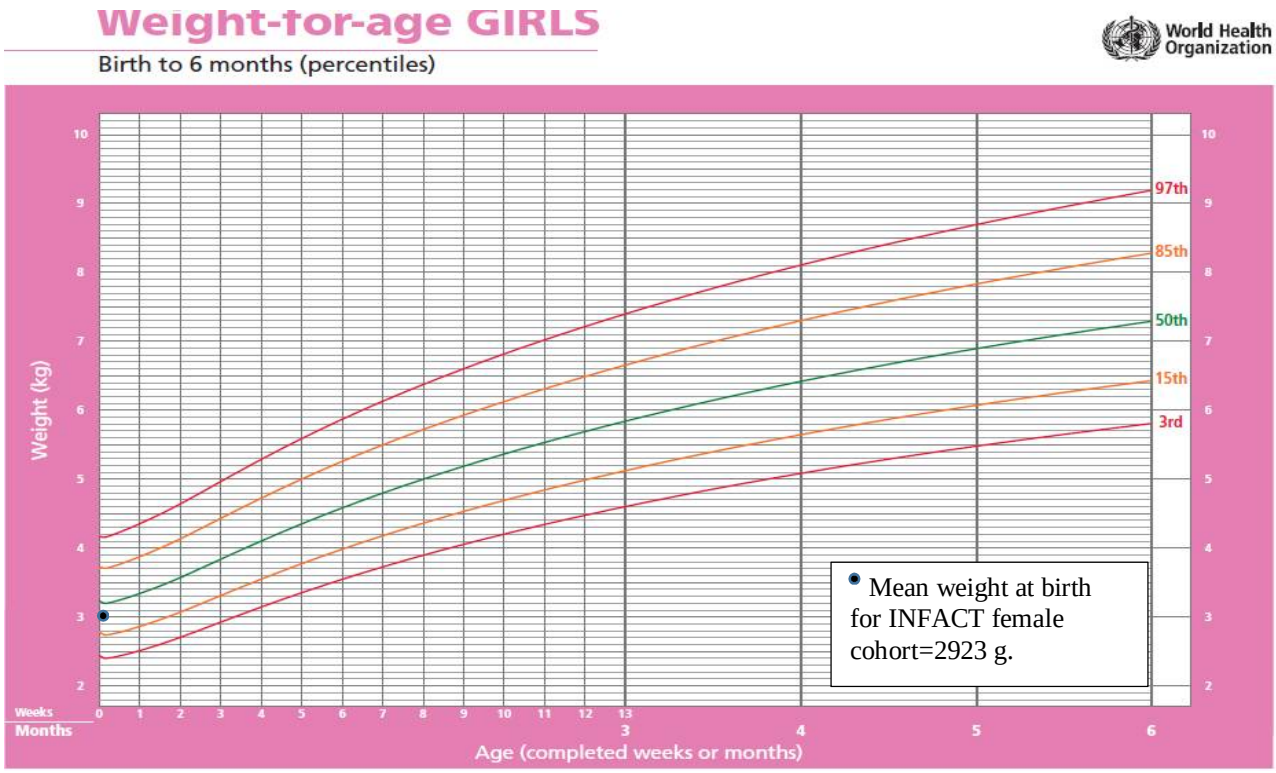
Gestational age (weeks)	Number of births	Mean (SD) birthweight (g)	Birthweight percentile (g)										
			1st	3rd	5th	10th	25th	50th	75th	90th	95th	97th	99th
20	197	333 (65)	190	210	230	265	290	320	374	410	450	490	525
21	256	386 (69)	210	250	270	300	340	390	433	470	510	515	530
22	333	474 (72)	260	325	355	400	425	480	520	560	589	610	620
23	376	558 (89)	320	375	400	445	506	560	615	660	700	725	800
24	528	637 (95)	380	430	480	520	580	641	700	754	793	815	860
25	599	730 (128)	410	470	498	559	645	740	817	884	940	975	992
26	809	825 (166)	428	490	520	594	717	840	940	1026	1072	1106	1186
27	879	949 (188)	500	568	598	675	840	965	1077	1175	1240	1280	1390
28	1136	1073 (230)	495	622	675	764	928	1090	1230	1347	1410	1470	1610
29	1188	1215 (252)	572	712	790	870	1055	1240	1380	1494	1595	1680	1840
30	1656	1394 (277)	725	870	918	1030	1220	1400	1571	1715	1840	1920	2130
31	2052	1582 (302)	880	1000	1060	1190	1385	1590	1780	1948	2065	2146	2338
32	3119	1772 (322)	970	1140	1230	1348	1570	1780	1970	2170	2290	2400	2620
33	4 421	2014 (356)	1180	1330	1424	1560	1790	2011	2235	2450	2616	2746	2970
34	8108	2242 (375)	1331	1525	1615	1764	2005	2240	2470	2705	2870	2995	3220
35	13104	2486 (403)	1525	1710	1820	1980	2230	2480	2735	2995	3175	3300	3516
36	28386	2720 (420)	1750	1940	2040	2198	2445	2710	2980	3250	3450	3575	3810
37	66928	2979 (439)	1970	2175	2275	2430	2690	2965	3255	3545	3735	3865	4100
38	214002	3215 (425)	2256	2440	2540	2690	2930	3200	3490	3770	3945	4062	4290
39	282046	3351 (415)	2420	2600	2690	2830	3070	3340	3620	3890	4060	4175	4390
40	398257	3493 (416)	2566	2740	2830	2975	3210	3480	3765	4030	4200	4316	4525
41	181434	3619 (424)	2680	2855	2945	3090	3330	3605	3900	4170	4340	4455	4670
42	17701	3665 (445)	2670	2850	2950	3110	3360	3650	3955	4240	4420	4545	4760
43	801	3579 (463)	2660	2800	2865	3010	3240	3560	3880	4210	4385	4560	4760

Reference: Dobbins et al 2012, Australian national birthweight percentiles by sex and gestational age, 1998–2007.

Appendix 17: Comparison of INFACT male cohort weight at birth with WHO standard (weight for age percentile)

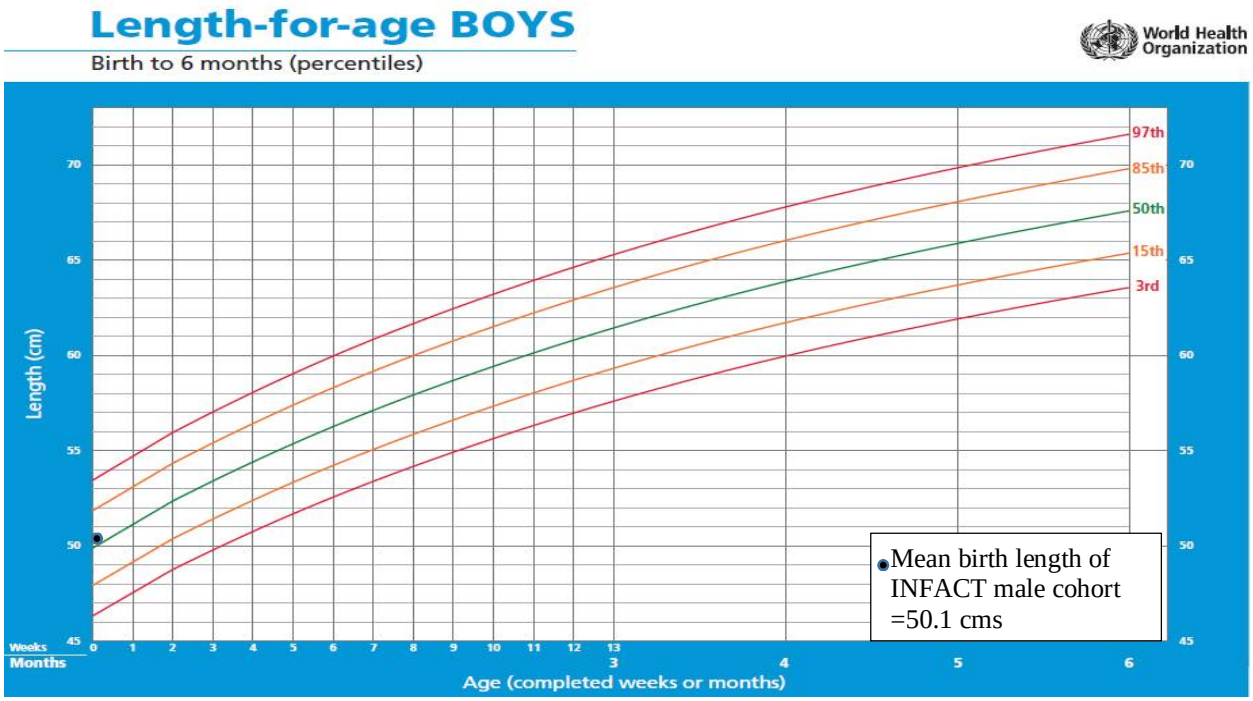


Appendix 18: Comparison of INFACT female cohort weight at birth with WHO standard (weight for age percentiles)

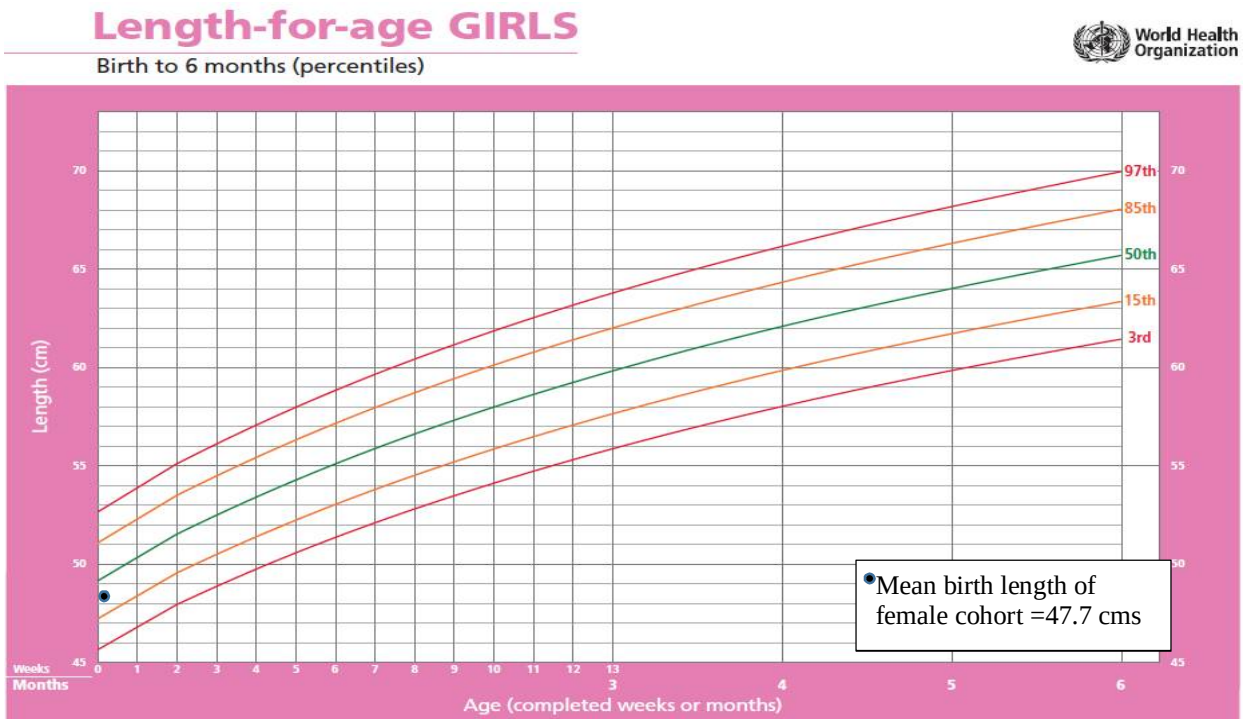


Reference: (http://www.who.int/childgrowth/en/, 2016)

Appendix 19: Comparison of DADHI male cohort birth length with WHO standard (length for age percentiles)

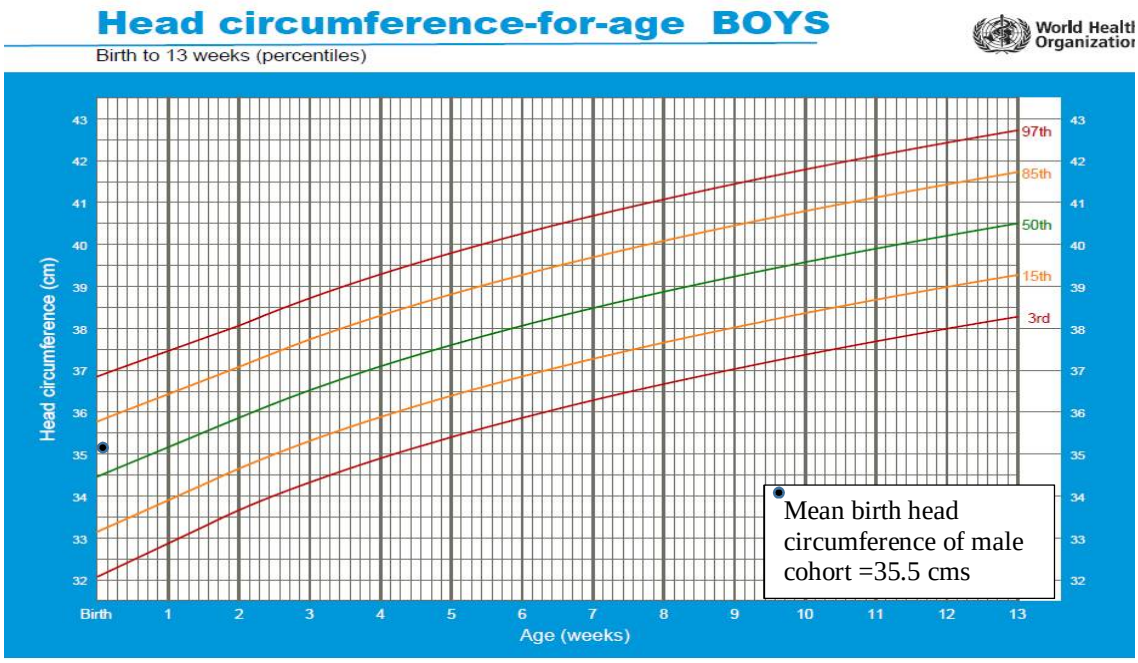


Appendix 20: Comparison of DADHI female cohort birth length with WHO standard (length for age percentiles)



Reference: (http://www.who.int/childgrowth/en/, 2016)

Appendix 21: Comparison of DADHI male cohort birth head circumference with WHO standard (length for age percentiles)



Appendix 22: Comparison of DADHI female cohort birth head circumference with WHO standard (length for age percentiles)