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Intrauterine Growth Retardation Among Preterms In Shri Lanka

Clinical, Endocrine And Molecular Aspects Of Folate Metabolism

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Intrauterine Growth Retardation Among Preterms In Shri Lanka. Clinical, Endocrine And Molecular Aspects Of Folate Metabolism

Abstract (*English*)

Background and Objectives: Intrauterine growth retardation (IUGR) is a major cause of neonatal mortality and long-term morbidity. Risk factors for IUGR could be infections, nicotine and ethanol abusus, genetics (low growth potential), and pre-gravid maternal morbidity such as renal disease. Environmental factors have been of central interest in recent years as well as the interaction between genome and environment on the risk of complex diseases. In developed countries, IUGR secondary to prematurity is attributed in the majority of cases to pregnancy-induced hypertension and pre-eclampsia, *inter alia* caused by disorders in homocysteine metabolism; also, cigarette smoking, substance abuse, and intrauterine infections have to be considered. Malnourishment and undernutrition of pregnant women, which are globally the main cause, only play a minor role in developed countries.

Hyperhomocysteinaemia is defined as abnormally increased fasting total homocysteine concentration in blood. Elevated homocysteine levels belong to the biomarkers thought to reflect a higher risk for cardiovascular disease, for atherosclerosis and thrombosis. Methylenetetrahydrofolate reductase polymorphism C677T exhibits decreased enzymatic activity leading to mild hyperhomocysteinaemia in homozygous C677T individuals especially during times of folate insufficiency. It has been advocated that it should be possible to overcome hyperhomocysteinaemia caused by the 677T allele by folic acid supplementation, furthermore, the prophylactic use of folic acid is administered to prevent neural tube defects.

Materials and Methods: In a monocentric study in Shri Lanka, we enrolled pregnant women who belonged to the three main ethnic groups in the island. All participating gravid women received a standard supplementation with folic acid tablets (5 mg / d) after their first visit in an antenatal care institution. The study population included 193 consecutive preterm neonates of less than 37 gestational weeks at Castle Street Hospital for Women in Colombo. There were 182 singletons (120 eutrophic / appropriate-for-gestational age [AGA], 62 hypotrophic / small-for-gestational age [SGA]), 10 twin pairs, and one triplet birth. SGA was defined as below the 10th percentile according to a population-based percentile curve Gestational age ranged between 28.2 weeks and 36.6 weeks, birth weight between 760 and 4,000 grams (values are median).

Umbilicial venous cord blood was collected immediately after birth. DNA was extracted from dried blood spots on capture cards using the QIAmp® DNA extraction procedure. Two MTHFR polymorphisms, C677T and A1298C, were genotyped with the use of TaqMan™ polymerase chain reaction. Total homocysteine (tHcy) status was determined by fluorescence polarisation immunoassay; folate and vitamin B_{12} concentrations were analysed via competitive chemoluminescence-ligand immunoassay technique. All statistical analysis were performed using SPSS for Windows, version 15.0. Approval was obtained from the "Ethik-Kommission der Ärztekammer des Saarlandes" (Germany) and from the Ethical Review Committee of the Faculty of Medicine, University of Colombo (Shri Lanka).

Results: Weight gain during pregnancy was lower in mothers who delivered SGA singletons than in those with AGA infants (6.5 *vs.* 8.0 kg; $p = 0.005$). Total Hcy concentration in umbilical cord blood was significantly increased in SGA singletons when compared to AGA singletons (5.17 *vs.* 4.58 µmol / L; $p = 0.016$); the difference was even more pronounced in the SGA group < 36 gestational weeks ($p < 0.005$). When comparing mothers with pregnancy-induced hypertension to mothers who did not develop hypertension during pregnancy, the SGA singleton offspring had elevated folate concentrations (77.6 *vs.* 46.9 nmol / L; $p = 0.054$) as well as significantly higher tHcy (6.54 *vs.* 4.77 µmol / L; $p < 0.001$) and vitamin B_{12} concentrations (494 *vs.* 362 pmol / L; $p < 0.001$).

We detected significantly increased folate concentrations in infants whose mothers suffered previous miscarriages (51.4 *vs.* 43.5 nmol / L; $p = 0.013$). With view to A1298C MTHFR polymorphism, vitamin B_{12} concentrations were decreased in SGA singletons carrying the C-allele compared to the AA 'wild type' group (353 *vs.* 548 pmol / L; $p = 0.001$); apart from that, we could not demonstrate an association between homocysteine concentrations and analysed gene polymorphisms.

Conclusions: Despite a relatively high vitamin B_{12} status, concentrations of tHcy in umbilical cord blood of SGA preterm neonates from folate-supplemented mothers were higher than in comparable collectives; this seems to be related to prematurely decreased activities of enzymes that catabolise Hcy or to the inability to utilise the available cofactors such as folate and vitamin B_{12}. As the difference disappeared after 36 gestational weeks, it should be discussed whether supplementation with folic acid may induce the expression of stability of metabolising enzymes (methionine synthase, methylentetrahydrofolate reductase), thus compensating for the premature activities of enzymes in the SGA cohort. Another novel finding was the increased plasma tHcy status in SGA preterms from mothers with pregnancy-induced hypertension compared to SGA infants from

normotensive mothers. Possible mechanisms could be: hypertension may reduce the excretion of homocysteine via the maternal kidney or it may reduce the removal of homocysteine from the foetus to the mother via arterial blood.

The present study makes a new contribution towards the pathophysiologic characterization of pregnancy-induced hypertension. Additional studies with larger sample sizes are needed to further differentiate our observations.

Keywords: intrauterine growth retardation; preterm birth; birth weight; methylenetetrahydrofolate reductase; folate; homocysteine; pregancy-induced hypertension

Intrauterine Wachstumsretardierung bei Frühgeborenen in Shri Lanka. Klinische, endokrine und molekulare Aspekte des Folat-Metabolismus

Abstract (*Deutsch*)

Hintergrund und Ziele: Die intrauterine Wachstumsretardierung ist eine relevante Ursache der neonatalen Mortalität und für langfristige Morbidität. Risikofaktoren für eine intrauterine Wachstumsretardierung können unter anderem Infektionen, Nikotinabusus und Ethanolkonsum, genetische Faktoren (vermindertes Wachstumspotenzial) sowie eine prägravide materne Morbidität wie zum Beispiel Nierenerkrankungen sein. Umwelt-bedingte Einflussfaktoren rückten in den vergangenen Jahren in den Mittelpunkt des Interesses ebenso wie die Interaktion von Genom und Umwelt auf das Risiko komplexer Erkrankungen. In entwickelten Ländern wird bei Frühgeburtlichkeit die intrauterine Wachstumsretardierung in der Mehrzahl der Fälle auf Gestationshypertonus und Präeklampsie, unter anderem verursacht durch Störungen im Homocystein-Stoffwechsel, zurückgeführt; zudem sind Tabakkonsum, Substanzabusus und intrauterine Infektionen zu berücksichtigen. Dahingegen haben in entwickelten Ländern die Fehl- und Mangelernährung der Schwangeren, die weltweit die Hauptursache einer intrauterinen Wachstumsretardierung darstellen, nur einen sekundären Stellenwert.

Die Hyperhomocysteinämie wird als abnorm erhöhte Konzentration des Gesamthomo-cysteinspiegels im Blut bei Nüchternheit definiert. Erhöhte Homocysteinwerte zählen zu den Biomarkern für ein erhöhtes Risiko von kardiovaskulären Erkrankungen, von Atherosklerose und Thrombose. Der Methylentetrahydrofolatreductase-Polymorphismus C677T manifestiert sich in einer verringerten Enzymaktivität, die bei homozygoten C677T-Individueen insbesondere bei gleichzeitig bestehendem Folatmangel zu einer milden Hyperhomocystinämie führt. Deshalb wird eine Supplementierung mit Folsäure als wirksame Maßnahme zur Behandlung einer durch das 677T-Allel verursachten Hyperhomocystinämie vorgeschlagen, darüber hinaus findet die Folsäure-Prophylaxe zur Prävention von Neuralrohrdefekten Anwendung.

Material und Methoden: In eine monozentrische Studie in Shri Lanka wurden Schwangere einbezogen, die den drei wichtigsten ethnischen Gruppen der Insel angehörten. Alle Schwangeren erhielten nach der ersten Vorstellung in einer Schwangerenvorsorgeeinrichtung routinemäßig eine Substitution mit Folsäuretabletten (5 mg / T). Die Studienpopulation umfasste 193 konsekutive Frühgeborene von weniger als 37 Schwangeschaftswochen im Castle Street Hospital for Women in Colombo. Davon

waren 182 Einlinge (120 eutroph für das Gestationsalter [AGA] und 62 hypotroph [Small-for-Date-Frühgeborene; SGA]), 10 Zwillingspaare und eine Drillingsgeburt. SGA wurde als Geburtsgewicht unter der 10. Perzentile einer populationsbezogenen Perzentilenkurve definiert. Das Gestationsalter der einbezogenen Frühgeborenen lag zwischen 28,2 und 36,6 Wochen, das Geburtsgewicht zwischen 760 und 4 000 Gramm (Werte jeweils median).

Venöses Nabelschnurblut wurde unmittelbar nach der Geburt abgenommen. DNA wurde aus auf Filterkarten aufgebrachtem Blut mit Hilfe des QIAmp® DNA-Extraktionsverfahrens gewonnen. Zwei MTHFR-Polymorphismen, C677T und A1298C, wurden mittels TaqMan™-Polymerase-Kettenreaktion genotypisiert. Der Gesamthomocystein-Status wurde mittels Fluoreszenz-Polarisations-Immunoassay bestimmt; die Analyse der Folat- und Vitamin B_{12}-Konzentrationen erfolgte via kompetitiver Chemolumineszenz-Liganden-Immunoassay-Technik. Alle statistischen Analysen wurden mit SPSS für Windows, Version 15.0, durchgeführt. Die Studie wurde sowohl von der Ethik-Kommission der Ärztekammer des Saarlandes (Deutschland) als auch vom „Ethical Review Committee“ der medizinischen Fakultät, Universität von Colombo (Shri Lanka), genehmigt.

Resultate: Die Gewichtszunahme während der Schwangerschaft fiel bei Müttern von SGA-Einlingen geringer aus als bei jenen mit AGA-Frühgeborenen (6,5 *vs.* 8,0 kg; $p = 0{,}005$). Die Gesamthomocystein-Konzentration im Nabelschnurblut war bei SGA-Einlingen im Vergleich zu AGA-Einlingen signifikant erhöht (5,17 *vs.* 4,58 µmol / L; $p = 0{,}016$); dieser Unterschied zeigte sich bei der SGA-Gruppe < 36 Schwangerschaftswochen sogar noch deutlicher ausgeprägt ($p < 0{,}005$). Bei SGA-Neugeborenen von Müttern, die während der Schwangerschaft eine Hypertension entwickelten, konnten wir im Vergleich zu jenen von Müttern ohne Gestationshypertonus jeweils erhöhte Folatkonzentrationen (77,6 *vs.* 46,9 nmol / L; $p = 0{,}054$) sowie signifikant erhöhte Gesamthomocystein- (6,54 *vs.* 4,77 µmol / L; $p < 0{,}001$) und Vitamin B_{12}-Konzentrationen im Nabelschnurblut (494 *vs.* 362 pmol / L; $p < 0{,}001$) nachweisen.

Signifikant erhöhte Folatkonzentrationen (51,4 *vs.* 43,5 nmol / L; $p = 0{,}013$) wurden bei Frühgeborenen von Müttern mit vorhergehenden Fehlgeburten analysiert. Hinsichtlich des A1298C-Polymorphismus wurden verringerte Vitamin B_{12}-Konzentrationen bei SGA-Einlingen mit dem C-Allel im Vergleich zur entsprechenden AA-‚Wildtyp’-Gruppe festgestellt (353 *vs.* 548 pmol / L; $p = 0.001$), ansonsten ergaben sich keine Zusammenhänge des Homocysteinstatus zu den untersuchten Genpolymorphismen.

Diskussion: Die Gesamthomocystein-Konzentrationen im Nabelschnurblut von SGA-Frühgeborenen waren - da alle Mütter eine Folatsubstitution erhielten – trotz eines

relativ hohen Vitamin B_{12}-Status höher als in vergleichbaren Kollektiven; dies könnte bedingt sein durch prämatur verminderte Aktivitäten von Enzymen, die das Homocystein katabolisieren, oder durch die Unfähigkeit, die verfügbaren Kofaktoren wie Folat und Vitamin B_{12} zu verwerten. Da der Unterschied nach 36 Schwangerschaftswochen nicht mehr auftrat, ist zu diskutieren, ob eine Folsäure-Supplementation eine Stabilisierung der metabolisierenden Enzyme (Methioninsynthase, Methylentetrahydrofolatreductase) induziert und so die prämatur verminderten Enzymaktivitäten in der SGA-Kohorte ausgleicht. Ein weiteres erstmalig beschriebenes Forschungsergebnis stellt der erhöhte Gesamthomocysteinstatus im Plasma bei SGA-Frühgeborenen von Müttern mit einem Gestationshypertonus im Vergleich zur SGA-Vergleichsgruppe von normotensiven Müttern dar. Potenzielle Mechanismen könnten hierbei sein: Die Hypertonie könnte entweder eine reduzierte Ausscheidung von Homocystein über die Niere der Mutter oder einen verminderten Abbau von Homocystein über das arterielle Blut vom Fötus zur Mutter bewirken.

Die vorliegende Studie ist ein weiterer Baustein zur pathophysiologischen Charakterisierung des Gestationshypertonus. Zusätzliche Studien mit größeren Fallzahlen zur weiteren Differenzierung unserer Ergebnisse sind erforderlich.

Schlüsselwörter: intrauterine Wachstumsretardierung; Frühgeburtlichkeit; Geburtsgewicht; Methylentetrahydrofolatreductase; Folat; Homocystein; Gestationshypertonus

1 Introduction

Intrauterine growth retardation (IUGR) is a leading cause of perinatal morbidity and mortality, followed only by prematurity[127] and a common cause of long-term morbidity and mortality in childhood age. Risk factors for IUGR vary according to lifestyle, genetic factors (low growth potential), maternal morbidities, among others. Environmental factors have been of central interest in recent years as well as the interaction between gene environment on the risk of some human disease. In developed countries, IUGR is attributed in the majority of cases to pregnancy-induced hypertension (PIH) and pre-eclampsia in preterm birth whereas in near-term or term neonates, preterm labour, cigarette smoking, and substance abuse have to be considered; in addition, intrauterine infections have to be monitored during the whole duration of gestation. Malnourishment, which is globally the main cause, only plays a minor role in developed countries. Genetic factors as a cause of IUGR have also to be mentioned and may be challenging the concept of catch-up growth. Hyperhomocysteinaemia (HHcy) denotes the presence of abnormal elevation in fasting total homocysteine (tHcy) levels. Elevated homocysteine (Hcy) levels belong to the biomarkers thought to reflect a higher risk for cardiovascular disease (CVD), for atherosclerosis and thrombosis. Methylenetetra-hydrofolate reductase (MTHFR) polymorphism C677T possibly exhibits decreased enzymatic activity leading to mild HHcy in homozygous C677T individuals especially during times of folate insufficiency. It has been advocated that it should be possible to overcome HHcy caused by the 677T allele by folate supplementation.

Although not within the scope of this study, we have taken the liberty to conclude the thesis with an excursus giving a brief overview of the island Shri Lanka, her public health care system and the study’s host institution Castle Street Hospital for Women in Colombo as we would like to serve the reader’s interest.

1.1 Intrauterine Growth Retardation

1.1.1 Research into “Intrauterine Growth Retardation” in Historical Retrospective

Almost seventy years ago, in 1941, the Australian ophthalmologist GREGG was the first to describe the causation of an illness by an external influence on foetal and embryonic development: rubella embryopathy due to maternal rubella infection. Until that time, the prevailing medical opinion had considered the foetus largely to be able to develop autonomously and independently of external influences. The initial interest in IUGR was

fostered by neonatologists two decades later. DÖRNER, FREINKEL, and BARKER, then, were the leading figures behind a paradigm shift in the understanding of multi-factorial disease pathogenesis.

G. DÖRNER (Charité, Berlin, Germany)[37] was the first to postulate a consequence of the intrauterine milieu and later deleterious endocrine consequences during the 1970s. This became widely known with the generic collocation of "functional teratology", *i.e.*, the study of abnormal development and congenital anomalies (teratophysiogenesis, teratopsychogenesis; prenatal and perinatal programming of the neuro-endocrine immunological system). This concept was taken up by N. Freinkel (Center for Endocrinology, Metabolism, and Nutrition / Department of Medicine and Biochemistry, Northwestern University Medical School, Chicago, USA) in his 1980 Banting Lecture[130], adding a wealth of references to substantiate his theorem; he broadened the concept in terms of a "fuel-mediated teratogenesis" (nutrient-dependent programming of the metabolism regulation), bringing the consequences of gestational diabetes mellitus for the offspring into focus. The understanding was further complemented by the "foetal origins of adult disease" hypothesis introduced by D. J. P. BARKER (MRC Environmental Epidemiology Unit, Southampton, United Kingdom) and his group of researchers[10] in 1989, opening up an new area of investigation with his pioneering epidemiological studies. He developed the theory that several of the chronic diseases associated with aging may be programmed in very early life, indicating adverse deleterious intrauterine conditions and impaired foetal growth to be a risk factor for cardiovascular mortality and other chronic diseases in adulthood; in short, he focused on the long-term adult consequences of foetal nutrient deprivation and IUGR.

Due to these landmark works, the problems of the impairment of intrauterine growth and their effects have more and more gained the awareness of the scientific community. According to these hypotheses, IUGR leads to a lifelong effective programming of various endocrine and autonomous pathways that result in an increased risk for cardiovascular and endocrine-metabolic diseases. Diseases, such as diabetes mellitus type II and arterial hypertension, arise, therefore, not only because of the individual's genetic background and external influences during life, but also because of events during the intrauterine and the early postnatal periods.

Strictly speaking, as to the history of ideas, the basic biological concept of an environmental "programming" of phenotypic features goes back to LAMARCK (1809: heredity of acquired features as starting point) and SAINT-HILAIRE (1837: structural teratology, teratomorphogenesis)[130]. On the other hand, the specific phenomenon of a non-hereditary, epigenetic, maternal-foetal transmission of acquired properties due to

intrauterine conditioning of the foetus has become the focus of recent scientific debates and research. These typological concepts were met with much scepticism and initially failed to achieve broad scientific acceptance. Even though some details remain controversial, there is no longer any question that intrauterine life is far more than just the execution of a genetic program of which the neonate is the final product, and the concepts of *functional teratology* and *foetal programming* have in the meantime made an international breakthrough and they have gained wide acceptance. Later, a number of epidemiological and experimental studies addressed the association of IUGR and hypertension[53].

The ensuing exposition intends to elucidate the multi-factorial nature of IUGR and the complex networks within which maternal and foetal genes have to act to determine birth weight.

1.1.2 Definition of Intrauterine Growth Retardation

The terms *IUGR* (synonyms: intrauterine growth restriction, intrauterine malnutrition) and *small-for-gestational age* (SGA) are often used interchangeably to designate a clinical entity in which the foetus has failed to attain its full growth potential[194], but IUGR and SGA are not synonymous. Due to confusing medical terminology, most authors do not distinguish between these terms without taking into consideration that these two clinical entities are not the same. IUGR as pathologic counterpart of SGA suggests a pattern of growth in the foetus over a period of time, whereas SGA is a term to describe a single point on a growth curve at the time of birth. Both IUGR and SGA have been associated with poor perinatal outcomes.

The significance of IUGR is well established, although the variables defining this condition remain a debatable issue. Today, IUGR is most widely defined as birth weight (BW) below the 10th percentile (*clinical definition*) or as a foetus who did not meet his full genetic growth potential *in utero* (diminished growth velocity)[17]. Foetal IUGR is considered as a clinical symptom of genesis and its diagnosis is based on the assessment of the exact gestational age (GA)[86] (*cf.* Chapter 2.2.5). The incidence of IUGR varies depending on the population under examination, including its geographic location, and the standard growth curves used as reference[86]. Other definitions and cut-off points have been applied, *e.g.*, infants weighing less than two standard deviations below the mean for those born at the same GA are considered abnormally grown, *i.e.*, growth-retarded[17]. The discordant definitions are a matter of controversy because they draw no distinction between foetuses who are constitutionally small, growth-retarded and small, and growth-retarded but not small. It is today recommended to restrict the use of IUGR to prenatal assessment of

growth and of SGA to the assessment of BW[109]. In *sensu strictu*, therefore, IUGR denotes the deviation of intrauterine growth from the genetically programmed target value, *i.e.,* below the 10th percentile with regard to the respective GA and gender with a pathologic retardation of foetal growth due to adverse genetic or environmental influences (*e.g.*, toxins, nutrient deficiency), with determination of cut-off values based on national population standards[45]. Birth weight below the 10th percentile for GA denotes a hypotrophic neonate, *i.e.*, SGA, in perinatology. This requires a tight control of the course of intrauterine growth at close intervals, for example, by means of ultrasound scans, which allows the non-invasive recognition of alterations in foetal growth. These definitions are applicable to both singletons and multiples. IUGR is an important reason for premature delivery.

In the narrow sense, three consecutive phases of intrauterine growth have commonly been described[36]. The first 16 weeks of gestation are characterised by a marked *cellular proliferation*, which is also termed *cellular hyperplasia* and which involves a rapid increase in cell number. Subsequently, *cellular proliferation* and *cellular differentiation* with increases in cell size and number and controlled by deoxyribonucleic acid (DNA) methylation, concomitantly run up to the 32nd week of gestation. During the last trimester, from the 32nd week of gestation on until term, *cellular hypertrophy* dominates with a maximum of increase in weight per unit of time. It is in this phase that foetal fat deposition is thought to take place. There is extensive remodelling of the placenta in the second half of gestation to increase its functional capacity to meet foetal demand for nutrients.

Low birth weight (LBW) is commonly used as indicator of IUGR. LBW may be result of either shortened gestation (preterm birth) or slowed rate of growth (IUGR; attributed to widespread maternal malnutrition)[86]. There has been a glaring inconsistency in the past regarding the definition of LBW. LBW is defined by the World Health Organization as a birth weight less than 2,500 g[171], since below this value birth weight specific infant mortality begins to rise rapidly; this definition is now widely accepted. The term LBW is being used as surrogate marker for IUGR, the complement of which is preterm birth (< 37 completed weeks of gestation). Preterm birth secondary to IUGR is operative in about 25 % of very preterm neonates born before 32 weeks of gestation[48].

LBW is a worldwide phenomenon but is most prevalent in developing nations, where it has been estimated that 13.7 million infants are born each year with the condition[32]. South Asia is most affected with 50 % of babies in Bangladesh defined as having LBW and about one third (28 %) in India[159]. According to estimates, 15.5 % of all births, or more than 20 million infants worldwide (varying data: 25 million babies a year[32]), are born with LBW. The incidence of LBW in developing countries is 16.5 %, which is about double the

rate in developed nations. According to the Berlin Perinatal Registry, the rate of newborns with LBW was 9.6 %[15]. As more than 95 % of LBW babies are born in developing countries[171] where perinatal and infant mortality is already high, this implies an obstacle to national development and a health priority in these countries. Most of the LBW babies in developed countries are premature; the main reason for LBW in India and in developing countries as a whole is IUGR[32]. However, reviews of low birth weight data and estimates consistently note their limitations[32], which renders a comprehensive examination of the data and estimation procedures timely.

The antenatal growth pattern overall depends on ethnical, genetic, medical, socio-economic and geographic influential factors. A generally accepted nomenclature of IUGR is still warranted.

1.1.3 Classification of Intrauterine Growth Retardation

Intrauterine growth retardation encompasses a highly heterogenous group in terms of aetiology, severity, and body proportionality; whenever possible, it should be assessed. There is a strong link between IUGR, chromosomal abnormalities, and congenital malformations. Regulation of foetal growth is multifactorial and complex. The multifactorial causes of IUGR create three possible scenarios: (a) abnormal placental function; (b) inadequate maternal supply of oxygen and / or nutrients; and / or (3) decreased ability of the foetus to use the supply.

Various schemes attempt to classify the aetiologic factors of IUGR. Some of these focus on classifying the insults as either extrinsic or intrinsic to the foetus (*cf.* Table 1). Other classification schemes view the pathophysiologic basis for the development of IUGR as being foetal, placental, or maternal in origin.

Historically, two types of IUGR are usually classified on the basis of discordant growth of head and abdomen - symmetric (*proportionate* or *hypoplastic* or *stunted* or *chronic* or *Type I*) *versus* asymmetric (*disproportionate* or *hypotrophic* or *subacute* or *wasted* or *late flattening* or *Type II*) growth retardation[188]; differences are outlined in Table 1. Perinatal problems associated with both distinct forms have been reported to

differ. Significant overlap among the two types makes a clear distinction difficult sometimes.

Symmetric growth retardation presents in early pregnancy and relates to a foetus whose entire body (head, femur, abdomen) is equally affected and proportionally small, *i.e.*, the ratios between weight, head circumference and length are equal. All growth parameters

are below the 10th percentile. Up to 10 % of growth-retarded infants are identified to have this type of foetal size (*cf.* Table 1, which presents divergent data). It results from early insult impairing foetal cellular hyperplasia. Because of the intrinsic foetal problem, cell division and cell growth is limited and is independent of substrate supply. Postnatal catch-up growth is rarely seen in this group of infants[127, 188], which may also be due to genetic programming.

An asymmetric pattern of foetal growth retardation, in contrast, may be caused by later insults in the second trimester. This is the most frequent type of IUGR. About two thirds of growth-retarded neonates are identified as having asymmetric retardation. This type of retardation refers to infants who - as skeletal growth and brain growth are less affected - have a relatively normal head dimension as a result of a physiologic adaptation, but a small abdominal circumference (due to decreased liver size), scrawny limbs (because of decreased muscle mass) and thinned skin (because of decreased subcutaneous fat) or to infants with LBW and (nearly) normal birth length and, therefore, a reduced ponderal index (PI)[127, 188], a pattern that has led to the concept of adaptive “sparing” of head and length growth and that is often termed the “brain-sparing process”[87]. Proportionality among IUGR infants is strongly confounded by the severity of the growth retardation, *i.e.*, disproportionate IUGR infants tend to be more severely growth-retarded[87]. Most of the asymmetrically growth-retarded infants have suffered chronic hypoxaemia and malnutrition *in utero* due to placental insufficiency[160].

ROHRER’s ponderal index[144] as a measure of the nutritional status or as an index of corpulence and as an efficient marker of disproportionate intrauterine growth describes the body proportionality at birth. PI essentially is an indirect measure of soft tissue and , inferentially, of fat accumulation. It allows the differentiation between symmetric and asymmetric growth retardation and measures the severity of asymmetry in growth-retarded neonates. It is defined as the ratio of birth weight to length[91] in order to evaluate the ratio of soft tissue to bone mass (PI = BW [kg] / length $[m]^3$), it correlates with direct measures of neonatal fat as estimated by skinfold thickness. An abnormal PI is a widely accepted measurement of disproportionate growth retardation by paediatricians worldwide[103]. In asymmetric IUGR infants, the PI is low. Identification of disproportionately grown SGA neonates by using the PI as a measure of the nutritional status at birth is important because they constitute a high-risk group among SGA neonates. Poor nutritional status of the mother could have a direct effect on the organs of the developing foetus and / or affect the endocrine milieu in the maternal foeto-placental unit resulting in an increased incidence of intrauterine growth-retarded SGA births[103]. Arrested head growth is of great concern to the developmental potential of the foetus[127]. Asymmetric

growth retardation implies an undernourished foetus who is directing most of his energy to maintaining growth of vital organs, such as the brain and heart, at the expense of the liver, muscle and fat. It is usually the primary result of placental insufficiency and it develops when oxygen or substrate supply to the foetus is reduced during the last trimester of pregnancy due to a reduced functional capacity of the placenta[127, 188]. Type II is progressive and it might induce severe damage. From the view of the infant, asymmetric growth retardation is of an extrinsic origin. Postnatal catch-up growth is frequently observed[188].

Foetuses with asymmetric growth retardation are at particular risk for intrauterine demise and foetal stress in labour. Small-for-gestational age is a marker for subsequent stillbirth. VASHVENIK *et al.*[178] conducted an retrospective analysis of data on 662,043 births, stillbirths and neonatal deaths in 1992 - 2002 recorded by the Victorian Perinatal Data Collection Unit in Australia. Stillbirths after 23 weeks were stratified by birth weight into AGA and SGA as main outcome measures; the stillbirth risk per 1,000 ongoing pregnancies was calculated. For the AGA, the overall stillbirth risk was 2.88 per 1,000; in the SGA group, the stillbirth risk was 15.1 per 1,000. The study reinforced that SGA foetuses are at much higher risk for stillbirth than those that are normally grown across all gestational ages, particularly with advancing gestational age. Some studies have reported that disproportionate infants with IUGR are at greater risk for neonatal mortality than proportional ones[124], although the interpretation of these findings seems to be limited by these studies' failure to control for the severity of IUGR[87].

KRAMER *et al.*[87] questioned the concept of "head and length sparing" and, as a result, of a proportionate *vs.* disproportionate neonate. The authors argued that symmetry among intrauterine growth-retarded infants is confounded by severity of growth retardation (*i.e.*, asymmetric intrauterine growth-retarded infants develop more severe growth retardation than their symmetric peers) and they suggested that in most cases IUGR is a continuum from asymmetry (early stages) to symmetry (late stages). If asymmetric growth retardation is sustained long enough or is severe enough, the foetus may lose the ability to compensate and will become symmetrically growth-retarded[127].

Table 1: Classification of IUGR

	Extremes of Type I (*symmetric IUGR*)	**Extremes of Type II** (*asymmetric IUGR*)
Incidence (*%*)	20 – 30	70 – 80
Timing (*weeks*)	< 24	> 28
Aetilology	(mostly) intrinsic: *chromosomal abnormalities and congenital malformations; drugs; infection; early-onset severe pre-eclampsia; pre-eclampsia < 30 weeks superimposed with chronic hypertension*	(mostly) extrinsic: *placental and maternal vascular factors* (e.g. *placental insufficiency*)
Pathophysiologic characteristics	impaired cellular embryonic division; impaired cellular hyperplasia ± hypertrophy	impaired cellular hypertrophy
Malformations	frequent	rare
Cell number	decreased	normal
Cell size	normal	decreased
Head circumference	decreased	normal
Ponderal index	normal	decreased
Catch-up growth	rare	frequent
Outcome	greater morbidity and mortality	lower morbidity and mortality

Source: WOLLMAN[188] (modified)

1.1.4 Aetiology of Intrauterine Growth Retardation

Intrauterine growth is an important predictor of perinatal and adult health. The underlying mechanism of IUGR still remains unknown, even though many determining factors have been described in the literature[86]. A large number of established aetiologic factors of IUGR can meanwhile be retrieved from the internet. This gives the impression that with respect to the aetiologies of IUGR the facts are clear, which is far from true. Until today, in at least 40 % of all cases of infants born too light and / or too short, no underlying pathology can be identified[188]. In terms of aetiology, a number of chromosomal and other congenital anomalities are associated with growth retardation. The incidence also varies according to the reference population. Based on aetiology, causes for IUGR may generally be divided into three large categories, foetal factors, placental factors, and maternal factors[17]; utero-placental dysfunction accounts for the majority of IUGR[48].

1.1.4.1 Foetal Factors

Foetal factors causing IUGR, although quantitatively rare, are often associated with severe retardation of growth and a bad prognosis as to long-term outcome. These factors include:

- Genetic conditions[157, 188]:

  crucial in determining the rate of intrauterine growth, such as chromosomal abnormalities, autosomal trisomies, metabolic errors such as mitochondriopathy. The presence of a chromosomal abnormality often results in the appearance of IUGR early in pregnancy (≈ 40 %). It may be assumed that a considerable number of those neonates with IUGR of unknown cause has an underlying genetic disease. In parenthesis, genetic factors as a cause of IUGR may be challenging the concept of catch-up growth[53].

- Foetal infections[36, 157, 188]:

  viral (IUGR is symmetric in this case): most important rubella, varicella zoster virus, cytomegalovirus; bacterial or parasitic: syphilis; protozoal: toxoplasmosis, malaria.

- Malformations[188]:

  syndromes / congenital anomalies, cardiovascular defects, gastrointestinal defects, or muscolo-skeletal dysplasias.

1.1.4.2 Placental Factors

The *placenta*[45, 48, 53, 188] as essential interface between mother and foetus regulates the transport of some metabolites to the foetus: glucose and fatty acids by passive diffusion, others, *e.g.*, amino acids, are transported actively. In addition, a large variety of active enzymes capable of metabolising amino acids by oxidation and protein synthesis are contained in the placenta. Detailed knowledge of regulative mechanisms in the placenta important for foetal growth still is scarce[188]. Utero-placental dysfunctional factors as a result of impaired vascular development that are strongly associated with IUGR include reduced blood flow and, thus, impaired oxygen and nutrient supply to the foetus, abnormalities of placental morphology (uterine perfusion of the placenta), recurrent abruption / placenta praevia; in severe cases, reduced blood flow may already start in the 2nd trimester.

1.1.4.3 Maternal Conditions

Maternal conditions associated with IUGR[83, 157, 188] exhibit:

- maternal morbidities and prescription medications:

  severe PIH (interestingly, a pre-existing, uncomplicated maternal hypertension does not reduce BW), pre-eclampsia, severe chronic infections (inflammatory bowel disease, malaria), hypoxia (asthma, cyanotic heart disease), diabetes, cardiovascular disorders, anaemia, immunological disorders, drug use (anti-metabolites, anticoagulants, anticonvulsants).

- environmental and lifestyle factors:

  malnutrition[45], abuse of toxic substances (alcohol, tobacco)[97] and use of illicit substances (marijuana, cocaine, amphetamines).

- certain demographic variables and socioeconomic characteristics such as poverty[83].

- geographic circumstances:

  high altitude because of decreased partial oxygen pressure (physiological changes in response to high altitude residence which reduce blood flow to the foeto-placental unit are detrimental to foetal growth) and north-south divide.

- other conditions:

  prior history of prematurity or spontaneous abortion; low pre-pregnancy weight, short maternal height, low pregnancy weight gain, previous pregnancy associated with IUGR (prior LBW infant), multiple pregnancy, also reproductive technologies[188] (*in vitro* fertilisation and gamete transfer).

Likewise, women who were themselves growth-etarded at birth or who have a sister who has had an IUGR pregnancy are at an increased risk for IUGR in the pregnancy[157]. In case of maternal age extremes, *i.e.*, among teenage (age < 15) and elderly mothers (age > 35), higher IUGR rates were also observed[194]; young maternal age confers a considerable risk for adverse pregnancy outcome, including LBW and prematurity. This latter association, however, is still controversial as available data are inconclusive; more studies are warranted to identify a clear explanation of these observations. Moreover, IUGR was found to be more prevalent among unmarried mothers and in minority ethnic groups[47, 194]. And the aetiology of growth retardation and preterm delivery may probably vary in developing or economically deprived countries and in different South-East Asian countries, as population data from Burmese, Thai, Chinese, and Vietnamese populations indicated[47].

1.1.4.4 Developed Countries: Smoking and Other Factors

In Europe and in developed industrial countries, cigarette smoking during pregnancy by far is the most important single aetiological determinant related causally to IUGR[86, 97] and one of the most preventable. The majority of the smokers in Germany were of low social status according to the self-report within the scope of the 1995 microcensus[59]; the marked social polarisation of smoking continued up to 2002[60]. There was an increase in the rate of smokers among women of childbearing age in the last years. In 1998, for instance, 44 % of all women aged 25 to 29 were smokers as against 41 % in 1990, in 1992, and in the years up to 2002[60, 79]. Yet only a part of the pregnant women gives up smoking. 27 % of the expectant mothers smoked according to the 1998 Perinatal Registry[79]. In order to restrain the spreading of IUGR in developed countries, it seems more likely to influence the determinants of unfavourable social circumstances, above all cigarette and alcohol consumption. In comparison to other European countries, there seems to be a great need for enhancing activities regarding prevention of smoking by adults in Germany[60]. Interestingly, a WHO meta-analysis[86] demonstrated that today 20 % - 40 % of IUGR cases among neonates may be attributed to maternal nicotine abuse.

Other factors follow in developed countries[166]:

- in preterm birth: moderate or excessive alcohol abuse and substance abuse, PIH, pre-eclampsia;
- in near term and term neonates: preterm labour, intrauterine infections.

1.1.4.5 Developing Countries: Incidence of Intrauterine Growth Retardation

In developing countries and in countries with low income, some of the main causes of IUGR are attributed to poor maternal nutrition suggested by a low pre-pregnancy weight, short stature, a slow rate of pregnancy weight gain[180], as well as infections, first of all malaria[89, 149]. Whereas malnourishment only plays a minor role in developed countries, it is globally the main cause of IUGR. Populations in developing countries have been demonstrated to have a higher incidence of chronically malnourished neonates (proportionate growth retardation) within their IUGR population (67 % - 79 %), whereas populations in developed countries have a predominance of subacute foetal malnutrition, with the proportion of chronically malnourished newborns being only 20 % - 40 % of the total IUGR population[180]. Malaria takes a clinically more critical course during pregnancy when compared to non-pregnant women who are in the same situation of life. Interestingly, a study demonstrated that a persistent chemoprophylaxis among primiparae led to a BW > 2500 g in almost 90 % of the cases under review[89, 149]. For Shri Lanka, the

following data on pregnancy outcome are available (in % of live births): LBW 18.4 %, IUGR 34.0 %, and preterms 14.0 %[88].

The high incidence of IUGR in the poorest countries of the world emphasises the aetiopatho-genetic implications of socioeconomic factors. The observation of increased incidences of cardiovascular diseases and diabetes mellitus in tropical developing countries indicates that disorders of intrauterine development likewise bring forth long-term effects[89 (nach UNWIN)]. In addition to infections and dystrophic influences, the growing importance of toxic substances in developing countries has to be taken note of. The increasing consumption of alcoholic drinks implies a high risk for low birth weight (39 %), IUGR (21 %), development of malformations (42 %; 9% cardial defects thereof), and prematurity (54 %) in the pregnant mothers or their infants, respectively[89]. The general improvement of the social condition should assumedly lead to a decrease of IUGR incidences in developing countries.

1.1.5 Short-Term and Long-Term Effects of Intrauterine Growth Retardation

IUGR leads to a reduced BW below the genetically determined weight and has serious sequelae during the neonatal period, even if today the long-term prognosis for most infants born with IUGR is known to be good; just as the advances in perinatal medicine lead to a continual increase of the survival rates for extremely premature infants[51], *i.e.*, those born at a GA of < 26 weeks or weighing ≤ 750 grams at birth. IUGR also is a leading cause of prematurity and a frequent cause of impaired growth during childhood; furthermore, short stature and catch-up growth are important features of growth-retarded foetuses during childhood.

Infants of LBW are at increased risk for short-term and long-term mortality and morbidity and other adverse outcomes; IUGR may, for instance, result in significant perinatal and neonatal morbidity and mortality in preterm and term neonates if not properly diagnosed[48]; a significantly high perinatal mortality has been shown to be a concomitant of reduction in BW. Perinatal counselling and decision-making should be based on reliable current mortality and morbidity information and the latest development in research[92]. If the growth-retarded foetus is identified and if appropriate management is instituted, perinatal mortality can be reduced, underscoring the need for assessment of foetal growth at each prenatal visit. Analyses by REISS *et al.*[141] highlighted BW below the 10th percentile to be associated with an about five-fold increased death rate during the neonatal period.

IUGR and perinatal factors including birth weight, birth length, and GA have also been associated with impaired intellectual and cognitive performance (speech, language) in childhood and adolescence as well as other medical problems later in life, but the the results are not uniform and the mechanisms for these associations remain unclear. Understanding the early life influences on intellectual performance may be important for developing initiatives aimed at preventing adverse outcomes in later life. Children born with IUGR were reported to have subtle long-term cognitive impairments, soft neurologic symptoms, and learning difficulties in school[16]. In an Australian study[96], IUGR (less than or greater than optimal weight) has been associated with development of intellectual disability. These reported findings are of great concern for developing countries, because they have a great proportion of children born with IUGR. The current data on psychomotor and intellectual developmental prognosis of hypotrophic preterms, however, still are disputed. Some studies could not entirely confirm a delayed intellectual development and growth[126], whereas, discrepantly, other studies reaffirmed an association between hypotrophic preterms and neurologic impairments and lower intelligence indices[46]. Symmetric growth retardation may have a different aetiology from asymmetric growth retardation and may lead to differences in intellectual performance later in life. These differences may be a result of the timing of the growth retardation. Symmetrically growth-retarded infants most likely experience their insult during a critical period of brain development, whereas asymmetrically growth-retarded infants experience their insult during a less critical period with regard to brain development. Those asymmetrically growth-retarded infants have larger head circumferences, indicating preferential perfusion to the brain[16].

The recent study by PAZ and joint authors (2001) which was free from clinical selection bias[126] may lead the debate on this aspect towards a new direction. This fundamental and possibly pioneering follow-up study on the intelligence of growth-retarded mature newborns at the age of 17 - 18 years and without adjusting for socio-economic differences examined recruits to the Israely army, using ≤ 3rd percentile of their study cohort as their SGA category. The study indicated evidence of only a small effect, though of marginal statistical significance, with slightly higher intelligence indices, among eutrophic infants when compared with SGA newborns; the deficits were < 5 intelligence quotient points, with scores still well within the normal reference range. The effect was more distinct among boys than among girls. The study included 13,454 consecutive singleton term infants born between 1974 and 1976; with regard to previous reports, it should set the standard for future investigations into the neurological and intellectual development of mature neonates born with IUGR. In summary, a review and evaluation of the findings of recent studies on this aspect by GORTNER *et al.*[49] pointed to a trend against SGA preterms

with regard to psychomotor and intellectual development, even though, on the basis of the known results, a concluding definition of the medium-term consequences of IUGR from infant age up to school age remains a necessary desideratum[49].

Inherited factors influence BW, but we know very little about the genes involved. Perinatal asphyxia involving multiple organ systems is one of the most significant problems in growth-retarded infants. Severe IUGR is often accompanied by oligohydramnion[105]. Additionally, an increased risk for severe long-term chronic pulmonary consequences of preterm delivery compared to age-matched controls has been demonstrated, *i.e.*, bronchopulmonary dysplasia (BPD)[50, 52, 141]. The pathogenesis of BPD is known to be multifactorial, its definition still remains difficult and the actual aetiopathogenic mechanisms remain incompletely understood, however[53]. Commonly accepted as definition today is a chronic pulmonary disease in very immature preterms (< 32 weeks of GA) with low birth weights which presents at 36 weeks of postmenstrual age with an adequate arterial oxygen saturation demand (SaO_2 > 90 %)[54]. Although BPD is most often associated with premature birth, it can also occur in infants born at term who need aggressive ventilator therapy for severe, acute lung disease.

Thrombophilia is believed to be a multifactorius gene disease with more than one defect and it is a condition characterised by a tendency for the occurrence of thrombosis, mostly as a consequence of inherited polymorphisms[74]. Studies on the role of thrombophilia have mainly focused on adverse pregnancy outcome, the role of gene-gene interactions between thrombophilic polymorphisms for pregnancy outcomes has received little attention. PETAJA *et al.*[128] have suggested that thrombophilia may play a role in the aetiology of intraventricular haemorrhage in preterm infants. For some time past, a potential association between IUGR and maternal thrombophilia has been postulated on the assumption that thrombophilic polymorphisms could affect placental vascular growth and circulation and, thus, foetal growth[93], but data on this topic are conflicting in the literature[69]. C. INFANTE-RIVARD / G. - E. RIVARD have carried out extensive research on this hypothesis[73, 74]. They reported on gene-environment interaction between MTHFR C677T and folate intake, potentially indicated by epigenetic mechanisms. It is well established that genetic effects are distinct across environments. Although the complex role of the gene-environment interaction between MTFHR C677T and periconceptional folate supplementation has so far received only little attention, recent reports have suggested the existence of a gene-environment interaction between the MTHFR C677T polymorphism and folate status. The protective effects of folate supplements have been shown to involve other environmental factors or gene-environment interactions. Findings by van BEYNUM *et al.*[173] provided a mechanism of the protective role of folate. A further

study[177] suggested a gene-environment interaction between maternal periconceptional folic acid supplement use and / or dietary folate intake and the MTHFR 677TT and MTHFR 1298CC genotypes of the mother on the risk of delivering cleft lip / palate offspring. Maternal genes may shape the early environment of the foetus, and genes that metabolise essential nutrients are particularly relevant. The findings of INFANTE-RIVARD *et al.*[73, 74] point towards a gene-environment interaction in thrombosis. Distinctions between arterial and venous thrombosis have to be made. The thrombus is platelet rich in arterial thrombosis and fibrin rich in venous thrombosis. Additionally, there is presence of atheroma in arterial thrombosis which represents vascular wall damage. Pre-eclampsia and hypertensive disorders of the pregnancy are still one of the leading causes of maternal mortality in developing countries. A prospective study with a multicentre, observational cohort design[40] was published to determine the impact of thrombophilia on the recurrence of PIH; 172 Caucasian patients with a previous singleton pregnancy complicated by PIH were observed in the next pregnancy. 60 women (34.9 %) showed the presence of a thrombophilic defect. They had a higher risk for the recurrence of PIH (OR: 2.5, CI: 1.2 – 5.1, *p*-value: 0.010), compared to patients without thrombophilia (n = 112). Similar findings were observed when only heritable thrombophilia was screened. In this case, thrombophilic patients were at an increased risk for the occurrence of very early preterm delivery (< 32 weeks of pregnancy; OR: 11.6, CI: 3.4 – 43.2, *p*-value: < 0.001). These observations indicated that screening for thrombophilia in Caucasian women with a history of PIH may be useful for preconceptional counselling and pregnancy management. The findings in only Caucasian women, most of them of Italian origin, also indicated that it is crucial to control for ethnicity when assessing the influence of genetic factors such as heritable thrombophilias.

1.2 Methylenetetrahydrofolate Reductase

1.2.1 Methylenetetrahydrofolate Reductase Enzyme and Its Genomic Structure

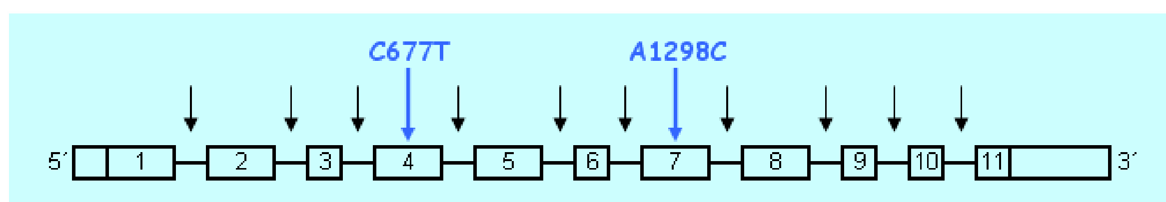
The human MTHFR gene maps to chromosome 1p36.6 (*cf.* Figure 1) and consists of 11 exons ranging in size from 102 base pairs (bp) up to 432 bp[55]; the entire coding region has a length of 1.980 bp with a predicted molecular mass of 74.6 kiloDalton. There is approximately 90 % similarity between the nucleotide sequences of the human and mouse MTHFR genes[174].

MTHFR plays a major role in the metabolism of Hcy and folates. The key enzyme MTHFR influences many cellular processes including methionine and nucleotide synthesis, methylation reactions, and maintenance of Hcy at non-toxic levels. It is required for the

reduction of 5,10-MTHFR to 5-methyltetrahydrofolate (5-methyl THF), the major form of folate in plasma, thus generating the active folate derivative (cosubstrate) for the remethylation of Hcy (regulation of methionine and Hcy concentrations in the circulation)[42]. MTHFR deficiency is inherited as an autosomal recessive trait[80]. Severe MTHFR deficiency as the most common inborn error of folate metabolism results in HHcy, homocystinuria, and hypermethioninaemia[44]. Patients with severe MTHFR deficiency (0 - 20 % residual enzyme activity) show a wide range of clinical symptoms in infancy or adolescence, such as developmental delay, severe mental retardation, perinatal death, psychiatric disturbances, motor and gait dysfunction, seizures, and profound later-onset neurodegenerative disorders; they are also at risk for premature vascular complication[148].

Many variations in the MTHFR gene have been reported, among others at bp 1059, bp 1289, bp 1317 (C1317T polymorphism), and bp 1793 (G1793A polymorphism)[183], although their functional relevance has not yet fully been investigated. In 2000, a report brought the total number of polymorphisms up to 24 mutations identified in severe MTHFR deficiency[20]. In this study, we have focused on two well characterised, commonly occurring single nucleotide polymorphisms (SNP) in the 5,10-methylenetetrahydrofolate reductase gene gene, C677T and A1298C that lead to altered amino acids. Both genes, MTHFR C677T and A1298C, have 11 exons of similar sizes and similar exon-intron boundaries[55] (*cf.* Figure 1), the alleles are approximately 2 – 3 kilobases apart.

Figure 1: Genomic structure of the MTHFR gene



Exons are numbered, black arrows indicate the position of the introns, the positions of the two analysed common polymorphisms are indicated

1.2.2 MTHFR Polymorphism C677T and Its Metabolic Effects

The C677T mutation is the best characterised MTHFR polymorphism. Briefly, nucleotide 677 in the gene has two possibilities. The most frequent is 677C leading to alanine at amino acid 222. 677T leads to a valine substitution at amino acid 222 and encodes a “thermolabile” enzyme with reduced activity. In the C677T polymorphism, the cytosine base cytidine is substituted for thymidine at the end of the short arm of gene locus chromosome 1 at 1p36.3 (bp 677 C → T). As result of this substitution, the affected codon GCC is altered to GTC and, consequently, the corresponding amino acids alanine

which is encoded in codon GCC is converted into a valine residue with its underlying codon GTC (A222V) within the predicted N-terminal catalytic domain in an evolutionary conserved region, rendering the enzyme thermolabile, because the activity of the encoded enzyme is reduced at 37°Celsius (C) or more [44]. The polymorphism lies at the folate-binding site for the MTHFR cofactor flavin adenine dinucleotide. The C → T substitution at bp 677 may impair folate status when folate intake is marginal, a particular concern of women of reproductive age.

The "thermolabile" variant of the MTHFR protein was already described in 1977[147]. The incidence of this variant was set at a frequency of ≈ 5 % of the population, but the rate varied between different populations and ethnic groups. In 1995, FROSST and colleagues[44] as well as ENGBERSEN[38] described a thermolabile variant (*genetic* identification) whose specific activity (TT) is diminished by about 50 % compared to the homozygous state of the mutation (CC 'wild type') resulting in slightly increased Hcy concentrations. It was not until 1996 that the encoding sequence by a point mutation was discovered[34]. Three different genotypes are distinguished (*cf.* Table 2):

Table 2: Genotypes 677 C → T and their characteristic features

Genotype	Type	Characteristic band features
CC	wild type	one band with 198 bp
CT	heterozygous	one band with 198 bp + one band with 175 bp
TT	homozygous	one band with 175 bp

Serum folate values > 15.4 nM appear to neutralise the effects of 677 C → T mutation on Hcy levels[77]. Individuals with the MTHFR 677TT genotype have been shown to have 30 % *in vitro* MTHFR enzyme activity compared with the wild type, whereas those with the heterozygous CT genotype have a decrease in enzyme activity to 65 %. Reduced activity of the mutated allele has been demonstrated in lymphocytes obtained from both peripheral blood[44] and placental tissue[31]. The common polymorphic MTHFR gene variant 677C → T has been associated with increased risk for neural tube defects (NTD): Subjects with 677TT genotype have higher total levels of Hcy in their plasma than 677CC homozygotes, they have an increased risk for birth defects such as NTD[20, 77, 176] or for CVD[21, 81]. NTDs are characterised by delayed or slow closure of the neural tube that occurs around the 28th day after fertilisation. They are complex traits with multifactorial aetiology encompassing both genetic and environmental components. The MTHFR C677T mutation as a perfect example exemplifies the interaction between nature and nurture in the risk of having a NTD pregnancy[174]. The presence of a thermolabile MTHFR is predictive of coronary artery disease, independent of other risk factors, such as age,

smoking, hypercholesterolaemia and hypertension[81]. The terminus coronary artery disease is sometimes equated with coronary heart disease denoting the failure of coronary circulation to supply adequate circulation to the cardiac muscle and the surrounding tissue. However, the term coronary heart disease is not consistently defined by all authors in the scientific literature.

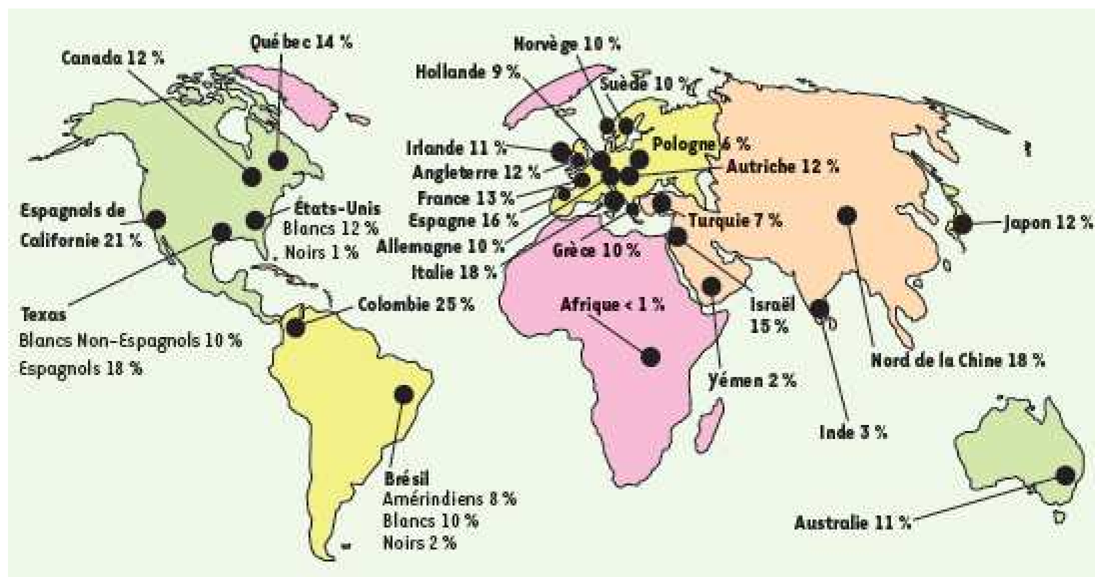
One other factor that may affect the frequency of the 677T allele could be the age of individuals as its prevalence is reportedly lower in older age groups, possibly because 677TT homozygous individuals are more prone to CVD[108]. The Hcy levels, however, depend in part on folate levels: in that Hcy is increased among those who have an inadequate intake of folate (indicated by low serum folate levels) while it is normal in others who have an adequate intake of folate[31, 44, 76]. Hence, it should be possible to overcome HHcy caused by the 677T allele by folate supplementation[101].

The literature is replete with investigations into the occurrence of the 677T allele in many parts of the world today. The findings established a wide variation of the allele frequency between geographical areas and ethnic populations as well as between different ethnic groups within geographical areas. Yet, current data on the distribution of this MTHFR mutation in some populations around the world still show gaps, and data for some small or isolated ethnic groups are limited, even for large geographical areas, *e.g.*, China[20]. Previous studies of the C677T mutation have mainly concentrated on European populations[150]. MTHFR polymorphism among Asians was studied especially in Indian populations, but also in Japanese and Shri Lankans. For other Asian populations, only limited data are available[150].

The prevalence of C677T ranges from 3 % to 40 % worldwide and in Western Europe (*cf.* Figure 2), it generally increases along a north to south gradient[185] (although this observation has not entirely been confirmed[24]). The prevalence was highest among in Italy and among Hispanics living in California and low among Blacks in the United States and in some areas of sub-Saharan Africa, whereas Caucasian Western Europeans and Chinese had frequencies intermediate between these two groups[150, 185]. The allele frequency in Europeans is 24 % – 40 %[176], the prevalence of the 677T allele among Caucasians in Britain is reported to range from 33 to 38 %[20], 26 % – 37 % in Japanese populations[150], ≈ 11 % in an Afro-American population in South Carolina[162], and 4.5 % in a group of Shri Lankans[150]. The reason for these variations remains unclear, although it has been suggested that they may reflect the ancestral origin of these populations[146]. The use of “within family” studies which are not affected by population differences in allele frequency should therefore advocated in order to test allele association while providing

matched controls. The T allele seems to be more common in regions with a higher dietary folate consumption[56].

Figure 2: Frequency of homozygous 677TT for different geographical regions, also the ethnic origin of some population groups are given



Countries / states and provinces from left to right [*French / English*] : Canada: *Canada*; Espagnols de Californie: *Californian-Hispanics*; Texas: Texas [Blancs Non-Espagnols: *non-Hispanic Whites*; Espagnols: *Hispanics*]; Québec: *Quebec*; États-Unis: *USA* [Blancs: *Whites*; Noirs: *Blacks*]; Colombie: *Colombia*; Brésil: *Brazil* [Amérindiens: *American-Indians*; Blancs: *Whites*; Noirs: *Blacks*]; Norvège: *Norway*; Hollande: *The Netherlands*; Irlande: *Ireland*; Angleterre: *Great Britain*; France: *France*; Espagne: *Spain*; Allemagne: *Germany*; Italie: *Italy*; Suède: *Sweden*; Grèce: *Greece*; Afrique: *Africa*; Pologne: *Poland*; Autriche: *Austria*; Turquie: *Turkey*; Israël: *Israel*; Yémen: *Yemen*; Inde: *India*; Australie: *Australia*; Nord de la Chine: *North China*; Japon: *Japan*

Source: LECLERC *et al.*[94]

1.2.3 MTHFR Polymorphism A1298C and Its Metabolic Effects

The other common SNP of MTHFR gene relevant to our study is a missense mutation, that in contrast to 677T mutation is characterised by an A → C exchange at position 1298 in exon 7 of the gene[143, 176, 183] (*cf.* Figure 1).

It has been suggested that the polymorphism affects folate status[183, 184]. In contrast to the 677C → T polymorphism, this polymorphism possibly is neither related to higher tHcy concentrations nor to lower folate levels. By itself, this polymorphism does not seem to be linked to HHcy in either the heterozygous or homozygous state compared to controls, although combined heterozygosity for both C677T and A1298C mutations, which produces a 677CT / 1298AC genotype, may result in significant plasma tHcy concentrations[176]. Some studies[176, 183] have shown that the two polymorphisms very rarely exist on the same allele, which renders it difficult to distinguish possible effects of the 1298C allele from those of the 677T allele. The combined MTHFR 677 TT and 1298 CC genotypes are extremely uncommon in the general population. These findings suggest a founder effect in which each alteration evolved on a separate wild-type allele[143, 146].

Overall, the population frequency of the A1298C allele is less documented than that of the C677T allele[20].

1.3 Homocysteine

1.3.1 Homocysteine: Historical Background

In historical context, BUTZ together with DU VIGNEAUD were the first to isolate and describe racemic homocysteine (Hcy) in 1932[110]. They demonstrated that demethylation of methionine *in vitro* led to the formation of Hcy and hypothesised on the possibility that demethylation might similarly occur *in vivo* and might participate in the catabolism of methionine to Hcy. They predicted that if this were true, Hcy, like methionine, would be capable of substituting for cysteine in the diet. Later on in 1962, CARSON and NEILL suggested an association between elevated Hcy levels and diseases[110]; in 1964, MUDD *et al.* identified a genetic defect of cystathionine-β-synthase (CBS) that causes severe elevation in plasma total homocysteine (tHcy), mental retardation, and early death because of atherosclerosis or thrombosis[110].

1.3.2 The Metabolic Pathways of Homocysteine

Hcy is an endogenous, non-protein-forming, sulphur-containing amino acid not found in foods. It is formed as an intermediate product during methionine catabolism via the S-adenosyl-methionine (SAM) and S-adenosyl-homocysteine (SAH)[161]. In plasma, Hcy exists in free (uncombined) reduced or bound oxidised forms: 70 - 80 % of the amino acid in plasma are bound to proteins, mainly albumin; about one third is bound via a disulphide bridge to either a cysteine or to Hcy (forming a homocystine). Less than 1 % of Hcy in plasma is found in reduced form, *i.e.*, as free Hcy.

Hcy can be catabolised via three pathways:

- First, Hcy can be remethylated into methionine, the precursor of SAM. This reaction is mediated via methionine synthase and requires methyl cobalamin as a cofactor and a methyl group supplied and a methyl group supplied by 5-methyltetrahydrofolate.
- Second, Hcy can be transsulphurated into cystathionine, then to cysteine. The last reaction is enhanced by cystathionine beta synthase and cystathioninase (*i.e.*, cystathionine beta lyase), both are vitamin B_6 dependent. Because cysteine is a precursor of glutathionine and taurine via the transsulphuration pathway, Hcy transsulphuration is thought to participate in the antioxidant capacity.

- Third, Hcy can be converted into SAH via a reversible reaction mediated by SAH-hydrolase. If the concentration of Hcy is elevated, the eventual pathway further progresses towards the production of SAH.

Figure 3 at the end of this chapter provides a brief overview of the Hcy metabolism.

Methionine is an essential amino acid and a component of many proteins in the human diet. The transsulphuration pathway is activated after the meal and is considered to be the main pathway for removing the amount of methionine ingested with the diet. On the other hand, the remethylation of Hcy into methionine dominates under fasting conditions.

The remethylation and the transsulphuration pathways are coordinated by SAM and oxidative stress. SAM acts as an allosteric inhibitor of MTHFR[154, 183]. By inhibiting MTHFR, SAM can reduce the influx of additional 5-methyltetrahydrofolate into the Hcy remethylation. SAM enhances the methylation of Hcy via betaine-Hcy-methyltransferase (BHMT). SAM has also been found to activate CBS, thus promoting the catabolism of Hcy via the transsulphuration pathway. The remethylation of Hcy to methionine via methionine synthase requires a reductive activation of the enzyme. The enzyme methionine synthase reductase is involved in the reductive activation of methionine synthase, whereas the transsulphuration pathway is activated under oxidative stress probably to provide additional amounts of cysteine.

Hcy is highly toxic. Remethylation and transsulphuration are unable to keep the intracellular Hcy concentration within a non-toxic range. Therefore, the amino acid is partially exported in stationary condition out of the cell and excreted via the kidney.

1.3.2.1 Homocysteine-Remethylation Pathway

The effective remethylation of Hcy to form methionine utilises 5-methylenetetrahydrofolate (MTHF) as a methyl donor and methylcobalamin as a cofactor. Betaine is an important alternative methyl group donor for Hcy remethylation to methionine; the reaction is mediated by BHMT. This pathway is widely expressed in the liver and the kidney and is possibly also present in the small intestine and pancreas tissue[41], but it is absent in heart and brain. BHMT is a SAM dependent enzyme, as SAM enhances the methylation of Hcy via BHMT.

1.3.2.2 The Transsulphuration Pathway of Homocysteine

In the transsulphuration pathway, Hcy can be catabolised first via CBS to cystathionine and then via cystathionase to cysteine. In the next phase, excess cysteine is oxidised to

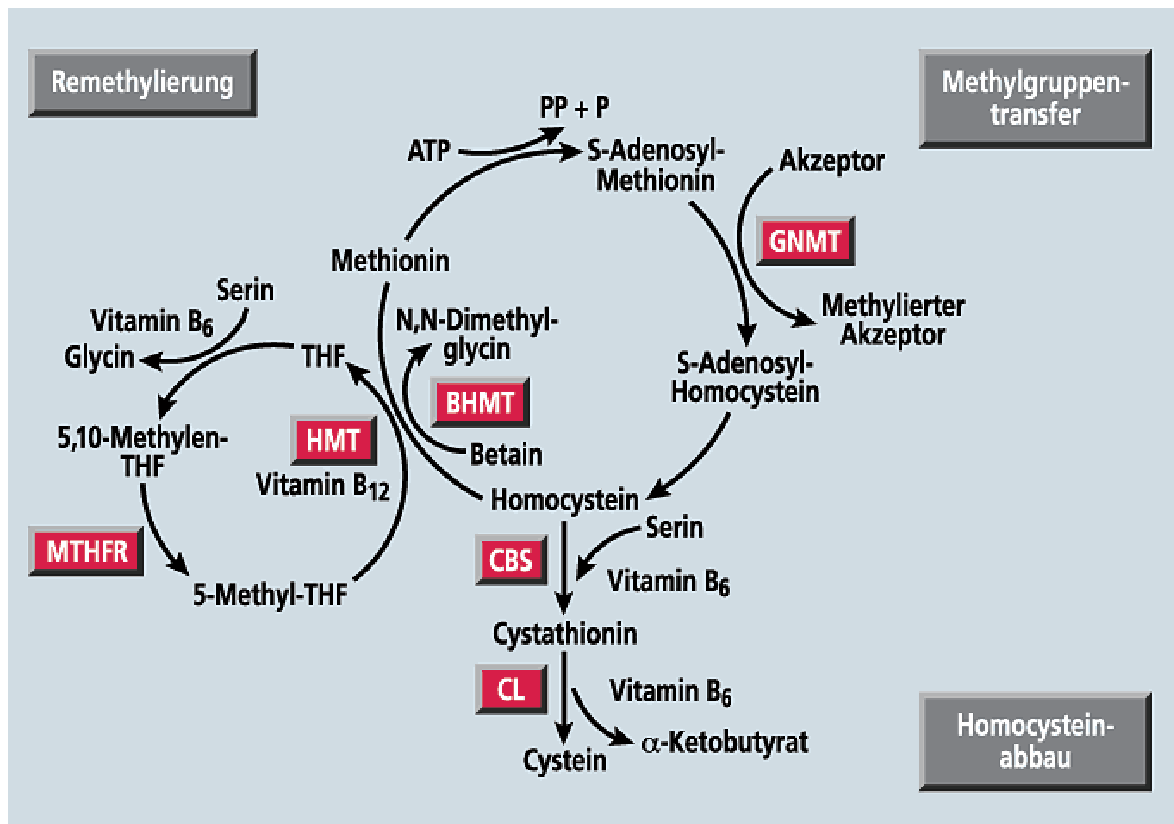
taurine, glutathione, or inorganic sulphate, or is excreted in the urine. This pathway is a major source of the important endogenous antioxidants glutathione and taurine.

Both CBS and cystathionase are vitamin B_6 dependent, thus, vitamin B_6 might have an indirect antioxidant function through its role in the transsulphuration pathway. CBS contains heme as a prothetic group necessary to bind pyridoxal-6-phosphate, the derivative active form of vitamin B_6[112, 113]. The transsul-phuration pathway is interestingly regulated by SAM which activates CBS and enhances the formation of glutathione. *In toto*, the transsulphuration pathway catabolises excess Hcy, which is not required for methyl transfer[22, 154].

1.3.2.3 The Transmethylation Pathway of Methionine

The transmethylation pathway of methionine is the only metabolic pathway known to produce Hcy from methionine. In this pathway, the enzyme methionine adenosyltransferase activates methionine by the transfer of an adenosyl group from adenosine-3'-phosphate (ATP) to the sulphur atom of methionine which as a result forms SAM. This reaction requires magnesium as a cofactor[113]. SAM is the primary methyl group donor in cellular metabolism to many acceptors, including DNA, myelin, membrane phospholipids, neurotransmitters, and nucleic acids[30, 161]. When methyl groups are transferred from SAM, SAH is formed as by-product of these methylation reactions. SAH is then hydrolysed by SAH-hydrolase to release adenosine and Hcy which subsequently becomes available either for the transsulphuration or for the remethylation pathways. The hydrolysis of SAH to Hcy is a reversible reaction and its equilibrium favours the synthesis of SAH when Hcy is elevated[154, 174]. SAH is a strong allosteric inhibitor of methyltransferases. Thus, Hcy and adenosine need to be metabolised rapidly in order to maintain low SAH levels; HHcy causes a secondary elevation of SAH that might lead to hypomethylation.

Figure 3: Homocysteine metabolism



Enzymes *(red)*: BHMT (betaine homocysteine methyltransferase); CBS (cystathionine-β-synthase); CL (cystathionine lyase); GNMT (glycine-N-methyltransferase); HMT (homocysteine methyltransferase); MTHFR (methylenetetrahydrofolate reductase)

Metabolites: ATP (adenosine-3'-phosphate); P (phosphate); PP (pyrophosphate); THF (tetrahydrofolate)

[*Translation: German - English*] Remethylierung - *remethylation*; Methylgruppentransfer – *methyl group transfer*; Homocysteinabbau – *homocysteine catabolism*

Source: HERTFELDER *et al.*[64]

1.3.3 Determining Factors of Homocysteine Plasma Blood Concentrations

There are a number of factors that influence plasma tHcy concentrations in humans. The main determinants include availability of folate, also - to a lesser extent - of vitamin B_6 and B_{12} status. The physiological, pathological, and genetic determinants are closely interrelated[181]. Higher Hcy levels are also reported in renal insufficiency, MTHFR 677TT individuals, smoking, ageing and gender. Supplementation with folic acid can reduce tHcy levels by up to approximately 25 %, and supplementation with vitamin B_{12} can provide a further 7 % reduction[67].

To summarise, the determining factors and conditions of tHcy concentrations in humans can be subdivided into:

physiological[154]	male gender [men have higher Hcy levels as compared to women of the same age]; increasing age [Hcy concentrations increase with age]; pregnancy; post-menopausal women
lifestyle[62, 119]	high coffee consumption; smoking; chronic high alcohol consumption; low physical activity; vegetarianism
genetic	polymorphisms in enzymes participating in Hcy catabolism (homo- / heterozygosity for CBS mutation; MTHFR defect [activity < 20 %]; thermolabile MTHFR [activity < 50 %]; methionine synthase; methionine synthase reductase; methyl cobalamin defective synthesis)
clinical[94]	renal insufficiency [is associated with increased Hcy concentrations]; folate deficiency; cobalamin deficiency; vitamin B_6 deficiency; thyroid function disturbances
medication prescriptions[161] *and various diseases*[140]	lipid lowering drugs; folate antagonists [methotrexate increases Hcy concentration]; nitrous oxide; hormons; antiepileptic drugs (phenytoin, carbamazepine), oral contraceptives; penicillamine [decreases Hcy concentration]
ethnic origin[168]	

1.3.4 Effects of Increased Concentrations of Plasma Total Homocysteine

1.3.4.1 Homocysteine during Pregnancy

Plasma tHcy is referred to as the sum of all Hcy forms in plasma / serum which generate this amino acid by reduction, including free and protein-bound forms[121]. Hcy plays an important role in pregnancy and has been connected with adverse pregnancy complications and poor outcome. The plasma tHcy concentrations decrease during pregnancy, probably due to changes in the renal handling of homocysteine or due to the hormonal changes associated with pregnancy[121]. Elevated maternal tHcy concentrations are known to be associated with pre-eclampsia, prematurity and LBW. HONGSPRABHAS and colleagues[68] examined tHcy levels in preterm and term infants and demonstrated lower levels in the preterm group. According to the findings by MURPHY *et al.*[115], mothers in the highest tHcy tertile at 8 weeks of pregnancy were three times (odds ratio [OR]: 3.26; 95 % confidence intervall [CI]: 1.05, 10.13) and at labour nearly four times (OR: 3.65; 95 % CI: 1.15, 11.56) more likely to give birth to a neonate in the lowest birth weight tertile.

1.3.4.2 Association of Homocysteine with Various Diseases in the General Population

In the meantime, numerous studies, both epidemiological and experimental, have concentrated on the role of Hcy and its association with various diseases: neuropsychiatric disorders such as Alzheimer's disease[155], immune activation[152], and, in particular, HHcy, which is regarded as a risk factor for atherosclerosis and thrombosis and, therefore, a factor in coronary heart disease, also as an established risk factor for cardiovascular disease [182].The exact mechanism for the atherogenic and thrombophilic tendencies of Hcy have not been fully elucidated. However, despite of higher Hcy concentrations observed in TT homozygotes, it still remains unclear whether the TT genotype is associated with increased risk for CVD. BRATTSTRÖM and co-workers[21] reported in a meta-analysis that the C677T polymorphism was not associated with increased CVD risk and the authors interpreted the results as lack of evidence for the association between HHcy and CVD. In a review of previously published meta-analyses on the association between MTHFR polymorphism and CVD, UELAND *et al.*[170] found none of the putative associations had sufficient statistical power to be conclusive.

The term HHcy denotes the presence of abnormal elevation in fasting tHcy levels. The normal range for tHcy concentrations has not clearly been defined. As a rule, normal tHcy is found in concentrations between 2 - 12 µmol / L in human plasma under physiological conditions. The *D.A.C.H.-League* [scientific society in Germany, Austria, and Switzerland conducting research on Hcy] considers a fasting tHcy concentration < 10 µmol / L as safe and a level between 10 – 12 µmol / L as tolerable and as threshold value for Hcy-lowering treatment in their consensus recommendations[129, 161]. In the literature, HHcy is in most cases defined in terms of values > 15 µmol / L. In the eyes of the *D.A.C.H.-Liga* guideline experts, epidemiologic studies clearly demonstrated a significantly increased risk for CVD already at levels of > 10 µmol / L. Differing cut-off values depend on the population group under review. Three types of HHcy are commonly identified in non-pregnant populations (*cf.* Table 3). Normal pregnancy is associated with lower tHcy levels compared to non-pregnant controls. Applying these values in the case of pregnant women would lead to misleading interpretation. So far, no cut-off value determining HHcy in pregnant women is available. In a recent study on poor nutritional status and hyperhomocysteinaemia in complicated pregnancy in Syria[75], a cut-off value of Hcy > 8.2 µmol / L, representing the 95th percentile of Hcy distribution in normotensive pregnant women with an adequate status of folate and vitamin B_{12}, was applied. There is some evidence that links increased tHcy plasma or serum concentrations, *i.e.*, HHcy (tHcy > 12 µmol / L) and vitamin B_{12} deficiency with vegetarianism[62].

Table 3: Classification of plasma homocysteine levels in non-pregnant populations by need to treat according to D.A.C.H.-League “Homocysteine”

Hyperhomocysteinaemia	Plasma total homocysteine concentration	a) Prevalence in general population b) Selected common causes c) Recommendation for treatment
safe status	< 10 µmol / L	a) --- b) --- c) no need to treat (target level of Hcy lowering intervention)
tolerable status	10 – 12 µmol / L	a) --- b) --- c) tolerable in healthy subjects; need to treat patients at increased risk
mild *or* moderate	12 – 30 µmol / L	a) 5.0 - 10.0 % b) unhealthy lifestyle; old age; mild folate or vitamin B_{12} deficiency; vegetarian diet; MTHFR 677 C → T mutation; renal insufficiency c) intervention required for all (apparently healthy individuals and patients)
intermediate	30 – 100 µmol / L	a) ≈ 1.0 % b) renal failure; severe vitamin B_{12} or folic acid deficiency; MTHFR mutations
severe	> 100 µmol / L	a) 0.02 % b) CBS mutation; homocystinuria; severe vitamin B_{12} deficiency; severe congenital disorders

1.4 Folic Acid

1.4.1 Folic Acid: Historical Background

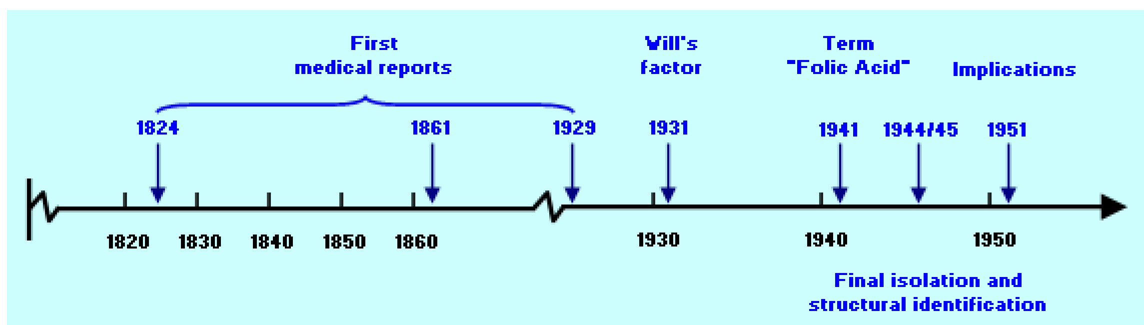
First reports on folate deficiency go back to CHANNING (1824), BARCLAY (1861), OSLER (1919), and MINOT and MURPHY (1926). The first attempts to come to terms with *folic acid* were based on the key findings by researcher WILLS in India in 1931[186] when she observed the curative effects of liver and of yeast and yeast extracts on tropical macrocytic anaemia in pregnant Hindu women in Bombay.

A first structural identification was accomplished by ANGIER *et al.* in 1945 when they reported the synthesis of a compound identical to the liver *Lactobacillus casei* factor. The publication of the complete skeletal structure of folic acid (final and successful isolation, decoding, proof by means of degradative reaction and synthesis) followed a year later again by ANGIER *et al.*[7]. The pteridine derivative which they discovered was named pteroylglutamic acid.

This compound was identical with DAY’s factor *vitamin M* (1938), HOGAN’s / PARROTT’s *vitamin* B_C (1940), SNELL’s / PETERSON’s *norite-eluate factor* (1940), MITCHELL’s *folic acid* (1941; introduction of the term *folic acid*), STOKSTAD’s / MANNING’s *factor U* (1943), and

PFIFFNER's *vitamin B_c* (1943; crystallisation of the acid). Most of the functions of folic acid involving the transfer of one-carbon units in the organism could be decoded until 1951. Folate-mediated one-carbon metabolism relates to a metabolic system comprising several interdependent metabolic pathways that use the cofactor tetrahydrofolate to chemically activate single carbons (referred to as one-carbon units) for cellular biosynthetic reactions.

Figure 4: Important developmental stages in folic acid research

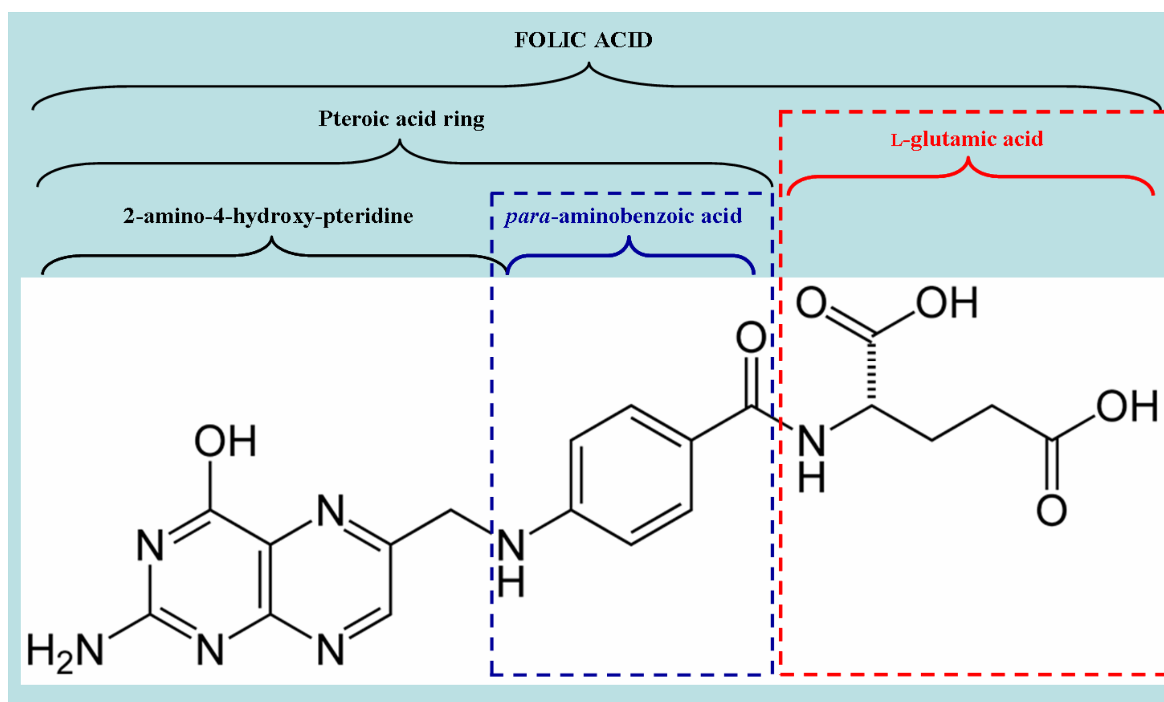


Source: HOFFBRAND[65], KOEBNICK[85] (modified)

1.4.2 Chemistry and Occurrence of Folic Acid

Folic acid (synonyms: folacin, vitamin B_C, vitamin B_9, *Lactobacillus casei* factor; molecular formula $C_{19}H_{19}N_7O_6$) is composed of three major subunits: a pteridine ring system, *p*-aminobenzoic acid and one molecule of glutamic acid (chemical name: pteroylglutamic acid). Naturally occurring folates are pteroylpolyglutamic acids with two to eight glutamic acid groups, *i.e.*, they are a variable mixture of different forms of mono- and polyglutamates, although polyglutamates are generally dominant (*cf.* Figure 5). Compounds which, such as folic acid, comprise the basic framework of the pteridines are widespread in nature.

Figure 5: Structure of folic acid



Folate is used as generic term for a water-soluble group of B vitamins including folic acid as well as folates that are prevalent in nature. Pteroylmonoglutamic acid (= folic acid) does not occur naturally in the human body; as the body is unable to synthesise folate *de novo*, it has to be obtained from the diet. It is a pure, synthetic folate compound commonly used in vitamin supplements and fortified food because of its increased stability and bioavailability. It easily disintegrates in acid and alkaline mediums as well as through action of light. The substance is scentless and tasteless[195].

Folates are found in every vegetable food (in high amounts in plant foliage) and in a great deal in food of animal origin. The largest concentrations occur in pig and cattle livers as storage organ of folate as well as in yeast. In particular, dark green leafy vegetables such as spinach and salad are rich in folate; furthermore, asparagus, tomatoes, mushrooms, potatoes, and some cabbage types (white cabbage) as well as some fruit variants (oranges) contain folic acid. Whole grains or cereals (wheatgearm and wheat bran), pulses (red lentils or *dhal*), beetroot, and cow peas contain folate in substantial quantities, too. The contents of folate in other meat as well as in fish and many fruit variants are relatively low[195]. The bioavailability of dietary folate is about half that of (unconjugated) folic acid.

The average food intake of folate amounts up to 0.26 mg / d. The human liver stores represent about half of the total body folate (5 – 10 mg) (*Expert Group on Vitamins and*

Minerals). In the United States, the major sources of dietary folate include cooked dry beans, leafy green vegetables, and fortified cereals[163]. Humans are entirely dependent on dietary sources or dietary supplements for their folate supply. Regarding today's usual eating habits worldwide, fruits and vegetables are consumed in far too small quantities. The estimated intake of folate is insufficient. A significant proportion of women of reproductive age have low dietary folate intake and do not use folic acid containing supplements or eat fortified cereals[151].

As folic acid is a highly sensitive vitamin, the folate content of nutriments also depends on how the food is prepared. Many folates are sensitive to heat, oxygen, light and extreme pH-values. Folic acid is highly susceptible to oxidative destruction during cooking; by prolonged cooking of vegetables, the losses of folate content can amount up to 50 - 90 %. Too intensive watering, too long storage and cooking times should, therefore, be avoided.

The reliability of folate contents of nutriments as listed in current databases is at present highly controversial. Problems with older methods of determination lead to folate contents being grossly miscalculated. More recent methods of analysis partially yielded up to double the previous results. The international introduction of the new term *folate equivalents* took the varying resorption into account. Since 2000, the *Deutsche Gesellschaft für Ernährung* ["German Nutrition Society"] applies a new definition after the introduction of the term *dietary folate equivalents* on the basis of new reference ranges[161]:

1 µg dietary folate equivalent = 1 µg food folate = 0.5 µg synthetic folic acid

(*pteroyl-monoglutamate*).

1.4.3 The Importance of Folate Status and Folic Acid Supplementation during Pregnancy

One of the first researchers to advance the importance of folate during pregnancy was HIBBARD[22], who suggested that the detection of megaloblastic anaemia in mid or late pregnancy implied an antedecent defect in folate metabolism. He hypothesised the existence of absolute and relative folate deficiencies during pregnancies.

Folate is a methyl donor required for Hcy remethylation to methionine and the most important determinant of tHcy plasma concentrations. Folic acid as a micronutrient of clear significance during pregnancy has been reported to be deficient in diets particularly of low-income groups. Increased demand for folate during pregnancy resulting in low folate status is well established and is a contributing factor to maternal anaemia.

A reference range for plasma folate has not been clearly determined. Cut-off points to indicate or separate subnormal from normal levels vary in the literature. For instance, 5 nmol / L has been used as a classification for those at risk for folate deficiency; a borderline level of 5 – 7 nmol / L has been reported in a study on an elderly population[29]. A serum folate level of < 6.8 nmol / L (< 3 ng / ml) is suggestive of a low folate status[85]. Particularly in the presence of low folate, homozygous MTHFR 677T is associated with raised plasma tHcy concentrations. Folate deficiency is implicated in the evaluated aetiology of nutritional anaemia, low folate status is associated with adverse pregnancy outcomes for the foetus, such as NTDs[22], other birth defects[164], congenital heart defects[173], LBW[98], and preterm delivery[158]. Low folate status may also lead to HHcy, which has been reported as independent risk factor for CVD[161]. Periconceptional and antenatal folic acid supplementation has been shown to improve micronutritient status during pregnancy, but it is not effective in correcting the pre-existing deficits[164]. Moreover, as folate status before conception determines folate status during pregnancy, it should be essential to maintain adequate levels of nutritional status in adolescent girls and women before pregnancy in order to meet the nutritional and physiological stresses of pregnancy and lactation. A database on micronutrient status in a particular population should be critical to the formation of national strategies to correct micronutrient deficiencies in developing countries such as Shri Lanka, as a seletive and tentative search of *MEDLINE* for micronutrients of interest in developing countries indicated.

A compromised maternal nutritional status is a major determinant of IUGR in developing countries. Women in the lowest quartile of both pre-pregnancy weight and weight gain during pregnancy are at highest risk of delivering an IUGR infant. Recent observations indicate that folate concentrations are 10 % lower in Indian Asians than in European Caucasians[27]. A prospective study conducted in rural West India showed a strong relationship between the maternal intake of green leafy vegetables and fruits in the second trimester and birth weight[136]. This relationship possibly was a consequence of folate intake, as a low maternal erythrocyte folate status was shown, which was also independently associated with LBW. The effect of micronutrients in significantly decreasing the risk of LBW has also been recently demonstrated in trials of multiple micronutrient supplementation in pregnant mothers in Nepal[123]. The weight gain during pregnancy could be increased and the risk for deficiency in foetal development could significantly be decreased when malnourished mothers in Gambia, West Africa, received nutrient supplements[25]. Another study[133] showed that an antenatal energy supplementation of 430 kilocalories per day consisting of ground-nut-based biscuits and a vitamin-fortified tea drink could influence the foetal weight development, although there was no effect on pregnancy weight gain.

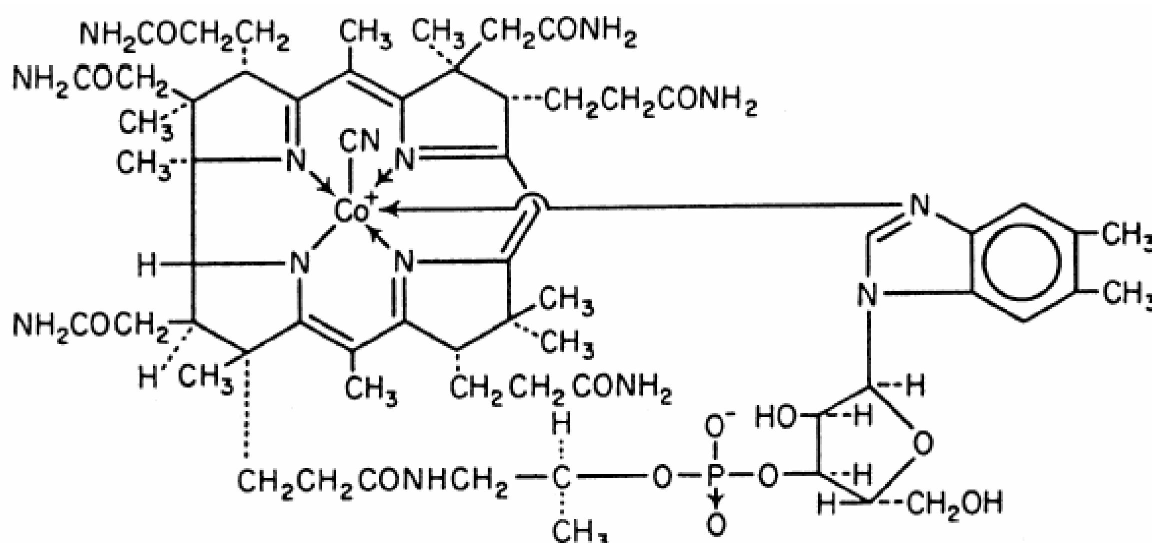
1.5 Vitamin B_{12}

Vitamin B_{12} (synonyms: cobalamin, antipernicious-anaemia factor, Castle's extrinsic factor, or animal protein factor) is the largest and most complex chemical structure of all vitamins. Interestingly, it is the only known biological compound to contain cobalt, the central metal ion, which gives this water-soluble vitamin its red colour. The name vitamin B_{12} is generic for a specific group of cobalt-containing corrinoids with biological activity in humans. This group of corrinoids is also known as cobalamins. The main cobalamins in humans and animals are hydroxocobalamin, adenosylcobalamin and methylcobalamin, the last two being the active coenzyme forms. Vitamin B_{12} was described for the first time in 1926 by Minot and Murphy, it was isolated from liver extract in 1948, its structure was elucidated in 1956 by Hodgkin *et. al.*, the total chemical synthesis of vitamin B1$_2$ was achieved by Woodward *et al.* in 1973.

Cyanocobalamin is an industrial form of vitamin B_{12} that is widely used clinically due to its stability, light, and heat resistance. It is transformed into active factors in the body. Acoording to the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (1974), the term vitamin B_{12} solely signifies cyanocobalamin. In the medical and pharmacologic literature, it is general practice to subsume all cobalamins which exert a biological effect in humans under the term vitamin B_{12}.

The chemical structure of vitamin B_{12} is based on a tetrapyrrol corrin ring, which has two of the pyrrole rings directly bonded (*cf.* Figure 6). Four of the six coordinations are provided by the corrin ring nitrogens, and a fifth by a dimethylbenzimidazole group. The sixth coordination partner varies, being a cyano group (industrial form: cyanocobalamin or CN), a hydroxyl group (OH), a methyl group (CH_3), or a 5'-deoxyadenosyl group.

Figure 6: Molecular formula of cyanocobalamin



There is evidence that vitamin B_{12} is required in the synthesis of folate polyglutamates (active coenzymes required in the formation of nerve tissue) and in the regeneration of folic acid during red blood cell formation. Because methylcobalamin is a cofactor for Hcy methylation to methionine, vitamin B_{12} treatment can lower elevated tHcy in plasma. However, folate is known to be more effective in lowering tHcy than vitamin B_{12}. Moreover, vitamin B_{12} deficient humans cannot utilise folate, therefore, they express secondary folate deficiency despite normal serum levels of folate (*cf.* also Chapter 1.5.3).

1.5.1 Absorption of Vitamin B_{12}

Vitamin B_{12} has multiple binding proteins that facilitate its absorption and transport. Vitamin B_{12} plays an important role in DNA synthesis and neurologic development. In the first enzymatic reaction in a human, methylmalonic acid is converted into succinyl-coenzyme A using vitamin B_{12} as a cofactor. In the second reaction, Hcy is converted to methionine by using vitamin B_{12} and folic acid as cofactors. Vitamin B_{12} deficiency can lead to increased levels of serum methylmalonic acid in the first reaction. In the second reaction, a deficiency of vitamin B_{12} or folic acid may lead to increased plasma tHcy. The acidic environment of the stomach facilitates the breakdown of vitamin B_{12} that is bound to food. Intrinsic factor, a glycoprotein which is secreted by the parietal cells in the stomach, binds to vitamin B_{12} in the duodenum. This vitamin B_{12}–intrinsic factor complex subsequently aids in the absorption of vitamin B_{12} in the terminal ileum. Approximately one percent of a large oral dose of vitamin B_{12} is absorbed by this second mechanism. This pathway is important in relation to oral replacement. Vitamin B_{12} binds to transcobalamin II and is transported to the blood and from there throughout the body to receptors on cell membranes. Transcobalamin II is the biologically active form of the vitamin. The interruption of one or any combination of these steps places a person at risk of developing deficiency.

1.5.2 Diagnosis of Vitamin B_{12} Deficiency

In recent years, there has been much interest in improving the specificity and sensitivity of the diagnosis of cobalamin deficiency. Vitamin B_{12} deficiency is more widespread in the population than has been assumed so far[63]. Cobalamin deficiency has often been mistaken for folate deficiency[61]. This complex inter-relationship between cobalamin and folate has been thoroughly researched[165] The diagnosis of vitamin B_{12} deficiency has traditionally been based on low serum vitamin B_{12} levels, usually less than 148 pmol / L (200 pg / ml), along with clinical evidence of disease. However, measurements of plasma concentrations of metabolites such as methylmalonic acid and Hcy have been shown to

be more sensitive in the diagnosis of vitamin B_{12} deficiency than measurement of serum vitamin B_{12} levels alone.

1.5.3 Aetiology of Vitamin B_{12} Deficiency

Causes of vitamin B_{12} deficiency can be divided into four main groups: nutritional deficiency, malabsorption syndromes, taking medication (especially antacids, which inhibit the production of gastric acid), and medication with the antidiabetic agent metformin, which ties up free calcium in intestines, also other gastrointestinal causes have to be screened. Normally, humans maintain a large vitamin B_{12} reserve, which can last two to five years even in the presence of malabsorption. Nutritional deficiency may result from inadequate intake. *e.g.* alcoholics. Food-bound B_{12} malabsorption can be assigned to the malabsorption syndromes: The phenomenon of food-bound malabsorption occurs when vitamin B_{12} bound to protein in foods cannot be cleaved and released. Any process that interferes with gastric acid production can lead to this impairment. The classic disorder of malabsorption is pernicious anaemia. Cystic fibrosis and short bowel syndrome have also to be mentioned in this context: pernicious anaemia is an autoimmune disease that affects the gastric parietal cells, destruction of these cells curtails the production of intrinsic factor and subsequently limits vitamin B_{12} absorption; the digestive transit of cobalamin in case of cystic fibrosis has been studied little until now. Other gastrointestinal manifestations may be: ileal malabsorption and biologic competition due to bacterial overgrowth or tapeworm infestation; Crohn’s disease; infection with Helicobater pylori; patients with stomach resection.

Vitamin B_{12} deficiency can lead to secondary folate deficiency that investigators have termed the “methyl trap”. Methionine synthase depends on vitamin B_{12} as a coenzyme and its activity is reduced during vitamin B_{12} deficiency. This results in the accumulation of 5-methylTHF, which gets “trapped” in this form and results in the decreased availability of THF and of all the other forms of folate. This can lead to megaloblastic anaemia due to insufficient folate coenzymes available for DNA synthesis[156].

The incidence of vitamin B_{12} deficiency appears to increase with age. Vitamin B_{12} deficiency is a common cause of macrocytic anaemia. Neurologic sequelae from vitamin B_{12} deficiency include paresthesias, peripheral neuropathy, and demyelination of the corticospinal tract and dorsal columns. It has also been linked to psychiatric disorders. In addition to these manifestations, it may exert indirect cardiovascular effects. This possibility becomes especially important when considering vitamin replacement therapy. Folic acid supplementation may mask an occult vitamin B_{12} deficiency and further exacerbate or initiate neurologic disease. Therefore, clinicians should consider ruling out

vitamin B_{12} deficiency before initiating folic acid therapy. Low vitamin B_{12} and folate concentrations are a risk factor for birth defects, poor pregnancy outcomes, and impaired neurocognitive performance.

The distinguishing feature of dietary vitamin B_{12} deficiency should briefly be mentioned in this context. Vitamin B_{12} in natural food is only provided in animal derived proteins; particularly good sources of the vitamin are liver, meat, free-range eggs, and and dairy products. Eating large amounts of raw liver, which contains high amounts of vitamin B_{12}, could save the life of previously incurable patients with pernicious anaemia. A vegetarian diet contains little vitamin B_{12}. Unfortunately, such a diet is also a poor source of methionine, thus, vegetarians may be at a particular high risk of developing conditions associated with reduced methylation activity. Low dietary intake or malabsorption of vitamin B_{12} may be the reason for the high risk for NTD in countries such as India and Mexico where the reported incidence in some regions is nearly ten times higher than that observed in the United States. Indians in India as well as those migrated abroad have high circulating tHcy concentrations compared to other ethnic groups. Low vitamin B_{12} concentrations in South Asian Indians are common, but the exact prevalence is not known. In a recent study of rural and urban Indian men living in and around Pune, Maharashtra, 67 % had a low vitamin B_{12} concentration (< 150 pmol / L) and 58 % had HHcy (> 15 µmol / L). Of the urban middle class, 81 % had a low vitamin B_{12} concentration and 79 % had HHcy. Urban middle-class residence was an additional independent risk factor of HHcy, compared to rural men[193]. In Caucasian populations not eating folic acid fortified food, HHcy is usually explained by low blood folate concentrations. In contrast, HHcy in Indians living in India is more attributable to low concentrations of vitamin B_{12}. HHcy has been implicated as an independent predictor of CVD. Notably, the harmful effect of vitamin B_{12} deficient mothers is expressed by low stores of vitamin B_{12}, and breast feeding may further aggravate the condition since maternal milk is low in vitamin B_{12}.

1.6 Objectives and Scope of the Study: Interactions between MTHFR, Homocysteine, Folate, Vitamin B_{12} and IUGR

Foetal growth varies between different populations due to ethnic and environmental factors[91]. SGA, with IUGR being one of the causes, has been proven to be a risk factor for increased morbidity and mortality among preterm neonates. Polymorphisms of the MTHFR gene together with increased tHcy levels may be associated with SGA, too. In addition, genetic variations of different populations and ethnic groups as well as nutritive and dietary intake, especially with regard to folic acid, may influence the level of tHcy and may play a role in the clinical course of pregnancies and SGA.

In the last two decades, scientists have focused on MTHFR polymorphisms and their effects in South Asian countries such as India and Shri Lanka. Whereas a subset of these polymorphisms have received some attention, others have not. The study populations were adults in these cases. Also, we are unaware of any study that aims to assess the effects of MTHFR polymorphisms in neonates or in preterm neonates in Shri Lanka.

Thus, the present study aimed at investigating the role of impaired Hcy metabolism as a risk factor for impaired intrauterine growth leading to birth weight < P 10, *i.e.*, SGA status. We also aimed to investigate genetic, ethnic and nutritive effects on Hcy concentrations and their influence on the rate and clinical course of SGA preterm neonates. In order to avoid stressful events following birth, umbilical venous cord blood was obtained.

Our objectives were to provide answers and explanations to the following issues:

1. Do the frequencies of the two most common mutations in the MTHFR gene, C677T and A1298C and their homozygous variants, differ among Shri Lankan preterm neonates with and without SGA? And do these frequencies differ among the three main ethnic groups in the island?

2. Is there a relationship between SGA and polymorphisms, as recent data indicate that there may be a link between IUGR and inherited thrombophilias, although with incoherent results[71, 73]?

3. Is there an association between tHcy concentrations and MTHFR polymorphisms in the study population, as some studies have reported an increased tHcy level among subjects who were homozygous for MTHFR C677T, whereas no connection has been established between an increase of tHcy level and subjects homozygous for MTHFR A1298C?

4. Furthermore, can we find an association between increased tHcy levels and SGA in preterm neonates? Does the occurrence of HHcy differ with regard to GA, *i.e.*, is there a correlation between a short gestation period and elevated tHcy concentrations?

5. Can our analyses confirm an association between HHcy and PIH? Can we establish a relationship between PIH, increased tHcy levels and SGA?

6. Is there an interrelation between folate status and vitamin B_{12} concentrations in SGA and appropriate-for-gestational age (AGA) preterms? Do folate and vitamin B_{12} function as determinants of tHcy status in the study group?

2 Materials and Methods

2.1 Materials

The equipment, consumables, substances, software, and conversion formulas employed in this study are compiled in Tables 4 - 8.

Table 4: Laboratory technical equipment

Equipment	Details
ADVIA Centaur®	Bayer HealthCare LCC [formerly: Bayer Vital GmbH (Diagnostics)], Fernwald, Germany
AxSYM® System	Abbott Diagnostics Division, Wiesbaden, Germany
Biophotometer	Eppendorf AG, Hamburg, Germany
Centrifuges	*Zentrifuge 5804*: Eppendorf AG, Hamburg, Germany *EBA 12*: Hettich GmbH & Co. KG Zentrifugen, Tuttlingen, Germany *Microcentrifuge Model IR*: Carl Roth®, Taiwan [*used in CSHW, Colombo*]
Puncher	*One-Punch Model II*: IEM Screening Systems, Inc., North Hollywood, CA, USA
7500 Real-Time PCR System	Applied Biosystems, Darmstadt, Germany
Thermoblocks	*Techne DRI-BLOCK® DB-3*: Techne Cambridge Ltd., Duxford, Cambridge, UK *Thermomixer Comfort*: Eppendorf AG, Hamburg, Germany
Vortexer	*Vibrofix VF 1 Electronic*: IKA-Werk, Staufen im Breisgau, Germany

Table 5: Other supplies and expendable consumables

Equipment	Details
AVDIA Centaur® base curve card	Bayer HealthCare LCC [formerly: Bayer Diagnostics], Fernwald, Germany
ADVIA Centaur® test tube rack	Bayer HealthCare LCC [formerly: Bayer Diagnostics], Fernwald, Germany
Cannulas	*BD Microlance™ 3 (sterile), 20G 1½" - Nr. 1, 0.9 x 40 mm, REF 301300*: Becton Dickinson GmbH, Heidelberg, Germany *BD Microlance™ 3 (sterile), 18G 1½" - Nr. 1, 1.2 x 40 mm*: Becton Dickinson GmbH Med. Technik und Labordiagnostik, Heidelberg (Neckar), Germany
Cuvettes	Sarstedt AG & Co., Nümbrecht, Germany
Isotherm System Cold Pack	Eppendorf AG, Hamburg, Germany
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems, Darmstadt, Germany
Microcentrifuge tubes	Eppendorf AG, Hamburg, Germany
Multi-Adapter for S-Monovette®	(*for blood collecting with S-Monovette®*): Sarstedt AG, Nümbrecht, Germany
Optical Adhesive Cover	Applied Biosystems, Darmstadt, Germany
Pipettes	*Eppendorf Reference Pipettes*: Eppendorf AG, Hamburg, Germany
Pipette tips	Sarstedt AG, Nümbrecht, Germany
QIAmp DNA Mini Kit	QIAGEN GmbH, Hilden, Germany
Screening cards	(*filter paper provided by*): Neugeborenen-Screening, Screeningzentrum Heidelberg, Universitätskinderklinik, Heidelberg, Germany
S-Monovette® blood collection system EDTA-tubes	*K3E* (*2.7 ml, 66 x 11.5 mm, for haemological testing, contains 1.6 ml EDTA*): Sarstedt AG, Nümbrecht, Germany
S-Monovette® blood collection system for serum separation	*Serum Z 1.3* (*1.2 ml, 66 x 8 mm, contains additive carrier and clot activator*): Sarstedt AG, Nümbrecht, Germany
Syringes	*Ecoject®* (*2 ml*): Dispomed Witt, Gelnhausen, Germany *Ecoject®* (*5 ml*): Dispomed Witt, Gelnhausen, Germany

Table 6: Solutions, buffers, primers and reagents

Items	Details
ADVIA Centaur® ReadyPack® Primary Reagents with VB12 Lite Reagent and Solid Phase	Bayer HealthCare LCC [formerly: Bayer Diagnostics], Fernwald, Germany
ADVIA Centaur® T3/T4/VB12 Ancillary Reagent ReadyPack	Bayer HealthCare LCC [formerly: Bayer Diagnostics], Fernwald, Germany
ADVIA Centaur® VB12 Diluent	Bayer HealthCare LCC [formerly: Bayer Diagnostics], Fernwald, Germany
AE Elution Buffer	QIAGEN GmbH, Hilden, Germany
Aqua ad iniectabilia Braun (1,000 ml)	B. Braun Melsungen AG, Melsungen, Germany
AL Lysis Buffer	QIAGEN GmbH, Hilden, Germany
ATL Tissue Lysis Buffer	QIAGEN GmbH, Hilden, Germany
AW1 Wash Buffer	QIAGEN GmbH, Hilden, Germany
AW2 Wash Buffer	QIAGEN GmbH, Hilden, Germany
DNA-free water (*Molecular Biology Grade, 10 x 50 ml*)	Eppendorf AG, Hamburg, Germany
Ethanol (absolute)	Riedel-de-Haën, Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany
Primers (C_{677}T and A_{1298}C) (*TaqMan® Genotyping Assays*)	Applied Biosystems, Darmstadt, Germany
Protein Kinase-K Solution	QIAGEN GmbH, Hilden, Germany
QIAmp® DNA Mini Kit (250)	QIAGEN GmbH, Hilden, Germany
TaqMan® Reagents: *TaqMan® 2X Universal PCR Master Mix (for Assays on Demand™)*	Applied Biosystems, Darmstadt, Germany

Table 7: PC programs

Software	Details
EndNote X.02	Adept Scientific GmbH, Frankfurt am Main, Germany
MS Office® (Word 2003, Excel 2003, Powerpoint 2003)	Microsoft Corporation Deutschland, Unterschleißheim, Germany
Sequence Detection Software SDS V 1.3.1	Applied Biosystems, Darmstadt, Germany
Statistical Package for Social Science for Windows (SPSS), version 15.0	SPSS GmbH Software, Munich, Germany

Table 8: Conversion formulas used in this study

	Formula	Decimal place
Homocysteine	---	2
Folate	nmol / L = ng / ml x 2.265	1
Vitamin B_{12}	pmol / L = pg / ml x 0.7378	---
Oxygen tension	mmHg = kPa x 7.5	---

2.2 Methods

2.2.1 Literature Search and Selection Criteria

The literature obtained includes original articles in journals, reports, meta-analyses, and books. The searches were conducted as follows:

We searched, with no date restrictions, the *MEDLINE* database of the National Library of Medicine in the United States (www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=PubMed or www.pubmed.org) for journal articles using the keywords *Sri Lanka*, *birth weight*, *gestational age*, *low birth weight*, *prematurity*, *intrauterine growth retardation* or *restriction*, *folic acid*, *folate*, *homocysteine*, *vitamin* B_{12}, *MTHFR C677T*, *MTHFR A1298C*, their determinants or metabolisms, and *ethics* or *bioethics*. This was supplemented by a similar search using the *SCOPUS* database (www.scopus.com). All search results were limited to the English and German languages; one article in French was the single exception. In addition to these initial searches, the *KARLSRUHER VIRTUELLER KATALOG* (www.ubka.uni-karlsruhe.de/kvk.html), the subject catalogue of the Saarland University and Regional State Library - Medical Part - (*Web-OPAC*) and of the Institution Library of the Paediatric Department were looked through for monographs and / or (edited) books. A “snowball” procedure was thereafter applied, whereby references cited in each article (pinpointed by this search strategy) were selectively scrutinised for additional relevant references. These were also reviewed to reveal any further secondary literature when thought particularly useful. Acknowledged review articles, seminars or book chapters were included as well, because they provided comprehensive overviews that are beyond the scope of this thesis. Although preference was given to the most recent publications, we did not exclude commonly referenced or highly regarded older articles or books. The manuscript was completed in May 2009; a great deal of the original material had to be abridged because of its considerable size.

2.2.2 Spelling of Some Terms

We would like to point out some remarks on the spelling of a few terms used throughout in this study as the spelling is inconsistent internationally as well as in Shri Lanka.

Shri Lanka: The term *Shri Lanka* is used when we are referring to the island, according to the pronunciation in the country and in India. ["Webster's Third New International Dictionary of the English Language Unabridged" (1981, Vol. III, S - Z, p. 2217): *shri* and *sri* both are correct and they are in official usage].

Singhalese: We have adopted this spelling when we refer to the members of this particular ethnic group. We have found various forms of spelling anthologised: Singhalese, Singalese, Sinhalese, also Cingalese and Cinghalese ["Webster's", Vol. III, S - Z, pp. 2123, 2125; YULE A / BURNELL AC: "Hobson-Jobson. The Anglo-Indian Dictionary", reprint 1996, pp. 838-839. DANIEL JONES lists only Singhalese in his classic pronunciation guide: "English Pronunciation Dictionary", 15 1997, p. 453].

Sinhala: We utilise the term *Sinhala* in reference to the language of this ethnic group [*cf.* "The New Encyclopædia Britannica in 30 Volumes, Micropædia, Vol. IX", 15 1983, pp. 229-230; "Webster's", Vol. III, S - Z, p. 2125; SWARNA PRAGARATNE: "Sinhala Phrasebook", 2 2003].

Moors: In English usage, by extension, *Moors* occasionally denotes Muslims in general, as in the case of the Moors in Shri Lanka or of the Philippines ["Encyclopædia Britannica, Micropædia", Vol. VII, p. 9; "Hobson-Jobson", reprint 1996, pp. 581-583]. The term has been adopted into colloquial language in Shri Lanka and is applied in the reports of the *Department of Census and Statistics*, by state sector health institutions and other governmental institutions in the country; they are also found in the *Demographic and Health Survey*. We have opted to use this term in our study, K. KULARATNAM's, former Professor and Head, Department of Geography, University of Shri Lanka, preference for the term *Muslims* instead of *Moors* notwithstanding ["Encyclopædia Britannica, Macropædia", Vol. 17, p. 521].

2.2.3 Ethical Considerations, Study Design and Study Location

H. BEECHER, a member of the research community (as opposed to an outside observer), in his 1966 milestone report in *The New England Journal of Medicine*[14] detailed 22 publications with unethical background; all of these papers were conducted by highly regarded researchers and published in prestigious medical journals. In response to

reported abuses of human participants in medical research, several ethical codes have been produced; notably, the *Nuremberg Code* (1948; voluntary informed consent, favourable risk / benefit analysis, right to withdraw without repercussions) [www.hhs.gov/ohrp/references/nurcode.htm], the *American Psychological Association's Code of Ethics* (1953, 1992, 2003), the *Belmont Report* (1979; three fundamental principles for human subjects research: respect for persons, beneficience, and justice); also the *Declaration of Helsinki* (World Medical Association, original version: 1964, resuming the principles of the Nuremberg Code; 6th revision: 2008) [http://www.wma.net/e/policy/b3.htm], which made the written consent of subjects a central requirement of ethical research involving human subjects, its revision emphasising that research is justified only if the population to be studied stands to benefit.

In Shri Lanka, there are ethical review committees in medical schools, national organisations, in the Sri Lanka Medical Association, and in the National Institute of Health Science. The Faculty of Medicine started formal ethical review at the Colombo Medical School already in the 1970s using international guidelines; this review institution was officially established in 1981. All medical schools teach some medical ethics, interest in medical ethics in the past was more or less confined to universities. In recent years, much effort has been invested in setting up binding and tight ethical regulations in Shri Lanka.

Approval in Germany: The research protocol of this study received approval from the "Ethik-Kommission der Ärztekammer des Saarlandes" (*Commission on Ethical Research in Human Subjects of the Medical Association of The Saarland*), No. SN 68/05, dated 24.05.2005.

Approval in Shri Lanka: Ethical clearance for all study procedures was granted by the Ethical Review Committee of the Faculty of Medicine, University of Colombo, No. EC/04/094, dated 10.02.2005. In addition, the Shri Lankan studies were also permitted by the Department of Health Services (Ministry of Health Care, Nutrition and Uva Wellassa Development), No. DDG(MS)01/2004, dated 11.01.2005, and by the Director of the partner institution, Castle Street Hospital for Women [Teaching] (CSHW) in Colombo 8.

After explaining in detail the purpose and benefits of the study in accordance with the ethical standards laid down in the Declaration of Helsinki, written and informed voluntary parental consent to participation and venous cord blood collection was provided and signed at enrolment by the mothers of the infants involved. Information leaflets with consent forms in both Sinhala and Tamil languages were used; the original German version was also translated into English (*cf.* Appendix). Enrolment took place during a three-and-a-half month period commencing January 2006.

A face-to-face interview with each mothers of neonates enrolled for the study, either in Singhalese or in Tamil, was conducted at the University Hospital in Colombo by the author to avoid any inter-observer error, in almost all of the cases before delivery. Only in case of emergencies, the interview took place within two days after delivery. The interview was based on a standardised detailed questionnaire which is contained in the "Appendix". Full data *inter alia* comprised the measure of potential confounding factors such as: demographic and socioeconomic factors (nicotine abuse, alcohol consumption; ethnicity, education, employment), anthropometric measures before and during pregnancy, pregnancy diseases like PIH, maternal and family diseases, obstetric history, diet and use of (multi-)vitamin supplements. In addition, we recorded living conditions such as number of family members or type of house (house, cottage, bungalow, apartment flat); family income per month was not noted. PIH met the following criterion as decribed by DANFORTH[153] and was defined as the development of new arterial hypertension in a pregnant woman after 20 weeks of gestation and in millimeter of mercury column: systolic blood pressure > 140 mm Hg and / or diastolic blood pressure > 90 mm Hg with debute during pregnancy without proteinuria and with no symptons occurring, measured twice with more than 6 hours apart. Blood pressure was measured with a mercury sphygmomanometer with the mother seated with feet supported for 2 - 3 minutes before blood pressure measurement (or, by way of exception, in the left lateral position), the cuff of the appropriate size placed on the upper right arm. Clinical characteristics of the newborns such as weight, length, head circumference or APGAR were also documented. The interviews took 20 to 40 minutes, they lasted longer only in exceptional circumstances.

All clinical assessments in Colombo were made by one observer (T. G.). Cord blood samples were obtained in CSHW from the umbilical vein after delivery of the placenta. This method of collection conveys no risk of physical injury to the mother or the newborn infant. During sampling in Shri Lanka, every care was taken that standard obstetric procedures not be altered to facilitate cord blood collections according to the cogent recommendations of the "American Academy of Pediatrics" and the "American College of Obstetricians and Gynecologists". In Germany, several guidelines jointly regulate the sampling of cord blood on the basis of the federal "Gesetz zur Regelung des Transfusionswesens - Transfusionsgesetz", a violation will be prosecuted.

2.2.4 Inclusion and Exclusion Criteria, Study Population, Percentile Curves

Enrolment criteria for study entry *included*: Outcome of interest was preterm birth, defined as a delivery < 37 completed weeks of gestation, over the period set for recruitment. Multiple gestations (twins and triplets) were included. The mothers were eligible regardless of family history or past pregnancy outcomes. Both vaginal and caesarean births were included. Neonates with severe or lethal malformations and chromosomal aberrations were to be *excluded* to avoid confounding (*e.g.* autosomal chromosomal aberrations are accompanied by an unspecific IUGR as a result of a primary impairment in skeletal growth).

Previous parallel studies[90] indicated that a total of at least 50 preterm SGA and AGA neonates had to be included to work up the study design for assessing causal factors of IUGR in Shri Lankan preterm neonates. One hundred and ninety three pregnant women (non-related) were recruited who gave birth to 205 preterm neonates. There were no *intra partum* deaths or maternal deaths. The co-operation of the volunteering parents was good; none of the parents refused to participate in the study, and all mothers who agreed took part and completed the study (100 %).

Because of the absence of standard percentile curves for Shri Lankan newborns, we adopted percentiles derived from presumably comparable populations th stratify data. These were the percentile curves established for Kuwait centres[4]. Apart from humans of Arab origin, the study population in Kuwait included a large group combining Indians and Southeast Asians. For corroboration and with regard to the geographical proximity to Shri Lanka, we compared the Kuwait percentiles with the BW standards published for South Indian newborns (customised for gender, parity, and height of the mother)[107]. As there were no substantial deviations after comparison, we used the Kuwait curves for this thesis on account of the larger study population (Kuwait: n = 35,768 *vs*. South India: n = 11,641) on the assumption that these study groups were comparable to the Shri Lankan population in our study. According to the findings, Indian-Asians had the lowest BWs, the highest prevalence of SGA birth weights and lowest prevalence of large-for-gestational-age birth weights[4].

2.2.5 Collection of Venous Cord Blood and Other Clinical Assessments

Immediately after delivery of the infant, the cord was clamped at both ends and cut. The cord was wiped free of any maternal blood. After clamping, the samples were collected under aseptic conditions into Monovette® EDTA tubes of either 2.7 ml or 1.3 ml containing K-heparin as an anticoagulant and also in serum tubes (both Sarstedt, Germany). Furthermore, blood drops were pipetted onto screening test cards (Screeningzentrum Heidelberg, Germany) for genetic (polymorphism) detection and air-dried.

The infants were weighed to the nearest 10 grams on a standard electronic scale balance immediately after birth. In parenthesis, UNICEF and WHO have estimated that 58 % of all newborn infants in the developing world are not weighed[171]; and overall, for infants who were weighed at birth, the mothers did not know or did not remember the weight for about 10 %.

An estimation and accurate knowledge of GA and the expected date of delivery is of value in the management of pregnancy. It is central to the social and clinical essence of pregnancy as this has considerable medical implications associated with the induction of labour. Likewise, distinguishing infants who are growth-retarded from those who are premature depends on valid GA measurements. To calculate GA, Naegele's rule, based on menstrual dates, is usually applied. For this thesis, the clinical determination of GA (in weeks / days) was carried out applying Naegele's rule. It was calculated according to the reported first day of the last menstrual period and estimation was subsequently validated by ultrasonographic measurements starting from the first trimester and again closer to the time of the delivery. Discrepancies of more than one week were corrected according to the ultrasound clearing. This was then confirmed after birth by a physical examination of the newborn.

It is customary to divide duration of gestation or pregnancy into three periods each of three calendar periods, *i.e.* three trimesters. The accepted duration of the trimesters is obtained by dividing 42 weeks into three periods of 14 weeks each. The first trimester is through to the completion of 14 weeks, the second through to 28 weeks, and the third trimester includes the 29th through to the 42nd week of pregnancy. Each of these trimesters may cluster its own specific type of complications although the division in trimesters seems rather crude.

2.2.6 DNA Extraction

DNA was extracted from dried blood spots on capture cards in the laboratory of the Paediatric Department of the University Clinics of The Saarland, Homburg, Germany. In brief, the QIAamp® DNA extraction procedure was carried out as described in Table 9.

Table 9: Procedure of DNA extraction

Procedure	Details
1. Breaking up the blood cells	Six circular screening card disk punches (3 mm Ø) were given into 1.5 ml microcentrifuge tubes (Eppendorf, Germany), 180 µl of lysis buffer ATL were added, followed by pulse vortexing (IKA-Werk, Germany) for 15 seconds.
	Then the reaction was incubated in Thermomixer Comfort (Eppendorf, Germany) at 90°C for 15 minutes, thereafter it was briefly centrifuged at 10,000 rpm to remove drops from the inside of the lid.
	20 µl stock solution protein kinase K (QIAGEN, Germany) was added, the reaction was vortexed for a short period, incubated for 60 minutes in Techne DRI-BLOCK® DB-3 (Techne Cambridge Ltd., UK) at 56°C and, aga in, briefly centrifuged at 10,000 rpm, in that order.
	Thereafter, 200 µl of AL buffer was added, the reaction was vortexed for 15 seconds, incubated at 70°C for 15 minutes in Th ermomixer Comfort, and centrifuged for 1 minute at 10,000 rpm, again in that order.
	Then, 200 µl of ethanol (Riedel-de-Haën, Sigma-Aldrich Laborchemikalien, Germany) was added to the reaction, mixed by pulse vortexing for 15 seconds, and the tube was briefly centrifuged again at 10,000 rpm to remove drops from the inside of the lid.
2. Adsorption	This reaction mix was then applied to a QIAmp spin column (QIAGEN, Germany) that was placed in a 2 ml collection tube, and the column was centrifuged at 8,000 rpm for 1 minute.
	Next, the QIAmp spin column was replaced in a new collection tube. 500 µl of wash buffer AW1 (QIAGEN, Germany) was applied to the spin column. Centrifugation followed at 8,000 rpm for 3 minutes. The QIAmp spin column was again replaced in a new collection tube. Lastly, 500 µl of wash buffer AW2 (QIAGEN, Germany) was applied to the spin column. Centrifugation followed at 14,000 rpm for 3 minutes, followed by centrifugation for 1 minute at 4,000 rpm.
	After each centrifugation step, the flow-through was discarded.
3. Elution of the membrane-bound DNA	Finally, the spin column was placed in a 1.5 ml collection tube and 120 µl of elution buffer AE (QIAGEN, Germany) was added. After incubating the column for 5 minutes at room temperature, DNA was eluted from the column by centrifugation at 8,000 rpm for 1 minute. The eluted filtrate was then reapplied to the same column and the same collection tube, followed by incubation at room temperature for 1 minute and centrifugation at 8,000 rpm for 1 minute.
	The concentration and the purity of the DNA was ascertained by measurement of the optic density of an aliquot in a bio-photometer (Eppendorf, Germany) at 260 nanometer (nm) as well as at 280 nm.
	Afterwards, DNA samples were labelled and stored at -20°C until testing for further studies.

2.2.7 Determination of MTHFR-Genotypes

2.2.7.1 Method of the Polymerase Chain Reaction

DNA fragments are needed in large quantities for most gene technological analyses and experiments. For this purpose, the polymerase chain reaction method (PCR) is used for the assay of the gene segment to be analysed. The reaction principle of PCR parallels the natural DNA replication (duplication) in the cell: The decisive principle of PCR is this cyclic

repetition of the constituent reaction steps which ideally duplicates the quantity of the DNA copies in each run. In doing so, it is possible to obtain a large homogeneous DNA population out of a small starting batch by means of relatively few production cycles. The DNA can then be identified and processed.

During PCR, a specific DNA reproducing enzyme, a thermostable DNA polymerase or Taq polymerase, synthesises new DNA along an existing nucleic acid matrix under controlled laboratory conditions. For that purpose, the enzyme requires a suitable nucleic acid molecule (two oligonucleotides) in the DNA strand as primer. The primer hybridises with the matrix strand and is used by the PCR polymerase as starter molecule. Also are needed: desoxy-nucleotide triphosphates (dNTP) as well as a suitable buffer system.

The entire chain reaction is based upon three partial steps which take place at varying temperatures and which are repeated many times (*cf.* Table 10).

- As absolute prerequisite for a PCR, the DNA has to be available in its single strands. Therefore, during the first step, called *denaturation*, the double-strand DNA molecule is splitted up in its single strands by means of heating it up to a temperature of 92°C. This cleavage is enabled because the hydrogen bonds lyse between the complementary nucleotide pairs.
- During the second step, known as (primer-)*annealing*, the temperature is cut down to the so-called melting temperature of 60°C, at whic h the pre-selected oligonucleotide primers hybridise with the two seperate DNA matrix strands. They are short one-stranded DNA molecules that are complementary to the defined ends of a DNA sequence matrix. Between them, these starter molecules flank a designated genomic region to be amplified using thermostable Taq DNA polymerase enzyme in the presence of deoxynucleotides and a reaction buffer.
- During the third step, the *extension*, the Taq polymerase enzyme extends the primers along the single-stranded denatured DNA matrix in the presence of dNTP[39]. So the extension of the primers is catalysed by the Taq (DNA) polymerase, while doing so the nucleotides that are dissolved in a suitable buffer act as building blocks.

Thereby, the DNA polymerase, originating from the 3'-end of the primer, is now enabled to synthesise the complementary strand with free dNTP. The optimum temperature for this selected polymerase centered around 60°C.

The cycle of the three partial steps described is subsequently repeated many times. Thus, an exponential amplification is obtained by means of the duplication of the selected DNA fragment in each cycle.

Table 10: Real-time PCR reaction conditions in this study

Reaction step		Temperature	Duration
	UNG activation	50° C	120 sec
	Initial denaturation	95° C	10 min
40 Cycles	Denaturation	92° C	15 sec
	Annealing	60° C	60 sec
	Extension		
	Final extension	60° C	

2.2.7.2 *Gene and Primer Sequences*

The design of the primers is essential for the PCR. They act as starter molecules for the polymerase and they determine the sequence to be amplified through bonding at the DNA matrix. The primers are specific as to sequence and length so that they flank only the target DNA in the genome and induce its amplification. Tables 11 and 12 list the primers employed together with their corresponding sequences and the PCR product properties.

Table 11: Gene and primer sequences in this study

Polymorphism	Identifier	Primer	Specific primer sequences
C677T	rs1801130	[*)]	[*)] [1)]
A1298C	rs1801131	forward	5´-GGA GGA GCT GCT GAA GAT GTG-3´ [2)]
A1298C		reverse	5´-TGG TTC TCC CGA GAG GTA AAG A-3´ [2)]

[*)] Assays were directly ordered from Applied Biosystems and came premixed in a 40 X concentration, gene sequence owned by Applied Biosystems, no sequence information available; Assay-ID: C__1202883_20

[1)] *cf.* Xu W-H, Shrubsole MJ, Xiang Y-B *et al.* (2007)[191]

[2)] *cf.* Rosen MP, Shen S, McCulloch CE *et al.* (2007)[145]

Table 12: PCR amplification product properties and quantities used for polymorphism discrimination

	Ingredients	Polymorphism	
		C677T	A1298C
Gene Expression Assay: Mastermix	*Reaction buffer:* TaqMan® Universal PCR Master Mix, Applied Biosystems, (5 ml vial; P/N 4304437), optimised for TaqMan® reactions and supplied at a 2 X concentration. The mix contains: AmpliTaq Gold® DNA Polymerase (*enzymes*), AmpErase® UNG, dNTP with dUTP, Passive Reference 1 (*internal reference dye*), and optimised reaction buffer components.	10 µl	10 µl
Dye labeled oligonucle-otide assay mix (*Primers and probes*)	AmpliTaq® MGB probes, 250 x 20 µl reactions, two primers and 20 X mix (MGB probes labelled with 6-FAM or VIC, Applied Biosystems [P/N 4316033]).	1 µl	0.50 µl
DNA free water	Aqua ad iniectabilia Braun	50 µl	5.5 µl

2.2.7.3 TaqMan® Assay

Our experiments and the reaction were performed on PCR System 7500 Real-Time (Applied Biosystems, Germany). The Sequence Detection software is used for instrument control, data acquisition in all 96 wells, on-line monitoring, auto-save function after run is completed and data analysis (analysis parameters can be included), set-up of four standard curves per run and relative quantitation analysis. Other features of the software are adjustable graphics for well position and standard curve plots as well as the ability to collect data from any step in the PCR cycle; manual calls can be persisted in allelic discrimination assays.

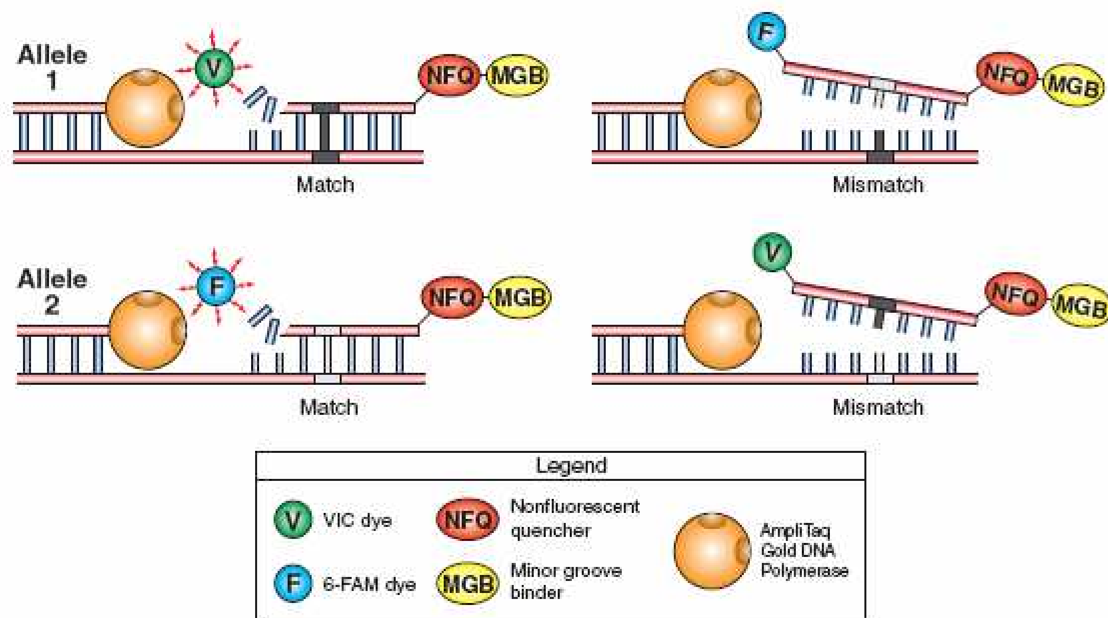
The TaqMan® method is based upon a combination of PCR and 5´-nuclease assay[102]. For the reaction, two allele specific DNA probes as well as a pair of primers are needed. The fluorogenic probes are complementary oligonucleotides to the DNA fragment around the polymorph position and their use in the 5´-nuclease assay combines PCR amplification and detection into a single step.

They are marked with two different dyes: *high energy* fluorescent reporter dye at the 5´-end and *low energy* nonfluorescent “dark quencher” at the 3´-end. Two different reporter dyes mark the two allele specific probes. The proximity of the “dark quenchers” to the reporter dye reduces the fluorescence of the latter if the probe remains intact. The Taq polymerase shows a 5´ → 3´-exonuclease activity that cuts a complementary probe which was hybridised along a DNA strand during PCR. The cutting of the fluorogenous probe leads to the spatial separation of the two dyes and as a result to an increase of the fluorescence intensity of the reporter dye. The probe fragments are now withdrawn from

the target segment and the PCR is taken up again. The genotype determination is carried out by means of an analysis of the allele specific fluorescence signals[111].

Real-time PCR allelic discrimination assays were conducted using the Assay-by-Design^{SM} service offered by Applied Biosystems, Germany. Figure 7 illustrates results from matches and mismatches between target and probe sequences.

Figure 7: Results from matches and mismatches between target and probe sequences



Source: APPLIED BIOSYSTEMS[8]

The TaqMan® method was used to identify both the carriers of homozygous DNA (CC_{677}, TT_{677}, AA_{1298}, CC_{1298}) and the carriers of heterozygous DNA (CT_{677}, AC_{1298}). The fluorescent reporter dyes, 6-carboxy-fluorescein (FAM™) or VIC® (dye owned by Applied Biosystems; chemical structure not published) were bound to the 5´-end of the probe molecules in order to ensure a differentiation of the alleles. Molecule structures were docked to the 3´-ends of the probe to enable a bonding of the probe with the small groove of the double helix (“minor groove binder groups” [MGB]) and to increase the sequence specifity. An isolated signal rise of FAM™ or VIC® fluorescence was characteristic of CC_{677} or TT_{677} homozygosity and respectively AA_{1298} or CC_{1298} homozygosity. A rise of both signals indicated heterozygosity of the analysed DNA (*cf.* Table 13).

Table 13: Correlation between fluorescence signals and sequences present in the sample

A substantial increase of …	Indicates …
VIC dye fluorescence only	homozygosity for allele 1
6-FAM dye fluorescence only	homozygosity for allele 2
both fluorescent signals	allele 1 - allele 2 heterozygosity.

Source: APPLIED BIOSYSTEMS[8]

2.2.8 Determination of Plasma Total Homocysteine Concentrations

Valid measurement of tHcy, *i.e.* the sum of free, protein-bound, and disulphide forms, can be performed by means of combined gas chromatography-mass spectrometry, immune-enzymetry, tandem mass spectrometry, or classically, high-performance liquid chromatography, a method that has been proven to be of equal high quality. Irrespective of the method employed, the samples have pre-analytically to be prepared with greatest care, because erythrocytes also develop Hcy after blood sampling. This is the reason why the plasma has to be separated as soon as possible and to be refrigerated until analysis.

Blood measurements of Hcy, which reflects the intracellular concentration of Hcy, generally refer to tHcy, because analysing the different forms of Hcy is difficult. In order for tHcy to be measured, free Hcy which is considered to be the main atherogenic proton, has to be released from its disulphides. This is an essential step, before the assay of tHcy can be made. As free Hcy is highly oxidative and unstable, it is, therefore, only practical in terms of concentration and stability to measure tHcy. Blood should be sampled after a fasting period of at least six hours in order to minimise alimentary influences. As has been already mentioned, the sample has to be immediately cooled and centrifuged.

We used the fluorescence polarisation immunoassay (FPIA) with the AxSYM®System manufactured by Abbott Diagnostics Division, Germany, for this study[1]. After determination, surplus samples were refrozen, as tHcy has been reported to be stable in plasma stored at -20°C for an extended period (*cf.* Chapter 4.2.3).

Hcy is reduced in the FPIA process, protein-bound Hcy and mixed disulphide forms are converted to free Hcy by dithiothreitol. Subsequently, the free Hcy is enzymatically transformed to SAH by S-adenosyl-L-Hcy-hydrolase and excess adenosine. SAH or an analogue of SAH (tracer) is identified and bound by a monoclonal antibody. This tracer may influence the polarisation of the fluorescence beam, the more tracer molecules are fixed to the antibody the stronger the influence. This depends on the Hcy concentration in the sample: the higher the Hcy concentration the weaker the measurement signal. Under physiological conditions, SAH-hydrolase transforms SAH into Hcy.

Up to now, there is no consensus about reference values for plasma tHcy concentrations, although age- and gender-dependent reference ranges of tHcy for normal adult populations have been known for some time. Reference intervals for healthy maternal and newborn or children populations are scarce. Mean tHcy values were quite different among these studies. REDDY[137]delineates reference values (in means and SD) for tHcy (µmol / L) in children aged 0.1 - 4.0 years as follows: *male* - 8.9, ± 0.9; *female* – 8.3, ± 0.7; *p*-value 0.2049.

2.2.9 Determination of Folate Status

A variety of methods have been used to assess folate status. Not all research groups use the same methods, and even within a given method, protocols may vary between laboratories. These differences limit the ability to directly compare results from different studies without an evaluation of interlaboratory differences. For this study, the folate values in blood plasma samples were measured with ADVIA Centaur® automated analyser (Bayer Diagnostics, Germany)[13] using its manufacturer's commercial assays. ADVIA folate assay is a competitve immunoassay using direct chemoluminescence technology. The principle of the assay is a competition of endogenous acridinium esther-labelled folate for a limited number of binding sites on a solid phase, *i.e.*, folic acid in the sample competes with folic acid marked with acridinium esther in the Lite Reagent for a folic acid binding protein (FBP) that is marked by biotin. The Solid Phase consists of biotin-labelled FBP and purified intrinsic factor, respectively. Biotin-marked FBP binds to avidin covalently bonded to paramagnetic particles in the solid phase. Prior to the incubation with acridinium esther-folate, samples are pretreated with ADVIA Centaur® folic acid test to release (separate) folatic acid from the endogenous binding proteins. An inversely proportional relationship exists between the folic acid quantity and the relative light units (RLU) measured by the system.

2.2.10 Determination of Vitamin B_{12} Concentrations

Vitamin B_{12} concentrations in blood serum samples were measured with ADVIA Centaur® automated analyser (Bayer Diagnostics, Germany)[12] using commercial reagents and consumables from Bayer as described in the manufacturer's manual. ADVIA VB12 assay is a competitve immunoassay using direct chemoluminescent technology in which vitamin B_{12} from the patient sample competes with vitamin B_{12} labelled with acridinium ester in the Lite Reagent, for a limited amount of purified hog instrinsic factor, that is covalently coupled to paramagnetic particles in the Solid Phase. The assay uses sodium hydroxide as releasing agent and dithiothreitol to release the vitamin B_{12} from the

endogenous binding proteins in the sample and cobinamide is added to the sample to prevent rebinding after the Solid Phase. An inversely proportional relationship exists between the vitamin B_{12} quantity in the sample and the RLU detected by the system. Preparation and loading of the reagents, calibration of the system and quality control were conducted keeping to the rules and regulations established in the manual.

2.2.11 Statistical Analyses

In a first step, data were extracted from the questionnaire and entered on an MS Excel spreadsheet; the entries were manually checked for transcription errors. Some variables such as pregnancy weight gain and body mass index (BMI) were calculated using MS Excel. These data then were read into SPSS for Windows, version 15.0 (SPSS GmbH Software, Germany).

All computer-assisted statistical analyses were conducted using SPSS for Windows, version 15.0. The distribution of all continuous variables was checked for normality, and skewed variables were log-transformed before applying tests that propose normal distribution of the data. Continuous variables are presented either as median (10th – 90th) percentiles or as geometric mean (SD). Some data are given as number of subjects and percentage. Differences between means of more than two groups were compared by analyses of variance (ANOVA) and post-hoc Tamhane tests. For few comparisons, we used multivariate analysis to adjust for possible confounding factors such as gender, gestational age, and birth weight. Differences between two groups were tested using the non-parametric Mann-Whitney test. Correlations between different variables were tested by using Spearman's rank test. Differences in a binary outcome between two groups were compared using chi-square test (χ^2). Correlation coefficients are reported. Statistical significance was interpreted assuming *p*-values of *p* below 0.05. During analysis, outlying points and any that seemed unlikely were checked. Outliers are displayed in box plots or scatter plots except where otherwise stated.

3 Results

At the outset of this chapter, we would again like to differentiate between the terms IUGR and hypothrophic preterms or neonates, *i.e.* SGA. SGA and IUGR are to be regarded as two distinct terms; although they often coexist. The majority of SGA children achieve sufficient catch-up growth to normalise their stature by two years of age, independently of whether they were born prematurely or at term. Genetically small infants who present a continual growth below the 10th percentile in their growth corridor are often erroneously included in many clinical studies which poses an inherent methodological limitation. Therefore, one has to consider that in the following sections of this study preterm infants are defined as SGA if their birth weight was below the 10th percentile adjusted gestational age.

3.1 Basic Perinatal Data

All 193 mothers belong to the three major ethnic groups in Shri Lanka (Singhalese, Tamils, and Moors). All women who participated were non-smokers and did not consume alcohol; they were given folic acid tablets (5 mg / d) as means of routine food supplementation starting from the first booking attendance at an antenatal clinic until delivery. Varieties of low-dose folic acid supplements is not available in Shri Lanka, these higher doses of supplementation are readily available in the country and over-the-counter. *Thriposha* (*cf.* Chapter 5.4) was also offered, but not all mothers took it. General descriptive and demographic characteristics of the mothers studied are presented in Table 14.

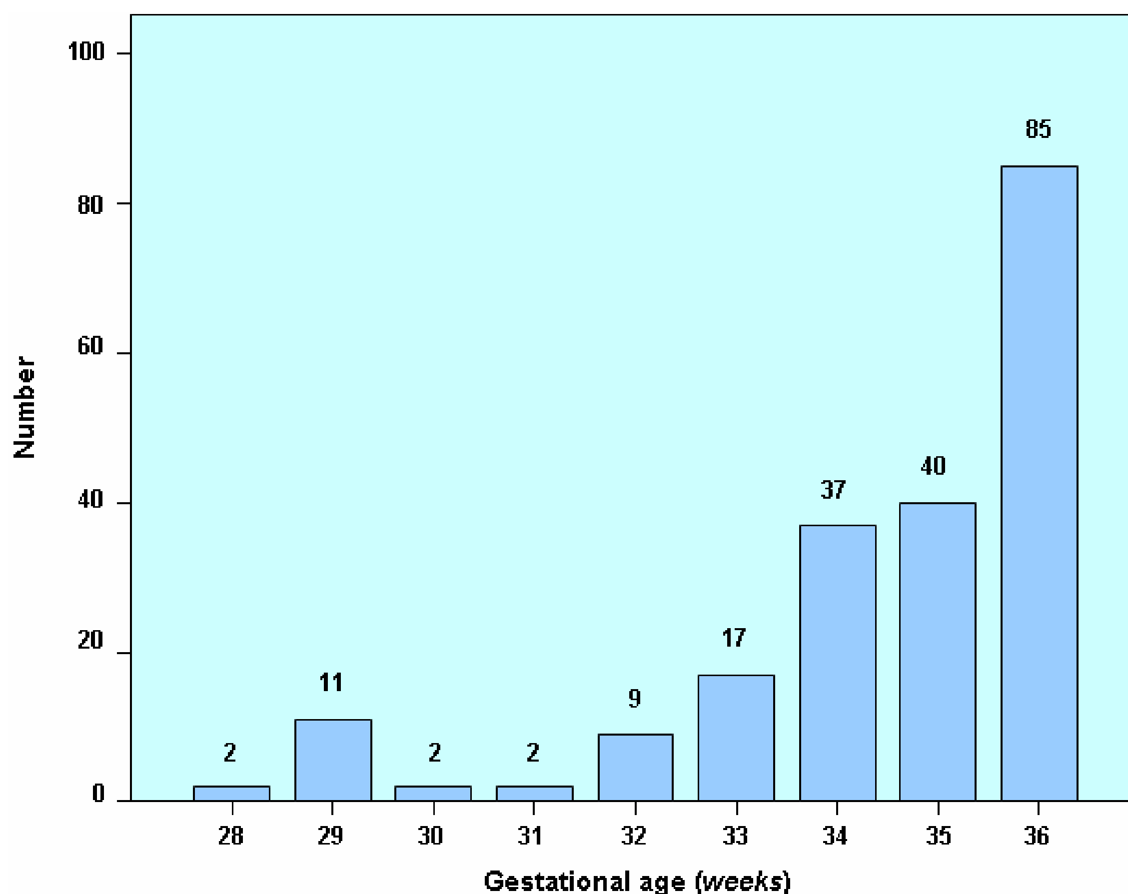
Table 14: Main maternal demographic data (n = 193)

Age distribution	**Age range**	**n**	**%**	**mean (± SD)** [*years*]
	< 18	1	0.52	
	18 – 23	24	12.44	
	24 – 29	77	39.90	
	30 – 35	54	27.98	29.9 (± 5.8 SD)
	36 – 41	30	15.54	
	> 41	7	3.64	
	20 – 34	149	77.01	
Ethnicity	**Ethnic group**	**n**	**%**	
	Singhalese	156	80.80	
	Tamils	19	9.80	
	Moors	18	9.30	
Education (*above 5 years*)	**Qualification**	**n**	**%**	
	O / L (*exam not passed*)	48	24.87	
	GCE (O / L)	87	45.08	
	GCE (A / L)	53	27.46	
	University degree	5	2.59	
Gravidae	**Pregnancy**	**n**	**%**	
	1	89	46.11	
	2	53	27.46	
	3	27	13.99	
	4	15	7.77	
	5	4	2.07	
	6	3	1.55	
	7	---	---	
	8	2	1.04	
Duration of gestation (*in weeks*)	**n**	**min.**	**max.**	**median**
whole population (*all mothers*)	193	---	---	35.1
induced labour (*sectio caesarea*)	130	28.2	36.8	---
spontaneous labour (*vaginal delivery*)	63	28.7	36.7	---
Pregnancy complications	**n**	**%**		
past deliveries with IUGR	38	19.7		
previous pregnancy losses or stillbirths	33	17.1		
pregnancy-induced hypertension	52	26.9		
cases of chronic hypertension	0	0.0		
BMI at beginning of pregnancy (kg / m^2)				**mean (± SD)**
				21.8 (± 3.5)

3.2 Basic Data of All Preterm Infants

The total number of preterms < 37 weeks in CSHW included during the three-and-a-half months study period was 205. The minimum GA was 28.2 weeks, maximum 36.6 (mean: 34.8, SD ± 2.0); most of the deliveries took place between 33 - 36 weeks of gestation. The number of deliveries according to the GA of the newborns is given in Figure 8.

Figure 8: Distribution of the gestational age of the preterm infants



BW ranged from 760 to 4,000 g (mean: 2,155, SD ± 613). In the study population, 105 deliveries resulted in male infants, 100 newborns were of female offspring. There were 182 singletons, 10 twin births, and 1 triplet birth. The mean APGAR score (5 minutes) was 9.5 mean (SD ± 0.72). A total of 61 preterms (29.8 %) were cared for in the intensive care unit, all of them survived without relevant postnatal complications.

An analysis of the BW among the male and female infants yielded the following results: the median BW of the male population was 2,250 g (range 815 - 3,475 g), the median BW of the female infants was 2,178 g (range 760 - 4,000 g).

The number of preterms born SGA and AGA as well as their distribution among the three ethnic groups are compiled in Table 15. There were no differences of statistical significance between the three ethnic groups ($p > 0.05$).

Table 15: Prevalence of SGA and AGA deliveries among the three main ethnic groups*)

		Ethnicity			***Total***
		Singhalese	**Tamil**	**Moors**	**(*n*)**
SGA	frequency (*n*)	54	11	8	*73*
	frequency within group (%)	32.3 %	55.0 %	44.4 %	*35.6 %*
AGA	frequency (*n*)	113	9	10	*132*
	frequency within group (%)	67.7 %	45.0 %	55.6 %	*64.4 %*
Total	**(*n*)**	*167*	*20*	*18*	*205*

*) included are singleton births, 10 twin pairs and 1 triplet pair

3.3 Results of Biochemical Variables

The analyses of concentrations of plasma folate, plasma tHcy, and serum vitamin B_{12} were conducted in the Central Laboratory at the Department of Clinical Chemistry and Laboratory Medicine of the University of The Saarland. In order to detect possible effects of IUGR in singleton and multiple preterm neonates, we assessed the values for singletons and multiples (twins and triplets) separately because of the disparate gestation times and other intrauterine growth conditions and because they have to be evaluated with specific percentile curves. To start with, the following assessments relate to singleton births.

As shown in Table 16, tHcy status remained significantly increased after adjustment for birth weight among preterms born SGA, there also was a tendency of increased vitamin B_{12} levels among SGA preterms although the values did not reach statistical significance.

Table 16: Main characteristics of SGA and AGA singleton births

Variables	AGA	SGA	*p*-value
Number	120	62	---
Age of the mothers (*years*)	30.00 (23.10 / 39.00)	28.00 (22.00 / 37.00)	---
Weight gain (*kg*)	8.00 (5.00 / 13.00)	6.50 (4.00 / 11.00)	0.005
BMI (*kg / m*^{2})	21.48 (18.22 / 27.53)	20.47 (16.61 / 26.53)	0.079
Gestational age (*weeks*)	35.57 (32.44 / 36.71)	35.71 (32.14 / 36.71)	0.656
Birth weight (*g*)	2,520 (1,510 / 3,050)	1,875 (1,180 / 2,299)	< 0.001
Birth length (*cm*)	50.0 (44.0 / 54.0)	46.0 (40.3 / 50.0)	< 0.001
Head circumference (*cm*)	32.0 (28.0 / 34.0)	30.0 (27.3 / 32.0)	< 0.001
APGAR	10.00 (9.00 / 10.00)	9.50 (8.00 / 10.00)	0.003
Homocysteine (*µmol / L*)	4.58 (3.27 / 6.72)	5.17 (3.66 / 8.87)	0.016
Folate (*nmol / L*)	45.1 (23.6 / 183.5)	44.4 (25.0 / 246.8)	0.655
Vitamin B_{12} (*pmol / L*))	356 (210 / 703)	405 (229 / 837)	0.098

Data are median, in brackets: 10th / 90th percentiles; *p*-values according to Mann-Whitney test, significance set at $p < 0.05$

The prevalence of the MTHFR polymorphisms C677T and A1298C among the preterm newborns (twins and triplets included; n = 205) are shown in Table 17.

Table 17: Frequencies of MTHFR polymorphisms C677T and A1298C in study group

MTHFR Polymorphisms	Genotypes	Frequency (*n*)	Frequency (%)
C677T	CC	162	79.0
	CT	40	19.5
	TT	3	1.5
A1298C	AA	67	32.7
	AC	96	46.8
	CC	42	20.5

Furthermore, we investigated the association between tHcy, folate and vitamin B_{12} status and the two polymorphisms C677T and A1298C, respectively (Tables 18 and 19). Because of the low number of TT frequencies, we pooled T allele carriers to be one group.

Table 18: Concentrations of plasma total homocysteine, folate and serum vitamin B_{12} of singleton SGA and AGA preterms according to MTHFR C677T polymorphism

		MTHFR C677T		
		CC	**CT + TT**	***p*-value**
SGA (n = 62)	**n**	48	14	---
	Homocysteine (*µmol / L*)	5.39 (± 2.14)	5.47 (± 2.03)	0.775
	Folate (*nmol / L*)	56.0 (± 96.32)	62.6 (± 180.75)	0.827
	Vitamin B_{12} (*pmol / L*)	395 (± 204.89)	468 (± 278.45)	0.198
	Gestational age (*weeks*)	34.7 (± 2.09)	35.2 (± 1.21)	0.980
	Birth weight (*g*)	1,667 (± 474.71)	1,808 (± 455.33)	0.533
AGA (n = 120)	**n**	95	25	---
	Homocysteine (*µmol / L*)	4.65 (± 1.69)	4.68 (± 1.65)	0.821
	Folate (*nmol / L*)	54.2 (± 77.13)	46.6 (± 60.46)	0.210
	Vitamin B_{12} (*pmol / L*)	381 (± 238.41)	337 (± 118.51)	0.393
	Gestational age (*weeks*)	34.7 (± 2.06)	35.6 (± 1.29)	0.102
	Birth weight (*g*)	2,266 (± 593.70)	2,612 (± 405.29)	0.007

Data are geometric mean (± SD); *p*-values according to Mann-Whitney test; significance considered to be $p < 0.05$

As for MTHFR A1298C polymorphism, vitamin B_{12} levels were found to be significantly lower in the C allele carriers compared with the wild type genotype (AA). This difference was observed only in the SGA group (Table 19).

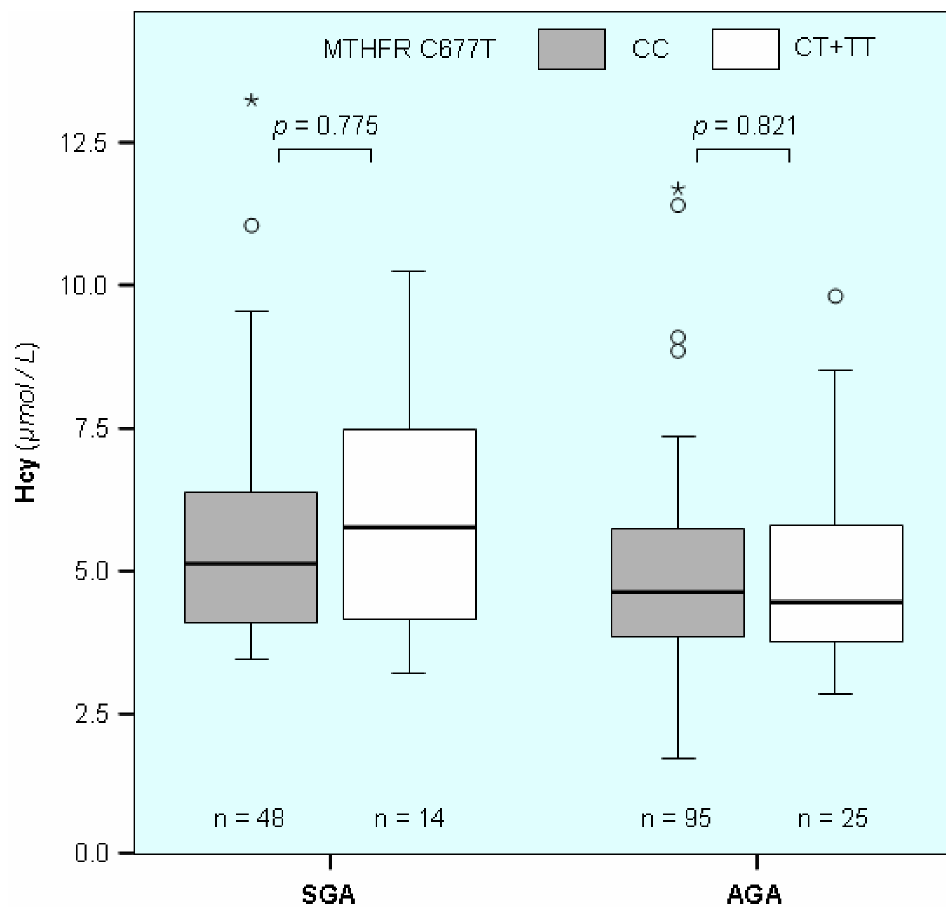
Table 19: Concentrations of plasma total homocysteine, folate and serum vitamin B_{12} in singleton SGA and AGA preterms according to MTHFR A1298C polymorphism

		MTHFR A1298C		
		AA	**AC + CC**	***p*-value**
SGA (n = 62)	**n**	23	39	---
	Homocysteine (*µmol / L*)	5.78 (± 2.21)	5.21 (± 2.02)	0.235
	Folate (*nmol / L*)	69.3 (± 161.79)	51.4 (± 84.15)	0.213
	Vitamin B_{12} (*pmol / L*)	548 (± 241.43)	353 (± 181.77)	0.001
	Gestational age (*weeks*)	34.4 (± 1.54)	35.0 (± 2.11)	0.030
	Birth weight (*g*)	1,583 (± 422.31)	1,770 (± 461.87)	0.048
AGA (n = 120)	**n**	39	81	---
	Homocysteine (*µmol / L*)	4.39 (± 1.38)	4.79 (± 1.79)	0.131
	Folate (*nmol / L*)	56.6 (± 83.17)	50.7 (± 65.98)	0.836
	Vitamin B_{12} (*pmol / L*)	366 (± 143.04)	374 (± 249.92)	0.987
	Gestational age (*weeks*)	35.2 (± 1.82)	34.8 (± 2.01)	0.302
	Birth weight (*g*)	2,372 (± 531.27)	2,316 (± 591.83)	0.566

Data are geometric mean (± SD); *p*-values according to Mann-Whitney test; significance considered to be $p < 0.05$

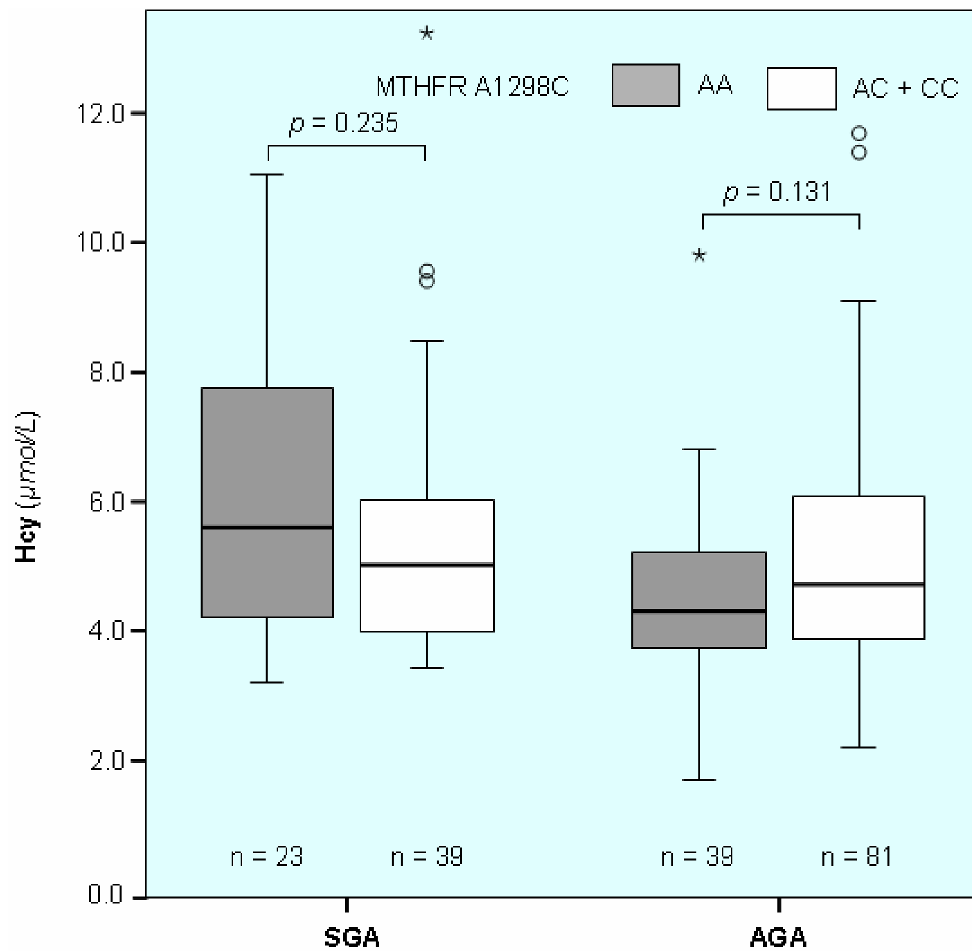
A significant association between MTHFR C677T and A1298C polymorphisms and tHcy was not observed. Furthermore, we did not find a significant association between MTHFR C677T and serum concentration of vitamin B_{12} or plasma folate (Figures 9 and 10).

Figure 9: Association between MTHFR C677T and plasma total homocysteine in SGA and AGA singleton preterms



Extreme values are not shown, *p*-values according to Mann-Whitney test

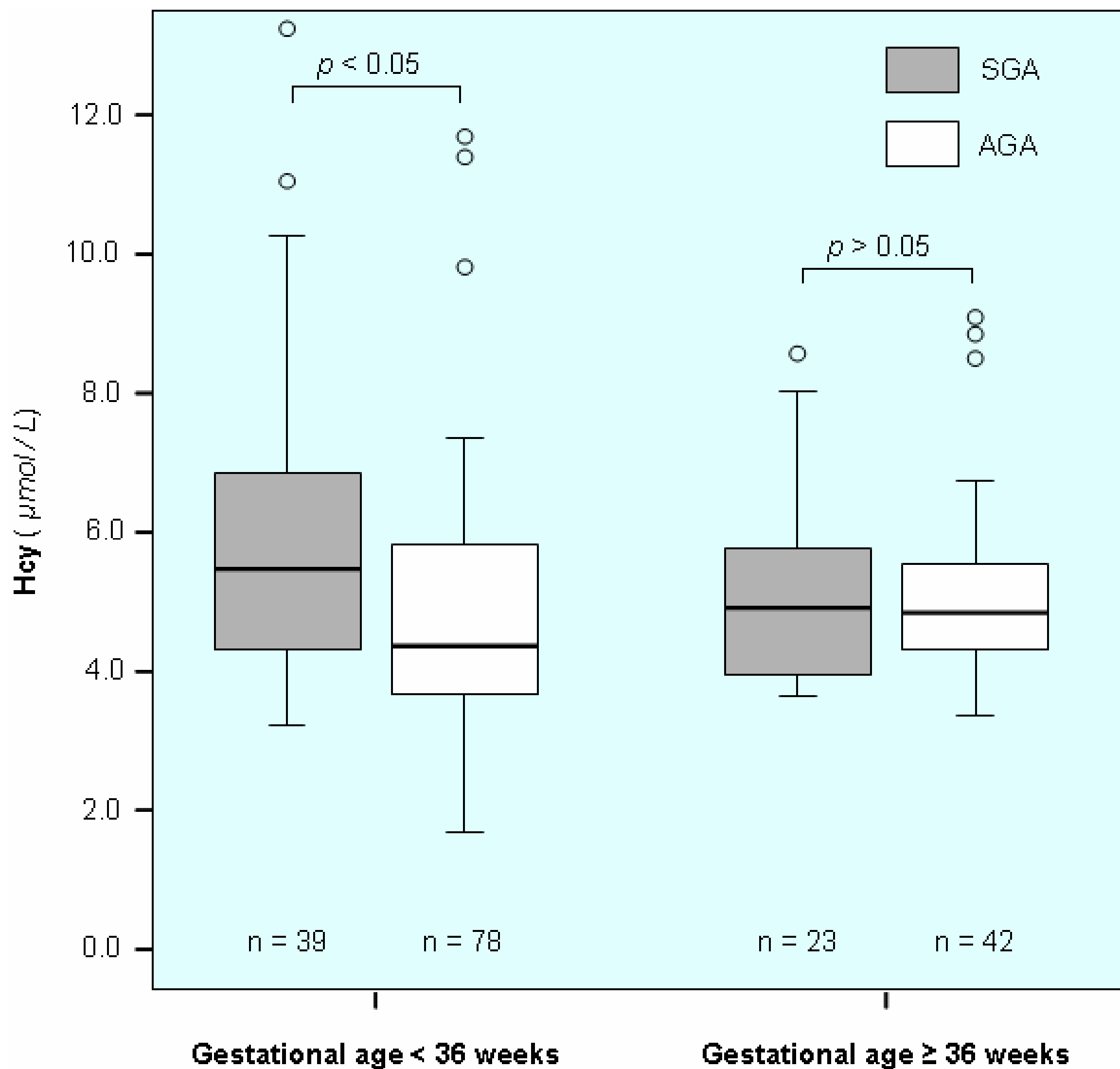
Figure 10: Association between MTHFR A1298C and plasma total homocysteine in SGA and AGA singleton preterms



Extreme values are not shown, *p*-values according to Mann-Whitney test

A multivariate analysis of the association between SGA and tHcy in singleton preterms < 36 GA provided statistically significant increased tHcy levels (Figure 11). An analysis of the association between plasma tHcy and GA showed a trend towards a decrease of plasma tHcy status when the gestation period lasted longer ($p > 0.05$); values were adjusted for gestational age.

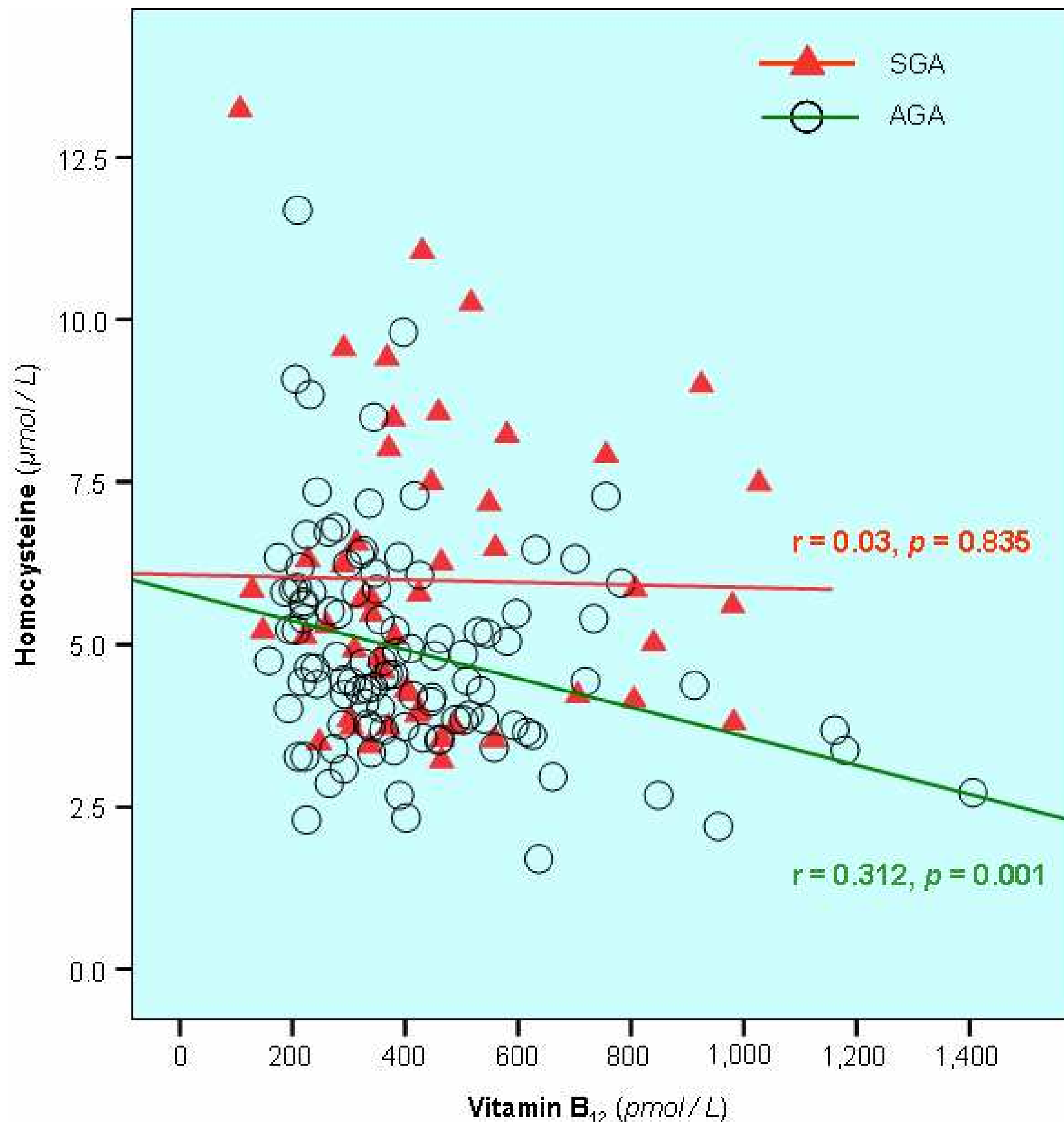
Figure 11: Association between SGA, AGA and elevated plasma total homocysteine status in singleton preterms < 36 and ≥ 36 weeks of gestation



Suggestive cut-off value = 36 gestational weeks; extreme values are not shown in the figure; *p*-values according to Mann-Whitney test

Figure 12 displays the correlation between vitamin B_{12} and tHcy in SGA neonates. Vitamin B_{12} status played a significant role as tHcy modulator in AGA preterms.

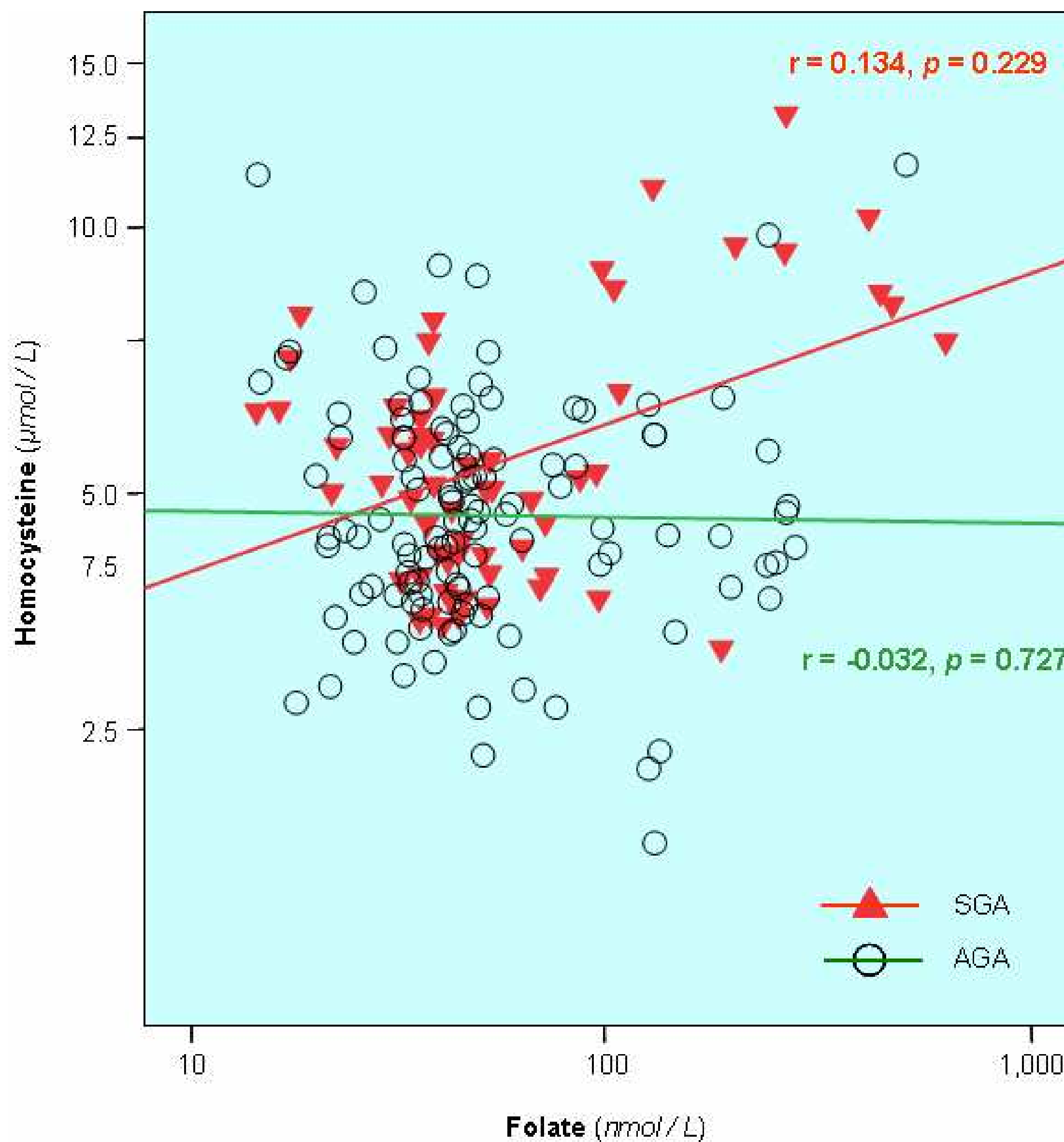
Figure 12: Correlation between vitamin B_{12} and plasma total homocysteine (singletons)



Normal scale; r: Spearman correlation coefficient; SGA: n = 62, AGA: n = 120

Figure 13 shows no significance between folate and tHcy in SGA and AGA singleton preterms.

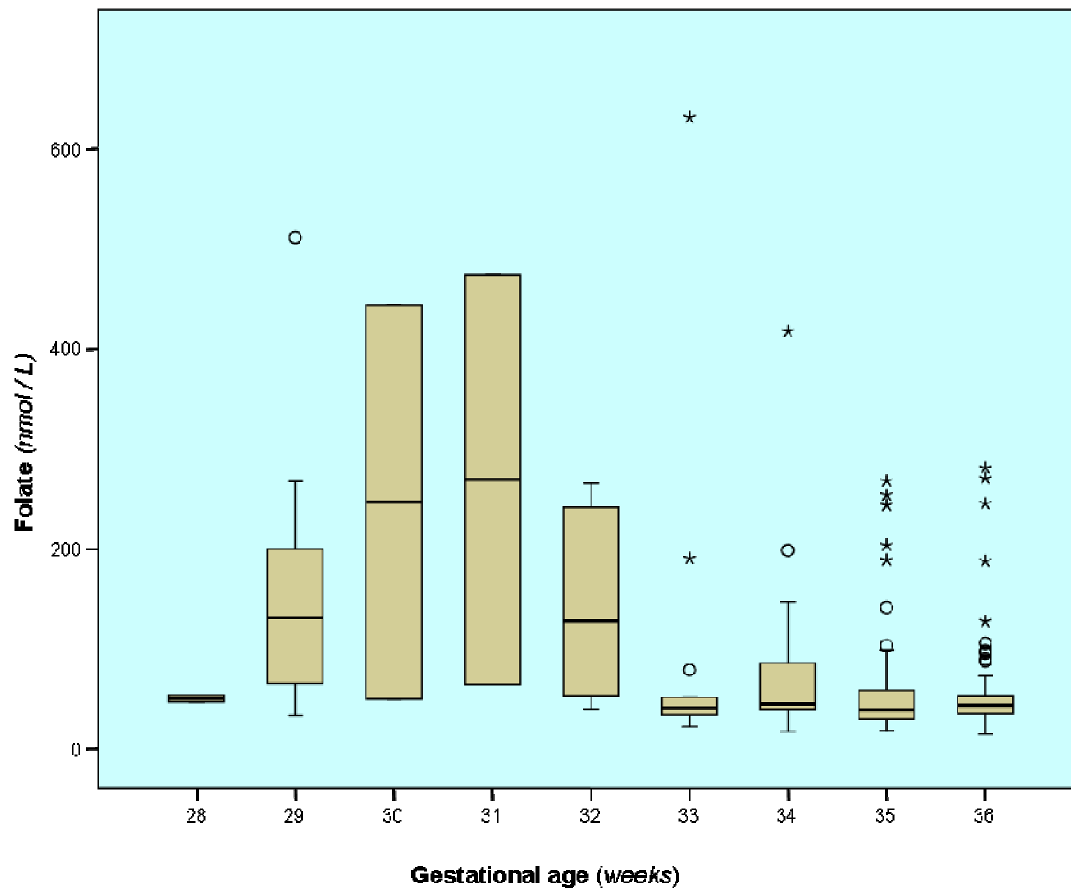
Figure 13: Correlation between folate and plasma total homocysteine in SGA / AGA neonates (singletons)



Anti-log scales; r = Spearman correlation coefficient; SGA: n = 62, AGA: n = 129

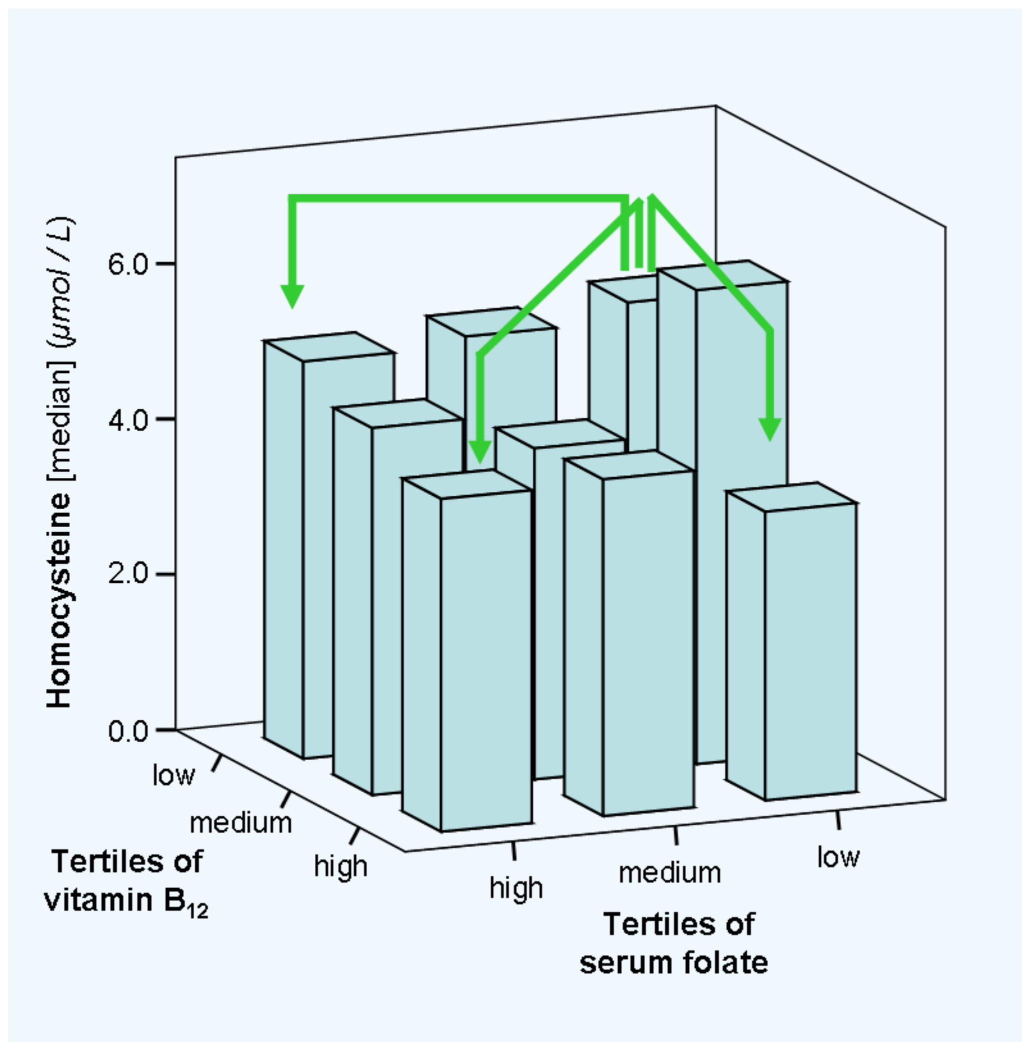
An analysis of the association between folate and GA (Figure 14) showed a tendency for lower folate levels in case of a longer gestational period ($p > 0.05$).

Figure 14: Association between folate concentrations and gestational age in singleton preterms (n = 182)



The highest tHcy levels were measured in AGA singleton preterms with both low folate and vitamin B_{12} status although the mothers received folate supplementation. The elevated tHcy values could partly be explained by a relatively low vitamin B_{12} status (Figure 15).

Figure 15: Interrelation between folate and vitamin B_{12} as determinants of plasma total homocysteine in AGA singleton preterms



Tertiles of vitamin B_{12} status [*pmol / L*]: low → 158 – 292; medium → 297 – 426; high → 431 – 1,406
Tertiles of plasma folate status [*nmol / L*]: low → 14.8 – 37.6; medium → 40.0 – 52.1; high → 53.0 – 511.0

Regarding ethnic differences (Table 20), we found that Tamil preterms born SGA showed a tendency toward slightly higher tHcy levels which is in accord with lower folate and vitamin B_{12} levels when compared with AGA Tamil infants. These distinctive features could not been shown either in the Singhalese nor in the Moorish preterms. An insufficient statistical power has to be conceded because the total number of subjects studied was relatively small, which renders it impossible to consider conclusions as representative or

final. Only in the Singhalese group, SGA preterms showed significantly higher vitamin B_{12} values when compared to AGA infants (mean: 445 pmol / L *vs.* 371 pmol / L, $p = 0.022$).

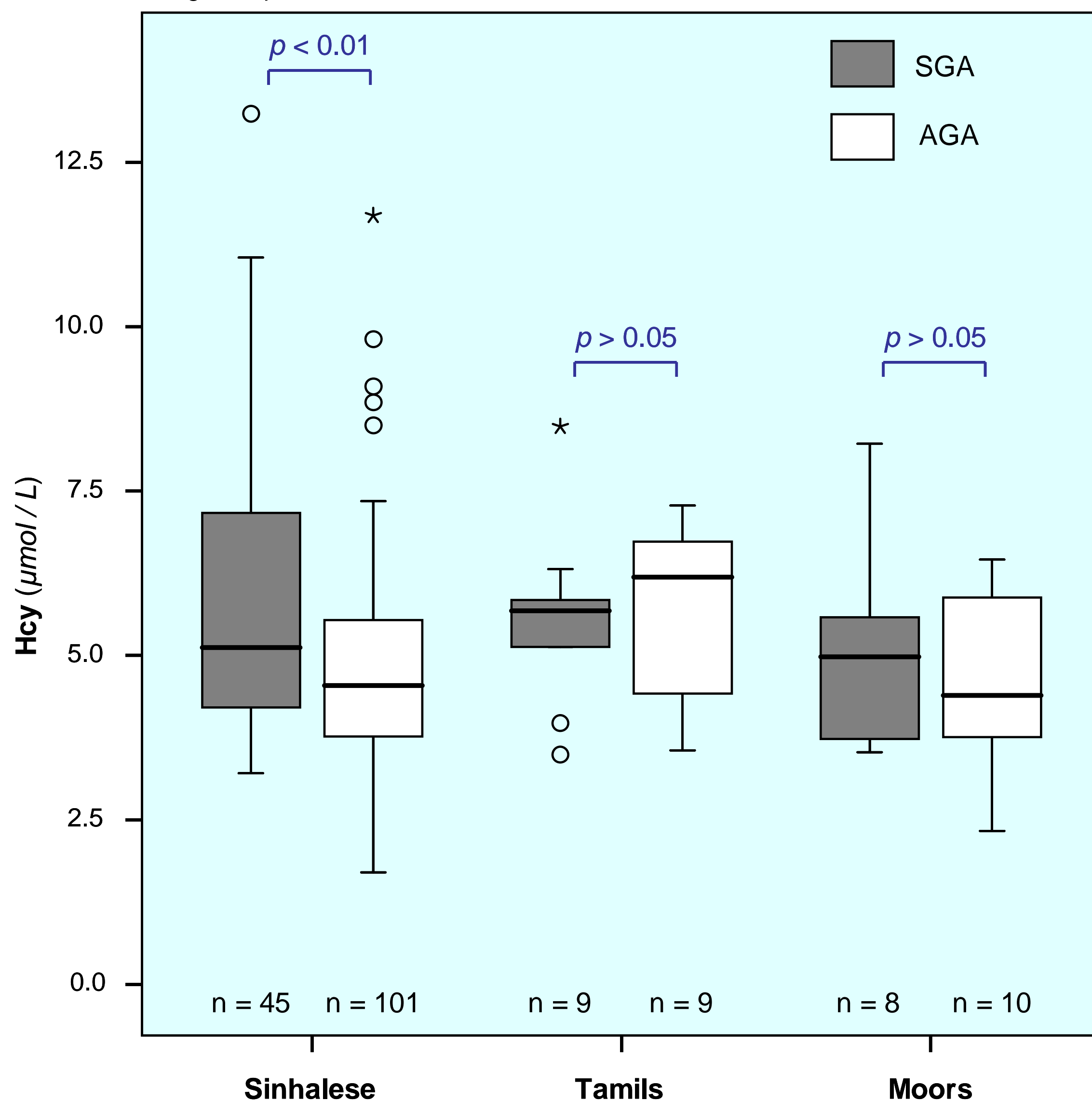
Table 20: Concentrations of homocysteine, folate and vitamin B_{12} among singletons according to the ethnic origin

	Ethnic origin					
	Singhalese		Tamils		Moors	
	SGA (*n* = 45)	AGA (n = 101)	SGA (*n* = 9)	AGA (*n* = 9)	SGA (*n* = 8)	AGA (*n* = 10)
Gestational age (*weeks*)	34.998 (± 1.708)	34.892 (± 2.049)	34.720 (± 505.842)	35.238 (± 1.484)	33.851 (± 2.909)	35.436 (± 1.158)
Birth weight (*g*)	1,741.77 (± 424.273)	2,308.36 (± 578.513)	1,703.19 (± 505.842)	2,470.58 (± 602.532)	1,465.19 (± 576.909)	2,476.15 (± 483.537)
Homocysteine (*µmol / L*)	5.52 (± 2.294)	4.62 (± 1.606)	5.40 (± 1.423)	5.68 (± 2.407)	4.86 (± 1.537)	4.28 (± 1.393)
Folate (*nmol / L*)	59.4 (± 113.741)	54.2 (± 77.789)	49.4 (± 135.509)	31.7 (± 23.213)	56.2 (± 152.631)	60.0 (± 57.649)
Vitamin B_{12} (*pmol / L*)	445 (± 241.720)	371 (± 231.403)	261 (± 135.509)	353 (± 199.414)	419 (± 108.852)	387 (± 134.799)

Data are geometric mean (± SD)

Total Hcy levels were significantly higher in Singhalese SGA preterms compared to AGA infants. This difference was not observed in the two other ethnical groups, probably because of the low number of subjects included resulting in low statistical power to detect differences of significance (Figure 16).

Figure 16: Association between homocysteine and the three main ethnic groups in SGA and AGA singleton preterms



Extreme values are not shown in the figure; significance was set at $p < 0.05$ according to Mann-Whitney test

We tested the effect of PIH on tHcy and related vitamins in singleton subjects according to the presence or absence of SGA. We have, therefore, compiled the values for tHcy, folate, vitamin B_{12}, gestational age, and BW in Table 21. After performing χ^2-test (Pearson and Fisher's exact test), we found significantly higher incidences of SGA preterm neonates among mothers with PIH. Hypertension was associated with increased concentrations of tHcy in SGA preterms but not in AGA infants. Folate levels were significantly higher in

preterms from mothers with PIH. These findings were confirmed in both SGA and AGA infants. Similar findings were observed in vitamin B_{12}, but the differences between PIH and non-PIH were significant only in SGA infants. Likewise, the analysis of the association between PIH and both of the MHTFR genotypes demonstrated no significant correlation ($p > 0.05$; data not shown in Table 21).

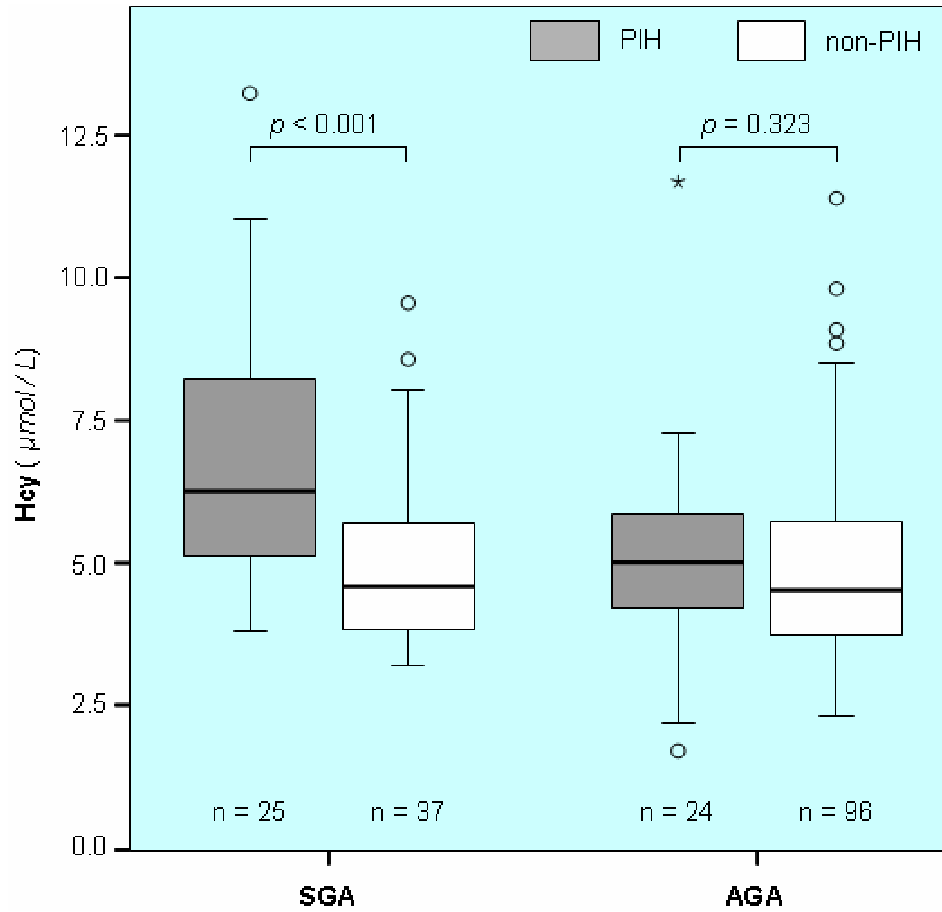
Table 21: Association between SGA, AGA and folate, plasma total homocysteine, vitamin B_{12} status with regard to pregnancy-induced hypertension (only singleton births)

		PIH	non-PIH	*p*-value
SGA	**n**	25	37	---
(n = 62)	**Homocysteine (*µmol / L*)**	6.54 (± 2.372)	4.77 (± 1.473)	< 0.001
	Folate (*nmol / L*)	77.6 (± 172.430)	46.9 (± 40.371)	0.054
	Vitamin B_{12} (*pmol / L*)	494 (± 264.088)	362 (± 169.283)	0.013
	Gestational age (*weeks*)	33.5 (± 2.243)	35.7 (± 1.045)	< 0.001
	Birth weight (*g*)	1,355 (± 367.900)	1,977 (± 332.091)	< 0.001
AGA	**n**	24	96	---
(n = 120)	**Homocysteine (*µmol / L*)**	4.80 (± 1.880)	4.63 (± 1.631)	0.323
	Folate (*nmol / L*)	76.5 (± 104.326)	47.8 (± 62.251)	0.006
	Vitamin B_{12} (*pmol / L*)	394 (± 209.907)	366 (± 224.519)	0.478
	Gestational age (*weeks*)	34.2 (± 2.553)	35.1 (± 1.747)	0.375
	Birth weight (*g*)	2,032 (± 773.914)	2,416 (± 494.883)	0.063

Data are geometric mean (± SD); *p*-values according to Mann-Whitney test; significance considered to be $p < 0.05$

PIH was associated with higher tHcy levels in growth-retarded singleton preterms but not in AGA newborns (Table 21 and Figure 17).

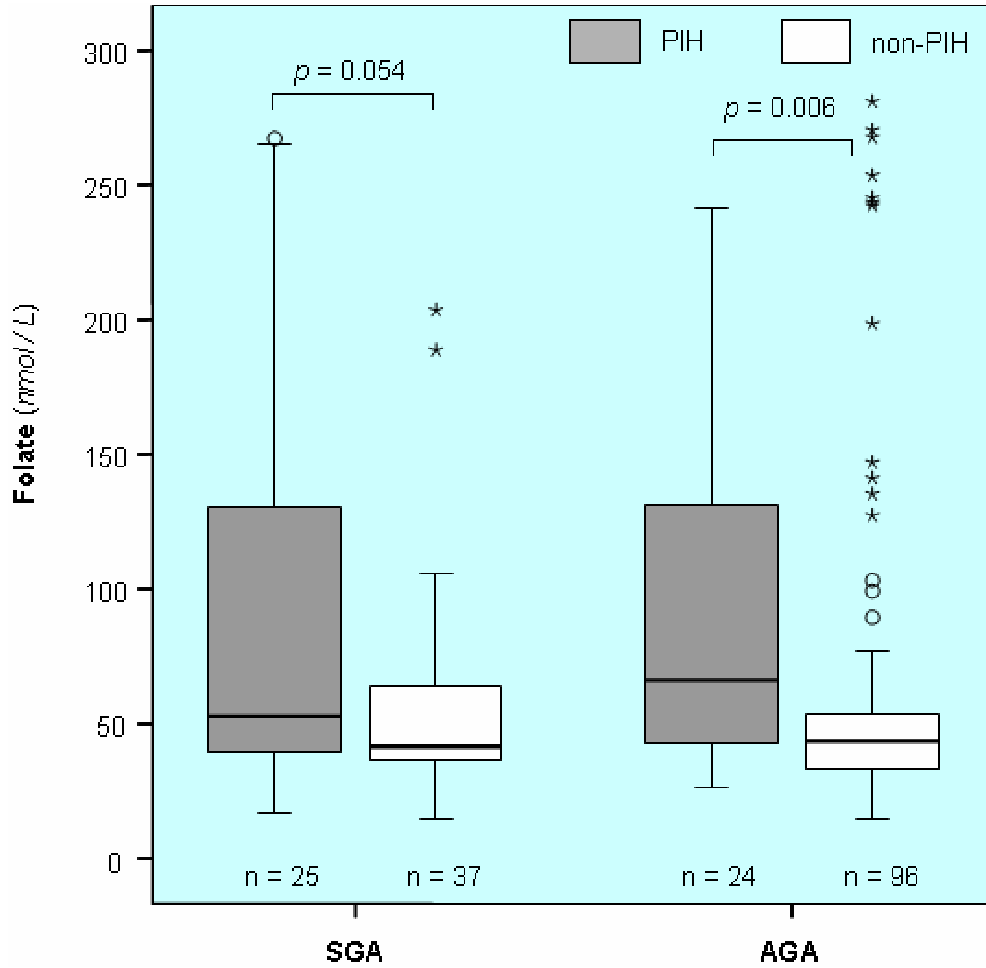
Figure 17: Association between elevated plasma total homocysteine levels and SGA, AGA singleton preterms



Significance considered to be $p < 0.05$

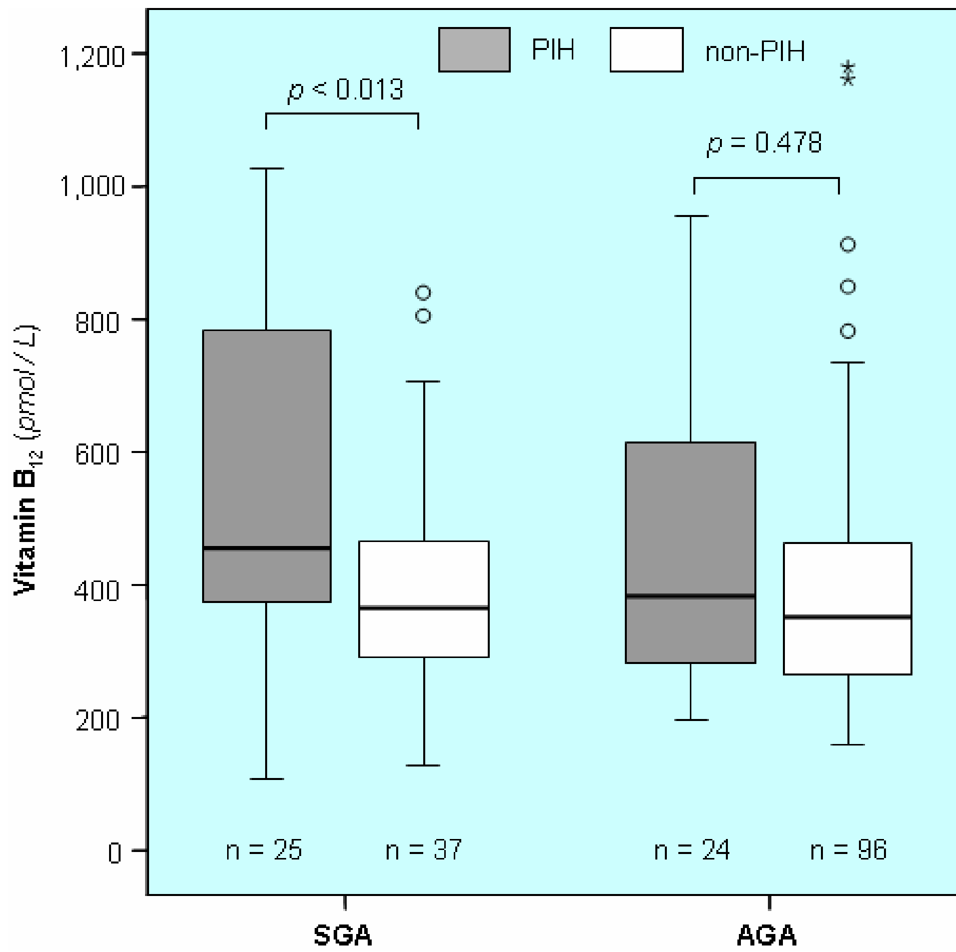
PIH was significantly associated with folate concentrations in AGA newborns but not in SGA singletons; on the other hand, PIH was significantly associated with vitamin B_{12} levels in SGA but not in AGA singletons (Figures 18 and 19).

Figure 18: Associations between folate and pregnancy-induced hypertension in SGA and AGA singleton preterms



Significance considered to be $p < 0.05$

Figure 19: Associations between vitamin B_{12} and pregnancy-induced hypertension in SGA and AGA singleton preterms



Significance considered to be $p < 0.05$

We perfomed a Mann-Whitney test to ascertain gender effects between AGA and SGA preterms with respect to the tHcy, folate, and vitamin B_{12} status. AGA preterm females had folate concentrations which reached the level of significance when compared to males (Table 22).

Table 22: Plasma total homocysteine and vitamin B_{12} status in singleton preterm AGA and SGA births according to gender

		AGA	SGA	p^1-value
Homocysteine (*µmol / L*)	male	4.52 (± 1.308)	5.69 (± 2.319)	0.167
	female	4.81 (± 1.977)	5.16 (± 1.845)	0.202
	p-value	0.426	0.229	
Folate (*nmol / L*)	male	47.0 (± 58.533)	57.3 (± 111.853)	0.022
	female	59.0 (± 86.248)	57.6 (± 129.506)	0.110
	p-value	0.036	0.816	
Vitamin B_{12} (*pmol / L*)	male	370 (± 222.980)	442 (± 240.776)	0.502
	female	373 (± 221.150)	382 (± 205.509)	0.272
	p-value	0.811	0.058	

Data are geometric mean (± SD); *p*-values according to Mann-Whitney test; significance considered to be $p < 0.05$; p^1-values adjusted for birth weight and gestational age in multivariate test

We further examined the effect of primiparous and multiparous pregnancies - among other variables - on tHcy, folate and vitamin B_{12} status in singleton neonates (Table 23).

Table 23: Association between plasma total homocysteine, folate and vitamin B_{12} levels and gravidae (only singleton births)

Variables	Gravida = 1	Gravida > 1	*p*-value
n (*total: 182*)	86	96	---
Weight gain (*kg*)	8.00 (4.00 / 13.00)	7.00 (4.00 / 12.30)	0.437
BMI (*kg / m²*)	20.26 (16.52 / 26.17)	22.51 (17.95 / 27.96)	0.001
Gestational age (*weeks*)	35.6 (33.0 / 36.7)	35.6 (31.8 / 36.7)	0.563
Birth weight (*g*)	2,148 (1,347 / 2,906)	2,298 (1,258 / 3,000)	0.190
Birth length (*cm*)	48 (42 / 52)	49 (42 / 54)	0.223
Head circumference (*cm*)	31 (28 / 34)	32 (28 / 33)	0.572
Homocysteine (*µmol / L*)	4.75 (3.38 / 7.39)	4.93 (3.40 / 8.49)	0.483
Folate (*nmol / L*)	46.5 (27.8 / 134.5)	43.2 (23.2 / 242.7)	0.474
Vitamin B_{12} (*pmol / L*)	360 (220 / 801)	380 (209 / 722)	0.857

Data are median, in brackets: 10th / 90th percentiles; *p*-values according to Mann-Whitney test, significance set at $p < 0.05$

With regard to previous miscarriages, the analysis showed no significant effect except for folate (Table 24).

Table 24: Association between plasma total homocysteine, folate and vitamin B_{12} levels and previous miscarriages (only singleton births)

Variables	Previous miscarriages (*no*)	Previous miscarriages (*yes*)	*p*-value
n (*total: 182*)	150	32	---
Weight gain (*kg*)	8.0 (4.0 / 12.9)	8.0 (5.0 / 12.7)	0.771
BMI (kg / m^2)	20.84 (17.23 / 26.63)	22.51 (17.34 / 27.91)	0.137
SGA (*n*)	53	9	0.286
Gestational age (*weeks*)	35.57 (32.29 / 36.71)	35.21 (31.40 / 36.67)	0.233
Birth weight (*g*)	2,250 (1,361 / 2,999)	2,165 (1,155 / 2,881)	0.474
Birth length (*cm*)	49 (42 / 53)	48 (51 / 55)	0.331
Head circumference (*cm*)	31 (28 / 34)	31 (28 / 33)	0.337
Homocysteine (*µmol / L*)	4.83 (3.45 / 7.47)	4.92 (2.97 / 9.05)	0.862
Folate (*nmol / L*)	43.5 (23.3 / 131.0)	51.4 (30.5 / 276.2)	0.013
Vitamin B_{12} (*pmol / L*)	363 (213 / 784)	389 (225 / 621)	0.459

Data are median, in brackets: 10th / 90th percentiles; *p*-values according to Mann-Whitney test, significance set at $p < 0.05$

Data were separately analysed for singletons and for multiple births in order to discover any potential differences in the effect of growth retardation between the two separate groups. The main characteristics of the AGA and SGA multiples (twins and triplets) are compiled in Table 25.

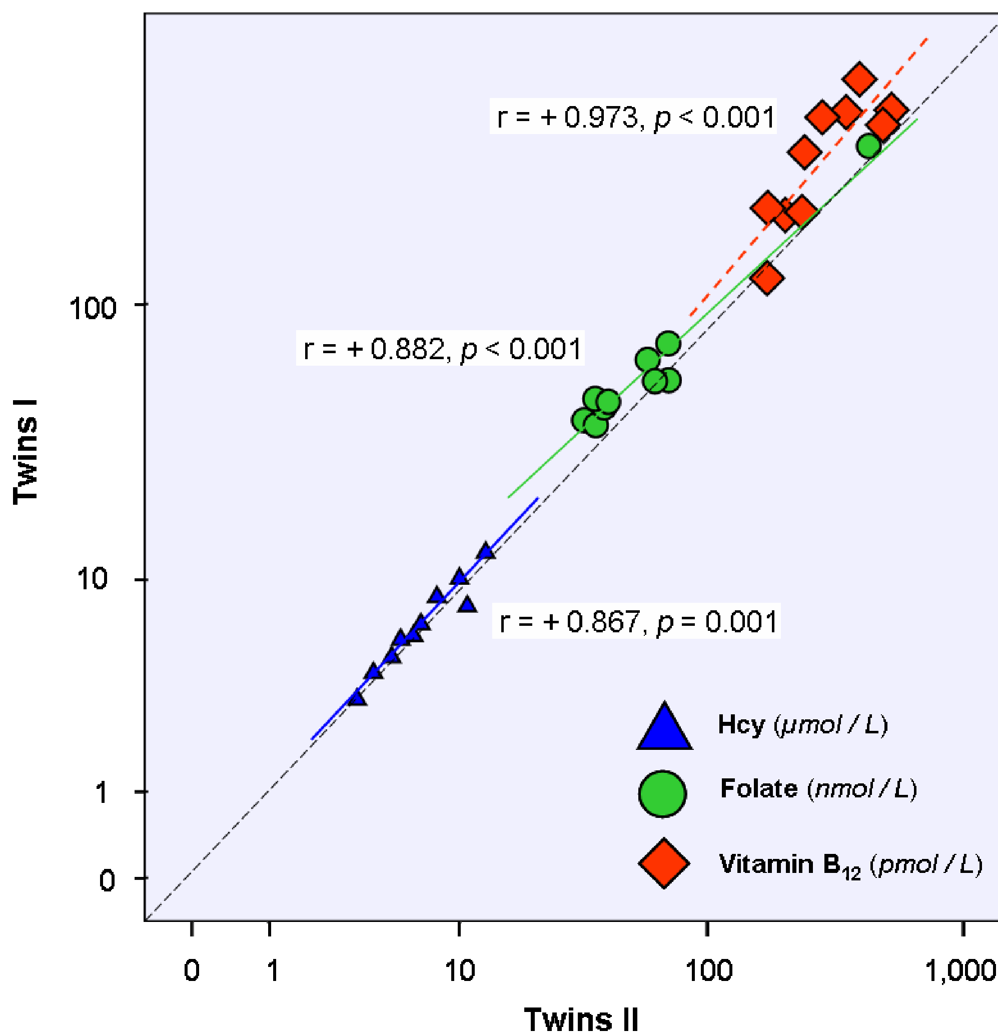
Table 25: Main characteristics of AGA and SGA multiple births

Variables	AGA	SGA
Number	12	10
Weight gain (*kg*)	11.5 (6.3 / 14.0)	9.0 (5.0 / 13.0)
Gestational age (*weeks*)	33.6 (29.0 / 36.7)	34.7 (34.0 / 36.7)
Birth weight (*g*)	1,970 (932 / 2,705)	1,700 (1,380 / 2,208)
Birth length (*cm*)	47 (36 / 53)	47 (43 / 50)
Head circumference (*cm*)	31 (26 / 34)	29 (26 / 32)
Homocysteine (*µmol / L*)	5.27 (3.26 / 12.86)	6.79 (5.28 / 10.61)
Folate (*nmol / L*)	50.3 (37.4 / 406.9)	50.0 (33.3 / 68.8)
Vitamin B_{12} (*pmol / L*)	394 (212 / 590)	235 (133 / 515)

Data are median, in brackets: 10th / 90th percentiles

The analysis of the correlation between the biochemical variables of twins yielded no statistically relevant differences as to tHcy, folate, and vitamin B_{12}, although in the case of vitamin B_{12}, slight variations were found between twin pairs. Also, tHcy values tended to be higher when at least one of the newborns was growth-retarded (Figure 20).

Figure 20: Correlation between plasma total homocysteine, folate, and vitamin B_{12} concentrations in twin pairs



r = Spearman correlation coefficient; n = 11 pairs. Values on the X- and Y-axis are anti-log values

4 Discussion

4.1 Study Group

For this mono-centre study, we prospectively enrolled 193 pregnant women belonging to the three major Shri Lankan ethnic groups, *i.e.*, Singhalese, [Shri Lankan] Tamils, and Moors (*cf.* Chapter 5.2). These are distinct groups with differences in lifestyle, religion, and, also, dietary habits. All mothers who met the inclusion criteria during the study period at Castle Street Hospital for Women in Colombo were selected without conscious bias in chronological sequence throughout the day. The mothers gave birth to 205 preterm newborns: 167 Singhalese neonates, 20 of Tamil and 18 of Moorish origin.

4.2 Method Validation

4.2.1 Pre-Analytical Factors

The blood samples were taken as described in Chapter 2.2.5. The EDTA tubes were immediately placed on ice to stabilise the Hcy (*cf.* Chapter 2.2.8). In order to avoid any Hcy analytical mistakes, the cord blood tubes were then centrifuged within 20 minutes at 3,000 rpm for 10 minutes. The resulting plasma and serum were separated and directly frozen in several aliquots and stored at -30℃ befo re shipment to Germany was carried out in dry ice by air courier and without interrupting the cold chain; laboratory analyses and data processing were later performed in Germany; 205 blood samples were collected altogether. All blood samples were analysed within one month.

The purification of the nucleic acids from dried blood spots on capture cards and the DNA extraction was done under sterile conditions using the commercial QIAamp® DNA Blood Mini Kit (QIAGEN, Germany). The procedure is described in Chapter 2.2.6. To ensure the quality of the results, we took random samples (about 35 - 40 %) for additional analyses. These results were consistent with the previous ones.

4.2.2 Genotyping

The determination of the MTHFR C677T and A1298C polymorphisms were performed in the laboratory of the Paediatric Department of the University Clinics of The Saarland. Our experiments and the reactions were performed on PCR System 7500 Real-Time (Applied Biosystems, Germany), random samples were taken (about 30 %).

The results were independently scrutinised by two blinded colleagues. The genotype determination was carried out without knowledge of clinical and personal data of the study

population. We used primers (Applied Biosystems, Germany; *cf.* Chapter 2.2.7.2) which have already been described elsewhere. Overall, our results about the prevalence of MTHFR polymorphisms agree with previous reports[3, 35, 150] (*cf.* Chapter 4.3.1).

4.2.3 Homocysteine, Folate and Vitamin B_{12}: Analytical Factors

For this study, we used FPIA with the AxSYM®System (Abbott Laboratories, Germany) for the determination of plasma tHcy (*cf.* Chapter 2.2.8). The sample collection for the Hcy assay was conducted according to previous studies. It is unlikely that pre-analytical conditions have affected the Hcy assay. The concentrations of folate values in plasma samples were measured with ADVIA Centaur® automated analyser (Bayer Diagnostics, Germany) using its manufacturer's commercial assays[13]. We used *serum* vitamin B_{12} levels to assess status principally for two reasons: First, serum vitamin B_{12} status reflects more recent intakes in the last three months (trimester), and, second, this measurement of vitamin B_{12} status has been used in previous studies[43, 138]. The measurement of vitamin B_{12} was also performed on the ADVIA Centaur® automated analyser[12] using commercial reagents and consumables from Bayer, Germany, as described in the manual. This assay is employed as routine determination of vitamin B_{12} at the Homburg University Hospital. Folate and vitamin B_{12} levels are comparable with values reported from the population.

All measurements were obtained in a blind manner to each other, and all analyses were performed under strong quality controls. The intraassay and interassay coefficients of variation for all measurements (tHcy, folate, vitamin B_{12}) were always < 5 %, respectively.

4.3 Clinical and Biochemical Results

4.3.1 MTHFR C677T and MTHFR A1298C Polymorphisms

A MTHFR deficiency is the most common inborn error of folate metabolism, and the common MTHFR C677T polymorphism of the human is associated with reduced specific MTHFR activity and elevated plasma tHcy levels[132]. The results of the analysis of the MTHFR gene C677T for all subjects in this study seem to be in general conformity with the findings reported in the literature. For instance, ALAGRATNAM *et al.* in his brief report[3] presents the following frequencies among healthy Shri Lankans of Tamil origin living in London: CC = 79.3 %, CT = 20.7 %, and TT = 0.0 %. Previous studies reported failure to find the homozygous mutant genotype TT in Shri Lankan Tamils and Moors[3, 35, 150] (*cf.* also ANGELINE *et al.*[5, 6] who examined South Indian Tamils). DISSANAYAKE[35] identified two 677TT homozygous Singhalese subjects (2.5 %). We found 2 Singhalese preterms, yet in

contrast and not in conformity with the previously known reports, we discovered 1 Tamil preterm being homozygous for the T allele; although this last observation has to be interpreted with caution as the number of the Tamil participants is to small to draw a general conclusion and can only give a first general indication. An individual with a 677TT haplotype has always been reported to have a 1298AA haplotype and *vice versa*[176]. In our analyses, all three participants with a 677TT haplotype had a 1298AA haplotype; however conversely, out of the subjects of the study population with a 1298AA haplotype (n = 75 / 36.6 %), 29 subjects (38.7 %) had 677CC haplotype and 25 (33.3 %) had a 677CT genotype. The low frequency of the 677T allele in DISSANAYAKE's study[35] coincides with the general population data of Shri Lankans in the previous studies mentioned above when compared with Caucasians. Although of no statistical significance, the 677T allele tended to be more common among the Singhalese than the Tamil or the Moorish population and the three main Shri Lankan ethnic groups are more closely related to each other when compared to European Caucasians. Overall, all these results are admittedly broadly in agreement with previously published works[125] on the population structure of the different ethnic groups in the Shri Lankan population. PRASMUSINTO *et al.*[132] in a study on MTHFR C677T and pre-eclampsia in two populations, too, found significantly lower rates of the MTHFR 677T allele among South-Asian patients (Indonesia), which were among the lowest reported for a human population. No association of the MTHFR C677T polymorphism with pre-eclampsia on the maternal or foetal level was reported in their investigation. Based on the low rates of MTHFR 677T homozygotes in Indonesians with normal and pre-eclamptic pregnancies, the investigators excluded a major influence of this genetic variant on the aetiology of pre-eclampsia in Indonesia. The findings overall further underline the need for a clear separation of samples according to ethnicity in the case of the study of disease associations of functional genetic polymorphisms.

Table 26: Overview of frequencies of C677T polymorphisms among Shri Lankan and Indian populations

Authors	**Objectives**	**Study Population and Locality / Setting**	**Findings** (*Genotypes and / or alleles*)	**Remarks**
SCHNEIDER *et al.*[150] (1998)	Screening of 881 unrelated individuals from 16 populations worldwide for the presence of the C677T polymorphism to complement existing data set	*Shri Lankans*: n = 67 Locality: Shri Lanka	*677T allele* 4.5 % (CC: n = 61 / 91 %; CT: n = 6 / 9 %; TT: n = 0 / 0 %) [MTHFR polymorphism in every population tested, C677T mutation with relatively high frequency worldwide (Africa: 6.6 %; Europe: 18.6 %; Asia: 20.8 %; indigineous Brazilian population: 44.9 %)]	No differentiation between the affiliation to one of the three major ethnic groups in Shri Lanka
ALAGRATNAM *et al.*[3] (2000)	Examination of Hcy, folate, MTHFR genotype for assessment of prevalence of abnormalities. Exclusion criteria: known diabetes	(healthy) *Shri Lankans*: n = 114 (male = 60, female = 54) Locality: London, UK	*677T allele* at 10 % (C/C: 79.3 %; C/T: 20.7 %; T/T: 0.0 %) [East Asian coastal communutes similar; ≈ 34 % frequency in various European communities]	No differentiation between the affiliation to one of the three major ethnic groups in Shri Lanka
DISSANAYAKE *et al.*[35] (2006)	Study to establish the frequency of the alleles of the MTHFR 677C→T poly-morphism in the three major racial groups in the Shri Lankan population, to establish whether the genetic susceptibility differs between these different Shri Lankan racial groups, and to compare the Shri Lankan frequency results with the frequency in white Western Europeans	*Shri Lankans*: n = 240 (Singhalese: n = 80; Indian Tamils: n = 80; Moors: n = 80) [male = 50 % each group] Locality: Shri Lanka	*genotype CC* (Sinhala: n = 61 / 77%; Tamils: n = 65 / 82 %; Moors: n = 65 / 82 %) *genotype CT* (Sinhala: n = 16 / 20%; Tamils: n = 14 / 18 %; Moors: n = 14 / 18 %) *genotype TT* (Sinhala: n = 2 / 3 %; Tamils: n = 0 / 0 %; Moors: n = 0 / 0 %) *677T allele* (Sinhala: 13 %; Tamils: 9 %; Moors: 9 %)	The authors were the first to differentiate between the three main Shri Lankan racial groups (*i.e.*, Singhalese, Tamils, Moors)
ASHAVAID *et al.*[9] (2002)	Gene polymorphism and coronary risk factors in Indian population (Review)	*Indians*	*Thermolabile heterozygous mutation (CT genotype) to be 4.34%* at 4.34 %	
ANGELINE *et al.*[5] (2004)	Analyses of MTHFR (A1298C and C677T) genotypes among Tamils and comparison of the frequency of the genotypes between patients with acute myocardial infarction (AMI) and normal individuals	*Tamils (in India)*: n = 72 (AMI patients: n = 52; healthy controls: n = 20) Locality: Tamil Nadu, South India	*CC genotype* (total: n = 58 / 80.6 %; AMI: n = 41 / 78.85 %; contr.: n = 17 / 85.0 %) *CT genotype* (total: n = 13 / 18.1 %; AMI: n = 10 / 19.23 %; contr.: n = 3 / 15.0 %) *TT genotype* (total: n = 1 / 1.38 %; AMI: n = 1 / 1,92 %; contr.: n = 0 / 0.0 %) *C allele* (total: n = 58 / 80.6 %; AMI: n = 41 / 78.85 %; contr.: n = 17 / 85.0 %)	
ANGELINE *et al.*[6] (2007)	Prevalence of C677T and A1298C polymorphisms in healthy Tamilis and in patients with acute myocardial infarction (AMI) with reference to plasma homocysteine concentrations, serum folate, serum cobalamin and riboflavin status; comparison of influence of genetic and nutritional factors on homocysteine levels in order to see whether MTHFR gene mutation could be risk factor for AMI among young and middle-aged Tamils	*Tamils (in India)*: n = 200 young, middle-aged (<48 years) (AMI patients: n = 100; healthy controls: n = 100) Locality: Tamil Nadu, South India	*CC genotype* (total: n = 165 / 82.5 %; AMI: n = 81 / 81 %; contr.: n = 84 / 84 %) *CT genotype* (total: n = 34 / 17 %; AMI: n = 18 / 18 %; contr.: n = 16 / 16 %) *TT genotype* (total: n = 1 / 0.5 %; AMI: n = 1; contr.: n = 0)	

In South India, with lower dietary intake of folate, MTHFR deficiency as risk factor may possibly be associated with a number of defects, for example vascular events and NTD[175] or Down's syndrome[134].

The analysis of the MTHFR gene A1298C in this study yielded the following results: n = 67 (32.7 %) for the wild type, n = 96 (46.8 %) for the heterozygous type, and n = 42 (20.5 %) for the homozygous type. The MTHFR gene variants we analysed did not seem to contribute to the occurrence of IUGR (data not given), but we cannot dispute the possibility that variants of these genes will act synergetically. Also, neither MTHFR C677T nor MTHFR A1298C polymorphisms were significantly associated with high tHcy and folate levels in SGA and AGA preterms, probably because of the maternal use of folic acid supplementation. In comparison, a study of term infants[114] also demonstrated no significant effects of foetal plasma folate and MTHFR 677 C → T genotype; in this case, the mothers were also given folic acid supplementation. Studies including higher numbers of neonates with thrombophilic disorders are definitely warranted in order to address this issue. Data regarding maternal thrombophilia were not available.

On the background of previous data which have demonstrated associations between thrombophilic polymorphisms in pregnant women and an increased risk for SGA in their offspring[11], we assessed the prevalence of polymorphisms associated with thrombophilia in the study population. Our results overall demonstrate neither significant associations between MTHFR C677T or A1298C and impaired intrauterine growth nor between these polymorphisms and tHcy concentrations. These findings seem to be in line with previously published data. In a study by van BEYNUM *et al.*[172], the MTHFR 677 C → T polymorphism did not significantly influence tHcy concentration. INFANTE-RIVARD *et al.*[71] reported no risk for elevated tHcy concentrations or IUGR in preterms being homozygous for C677T or A1298C. They also found little or no indication that thrombophilic genes have an effect on IUGR[73]. Furthermore, KENET *et al.*[82] reported no association of thrombophilic risk factors with increased risk of any perinatal complications.

4.3.2 Homocysteine in non-IUGR and IUGR Preterm Neonates

The metabolic pathways of Hcy have been described elsewhere (*cf.* Chapter 1.3.2). Plasma tHcy concentrations are known to be regulated by the levels of folate, vitamin B_{12}, vitamin B_6, and other determinants. Some supplemental protocols with these vitamins and folic acid have been proposed to be effective in patients with extremely high tHcy levels and certain obstetrical complications[33]. Plasma tHcy concentrations are generally lower in pregnant women than in non-pregnant individuals, probably as a result of hormonal

changes associated with pregnancy[66], although an elevated plasma tHcy concentration has been identified in pregnant women with a history of IUGR or with a diagnosis of various adverse pregnancy outcomes[22]. HHcy, defined as a moderately elevated tHcy concentration, is an established independent risk factor for arterial vascular disease and venous thrombosis in adults[169]. Data on this relation in childhood are scarce. KOCH *et al.*[84] linked elevated tHcy to an increased risk for venous thrombosis in children such as sinus venous thrombosis or renal venous thrombosis. NOWAK-GÖTTL *et al.*[118] in their prospective nationwide two year case registry study calculated the incidence of symptomatic neonatal thromboembolism in Germany to be 5.1 per 100,000 births (n = 79). 23 children received supportive treatment, 42 received heparin, and in 13 neonates, thrombolytic agents were administered; most neonates survived (91 %).

So far, detailed studies on the normal range of plasma tHcy concentrations have been published for adults[167]. UBBINK *et al.*[167] suggested a reference range of 4.9 - 11.7 µmol / L. Regarding tHcy concentrations in childhood, only few studies have been performed[137, 179]. A study from the Netherlands[172] cross-sectionally analysed samples from 234 Caucasian, apparently healthy children aged 0 - 19 years with the objective to describe age-specific tHcy concentrations and its predictors. The investigators observed great variations in tHcy concentrations during the first months of life. Therefore, for newborns, and particularly for those who are born prematurely, separate reference ranges should be established[95, 151]. REFSUM *et al.*[139] determined the serum concentrations of tHcy and related variables in 4,992 capillary blood samples collected anonymously as part of the routine metabolic screening program in newborn children in Norway. The mean tHcy concentration was 7.5 µmol / L (SD: 3.0; median: 6.8 µmol / L), 43 probands had a concentration of > 15 µmol / L. This signifies a relatively high mean value. The authors, however, did not discuss whether these tHcy values were still influenced by maternal metabolism as this did not form part of the study design[139]. They specified that data on metabolic changes during the first few days of life in full-term infants were not available, although in newborn premature infants, tHcy tends to increase, probably as a result of parenteral nutrition [68]. Furthermore, the authors had no information about the gestational age, the gender of the newborn infant, nor about the day after birth when the blood was collected and the time from blood collection until the samples were frozen; also, the samples in the Norway study were kept unfrozen for about 14 days, which could have affected concentrations of metabolites or vitamins and which may have weakened the associations. In conclusion, the authors suggested that screening for tHcy and related factors should be further evaluated in regions with high prevalence of homocystinuria and in newborns at high risk for vitamin B_{12} deficiency.

In this study, we found a significant association between elevated tHcy concentrations and SGA preterm neonates. SGA preterms (n = 62) exhibited a higher tHcy level when compared to AGA infants (n = 120) of comparable GA. The elevated tHcy status remained significant after adjustment for birth weight (median: 5.17 µmol / L *vs.* 4.58 µmol / L, $p = 0.016$). The differences in our study were more pronounced in SGA newborns of earlier GA (< 36 weeks, $p = 0.005$), there was no statistical significance in SGA infants born after 36 weeks of gestation. The following factors may be operative: All mothers in our study were prescribed folic acid supplementation according to standard prenatal care in Shri Lanka (*cf.* Chapters 3.1 and 5.4). The higher concentrations of tHcy in SGA neonates are, therefore, most likely related to premature activities of enzymes that catabolise Hcy or to the reduced capacity to utilise the available cofactors folate and vitamin B_{12}. The differences between SGA and AGA infants disappeared after 36 weeks of gestation which might suggest a higher tHcy concentration in spite of folate supplementation and a relatively high vitamin B_{12} status. However, the regular intake of the folate supplementation was not documented throughout, the intake at erratic intervals may, therefore, be reflected in the results.

Some studies have established such an association between increased tHcy levels and IUGR in newborns and others have not. Yet, the cord blood tHcy concentrations in the participants are broadly consistent with data from Western European study populations (*cf.* Table 27). In a study by MURPHY *et al.*[115], the foetal and maternal tHcy correlated significantly throughout the study. They demonstrated that the ORs for lower birth weight was significantly higher for infants born to mothers in the high tHcy tertile at 8 and 20 weeks of pregnancy and at labour and for infants in the highest foetal cord tHcy tertile compared with infants born to mothers in the low-medium tertiles and in the lower tertiles for foetal cord blood tHcy. The present study showed no correlation between plasma folate and serum vitamin B_{12} concentrations, neither in AGA nor in SGA preterms (data not given). We attribute this to the fact that folate was not only taken from food sources like vitamin B_{12}. This reinforces the results obtained by MURPHY *et al.* who did not find any correlation between their plasma levels. In addition, mothers who took folic acid supplementation during pregnancy had lower tHcy status at labour than unsupplemented mothers as did their neonates. Compared with this, however, HONGSPRABHAS *et al.*[68] have shown that the plasma tHcy concentration of preterm infants was significantly less than that of term infants ($p < 0.05$) albeit a small number of values. INFANTE-RIVARD *et al.*[72] suggest that mothers with small infants have lower tHcy concentrations than those giving birth to larger neonates.

By way of comparison, we present reported concentrations of tHcy, folate, and vitamin B_{12} concentrations in neonates from different countries in Table 27.

Table 27: Overview on concentrations of homocysteine, folate, and vitamin B_{12} in newborn infants from different countries

Population		Homocysteine (*µmol / L*)	Folate (*nmol / L*)	Vitamin B_{12} (*pmol / L*)	Reference
Shri Lanka (*this thesis*)	Term	---	---	---	---
	Preterm (*AGA*)	4.58 (3.27 – 6.72)	45.1 (23.6 – 183.5)	356 (210 - 703)	
	Preterm (*SGA*)	5.17 (3.66 – 8.87)	44.4 (25.0 – 246.8)	405 (229 – 837)	
Germany	Term	---	---	---	LINDNER U *et al.*[100]
	Preterm (*AGA*)	5.96 (1.92 – 15.27)	30.0 (12.8 – 90.2)	---	
	Preterm (*SGA*)	5.06 (3.11 – 10.7)	32.7(10.8 – 194.5)	---	
Germany	Term	5.37 (3.6 – 7.7)	58.6 (36.2 – 91.8)	253 (154 – 427)	OBEID R *et al.*[120]
	Preterm	5.6 (3.2 – 7.8)	60.0 (42.1 – 111.7)	210 (128 – 445)	
	IUGR (incl. > 37 wk)	5.2 (3.2 – 8.3)	57.1 (24.8 - …)	298 (186 - …)	
The Netherlands	Term	---	---	---	RAIJMAKERS MTM *et al.*[135]
	Preterm (*non-IUGR*)	---	---	---	
	Preterm (*IUGR*)	9.6 (4.8 – 17.4)	---	---	
The Netherlands	Term[c)]	5.1 (4.6 – 5.6)	79 (60 – 104)	439 (326 – 591)	van BEYNUM IM *et al.*[172]
	Preterm (*non-IUGR*)	---	---	---	
	Preterm (*IUGR*)	---	---	---	
USA	Term[a)]	4.49 (± 1.78)	---	---	MALINOW M R *et al.*[104]
	Preterm (*non-IUGR*)	---	---	---	
	Preterm (*IUGR*)	---	---	---	
Norway	Term[b)]	6.22 (5.00 – 7.48)	27.0 (20.4 – 36.3)	314 (238 – 468)	MONSEN A-LB *et al.*[19]
	Preterm (*non-IUGR*)	---	---	---	
	Preterm (*IUGR*)	---	---	---	
Brasil	Term	6.0 (2.3 – 15.3)	27.9 (15.2 – 37.8)	199 (80 – 1,350)	GUERRA-SHINOHARA EM *et al.*[57]
	Preterm (*non-IUGR*)	---	---	---	
	Preterm (*IUGR*)	---	---	---	

Data are median values; in brackets: 10th and 90th percentiles, except where otherwise stated
a) mean value ± SD (paired *t*-test, $p < 0.001$)
b) children, 4 days old
c) children aged 0 – 1 year, values are geometric mean, 95 % CI in parentheses
d) values are mean ± SD

4.3.3 Role of Folate and Vitamin B_{12} as Determinants of Homocysteine Concentrations

In children and adults, folate is a strong determinant of tHcy concentration[19]. Some[57], but not all studies[18], have also found a significant association in newborns. The generally high folate concentrations in infants may explain the relatively weak tHcy–folate relationship[19]. The MTHFR 677C → T polymorphism is a strong determinant of tHcy in older children and

adults[77], but not in children < 10 years. Our data now show that tHcy was not associated with serum folate or with the 677 C → polymorphism.

Vitamin B_{12} is an established essential nutrient in the diet of humans, particularly during pregnancy when it is necessary for normal foetal development. Malabsorption is a leading cause of cobalamin deficiency. If untreated, vitamin B_{12} deficiency results in an irreversible decline in cognitive function and memory[23]. Vitamin B_{12} deficiency has a role in elevating plasma tHcy and lowering methyl donor levels in pregancy and has methyl donor levels in pregancy and has been implicated in adverse pregnancy outcomes including LBW[181]. Methionine synthase is an enzyme that catalyses the methylation of tHcy to methionine using vitamin B_{12} as a cofactor and MTHF as a substrate. A deficiency of either vitamin B_{12} and / or folate is likely to affect this important pathway resulting in an elevation of plasma tHcy with a relatively low methionine level. Maternal vitamin B_{12} status has been demonstrated to correlate to neonatal vitamin B_{12} status, as measured by cord serum vitamin B_{12}[160].

We compared folate concentrations in SGA (median: 44.4 nmol / L) and AGA (median: 45.1 nmol / L) preterms and found no statistically relevant deviations. Also, the correlation between folate and tHcy status was not statistically significant. Our findings are not consistent with a study on pregnant women from Pakistan[98] largely belonging to the middle and low socio-economic strata. In that study, the folate status in preterm IUGR infants was 42 nmol / L (median) and in non-IUGR preterms 25 nmol / L (median). However, there was an inverse correlation between cord blood folate and tHcy levels (r -0.26, $p = 0.006$). A possible reason behind the diverging results of our and of the Pakistan study may be the high dose of folic acid received by the participating mothers (*cf.* Chapters 3.1 and 5.4), whereas such a medical intervention was obviously not available to the women in the Pakistan study. Another reason may also be the practice of prolonged cooking in South Asia and among South Asians in Britain, which destroys 90 % of the folates[98]. Interestingly, the results of an observational study[158] in North Carolina, USA, support the hypothesis that low maternal folate levels during the second trimester of pregnancy, as measured by both diet and biological markers, are associated with an increased risk of preterm birth; very little evidence of potential confounding by other factors was found. The mothers who participated in our study were given folic acid tablets (5 mg / d). In addition, all mothers delivered before the 37th week of gestation. Moreover, the singleton preterms born between 29 and 32 weeks of gestation in our study showed markedly higher folate concentrations when compared with the preterm neonates born at a GA > 32 weeks, although the differences did not reach statistical significance (*cf.* Figure 14). A possible explanation for the lower folate status after 32 weeks of

gestation may be the uptake of folate during proliferation of stem cells in foetuses and the growth of the foetus *in utero*. These differences clearly warrant further investigations and explorations of the exact biological mechanism for how maternal folate status may or may not lead to a preterm birth.

We did not measure vitamin B_{12} concentrations in maternal blood because the study was not designed for this purpose. However, when compared to normal B_{12} levels in cord blood and maternal blood in other studies, we might exclude that the volunteering pregnant women were vitamin B_{12} deficient. Our study yielded no significant difference between birth weight and vitamin B_{12} status (AGA [median]: 356 pmol / L *vs.* SGA [median]: 405 pmol / L, $p = 0.098$). Previous studies on vitamin B_{12} status and its effect on birth weight have produced conflicting results. In 2005, YAJNIK *et al.*[192] pointed out an association between higher plasma tHcy concentrations and LBW in rural Indian women, but the authors could not establish a relationship between maternal vitamin B_{12} and offspring size; although there was a significant inverse relationship between vitamin B_{12} status and plasma tHcy status. The authors concluded that the lack of an association might have been due to an overall low vitamin B_{12} status. Compared with this, a recent study[116] examined cord serum vitamin B_{12} concentrations in relation to the BW of Indian neonates. Low maternal vitamin B_{12} status translated into a low neonatal vitamin B_{12} status as evinced by cord serum vitamin B_{12} concentrations. Neonates with lower birth weights had significantly lower mean cord serum vitamin B_{12} concentrations when compared to those with a BW ≥ 3,000 g. Furthermore, cord vitamin B_{12} concentrations were significantly correlated with birth weight up to 40 weeks of gestation, but not beyond ≥ 40 weeks of pregnancy. The authors hypothesised that the neonatal vitamin B_{12} status - birth weight relationship seems to operate up to a term gestation of 40 weeks and that beyond this age, no relationship between neonatal vitamin B_{12} status and birth weight would be operative. Also, this observation seems to be confirmed by an earlier study from France by FRÉRY *et al.*[43], who found a weak negative correlation between BW and cord vitamin B_{12} levels ($p < 0.04$). Interestingly, the correlation between vitamin B_{12} status and BW was remarkable in mothers who continued smoking during pregnancy ($r_{cord\ B12}$: $p < 0.05$, $r_{maternal\ B12}$: $p < 0.02$). There is some amount of evidence that birth weight is not influenced by a first trimester smoking exposure (WHO)[cited by 43]. FRÉRY *et al.* confirmed this and also reported the observation of a significant difference between the mean cobalamin levels of women who smoked during the first trimester and of women who did not smoke during their pregnancy.

The effects of small maternal size before and during pregnancy on LBW are well established[189]. A recent study from India[117] demonstrated associations between

educational status, maternal weight and gestational weight gain with IUGR. Maternal weight < 45 kg, the lowest quartile in early pregnancy in the Indian study[117], carried with it a high risk for IUGR, which contrasts with the observation in our study as we could not establish a significant association between low BMI and an increased risk for SGA (SGA: 20.5 - AGA: 21.5; values are median). However, we found a strong significant association between mothers with low weight gain during pregnancy and SGA births ($p = 0.005$), which confirms earlier findings that low weight gain for even brief periods during pregnancy places the foetus at risk for SGA. Similarly, in the Indian study cited, the majority of the mothers who delivered IUGR neonates had low weight gain per week in the second trimester.

Furthermore, our analyses showed higher folate and vitamin B_{12} concentrations in SGA preterms, although they were not significant determinants of tHcy concentrations. A correlation between folate and tHcy was absent both in SGA and AGA preterms. Only vitamin B_{12} but not folate was a significant modulator of tHcy concentrations in AGA preterm group. In this context, contrasting results from a study in Brazil by GUERRA-SHINOHARA *et al.*[57] should be mentioned, even if they did not explicitly examine IUGR newborns or preterm infants (GA of infants: ≥ 37 and ≤ 42). They detected a significant correlation between maternal and cord blood tHcy. Neonatal tHcy levels were affected by neonatal vitamin B_{12}. These observations support the conclusion that lower vitamin B_{12} and folate levels are associated with higher tHcy in pregnant women and their newborn infants. No correlation was observed between birth weight and biochemical markers (*i.e.* vitamin B_{12}, serum folate, tHcy). A possible explanation could be that - projected onto the background of an underdeveloped country where chronic nutritional deficiency exists side by side with poverty and thus preventing the more underprivileged population from obtaining a diversified diet - pregnant women with low vitamin B_{12} levels seem to be unable to provide the necessary amounts of this vitamin to their foetuses, as occurrs in folate deficiency. The poverty condition during pregnancy and inadequate maternal nutritional status may have been the cause of lower vitamin B_{12} concentrations in cord blood, since the neonatal vitamin B_{12} value in the Brazilian study (median: 199 pmol / L) was lower than that reported by FRÉRY *et al.*[43], who showed that cord blood vitamin B_{12} levels of the newborns (median: 438 pmol / L) were two-to threefold higher than the maternal levels.

We found marked differences between the three ethnic groups of the population in this study. SGA Singhalese preterms had significantly higher tHcy and vitamin B_{12} concentrations than their AGA peers. This could not be observed in the Tamil and the Moorish study cohort. Remarkably, however, is the finding that only in the Tamil study

group a tendency towards higher tHcy levels with concurrent low folate and vitamin B_{12} levels could be observed, possibly because of the low number of subjects.

Regarding folate, tHcy and vitamin B_{12} levels, birth weight and GA, gender effects between male and female SGA infants were not observed. A gender effect between AGA male and female newborns was determined as female infants had significantly higher folate concentrations than their male counterparts. When comparing SGA and AGA male infants, we initially found significantly higher tHcy concentrations in SGA males, but there was no statistical siginificance after adjustment for GA.

When assessing the vitamin levels of the singleton preterms in relation to previous miscarriages among the parturients, the analysis showed no significant effect except for folate; thus, the cord blood levels of folate in the preterms were significantly higher when their mothers had endured previous miscarriages (median: 51.4 *vs.* 43.5 nmol / L, $p = 0.013$; *cf.* Table 24). We also calculated higher vitamin B_{12} levels in this cohort, they did not reach statistical significance (median: 389 *vs.* 363 pmol / L, $p = 0.459$; *cf.* Table 24). It has been reported in the literature that cord blood levels of folate, vitamin B_{12}, and tHcy in infants correlate significantly to the maternal levels. In 1965, MARTIN *et al.*[106] reported that serum folate was low in women who had a history of spontaneous abortion. HÜBNER *et al.*[70] determined significantly decreased serum vitamin B_{12} levels in Syrian patients with recurrent abortion when compared with controls (mean: 176 *vs.* 222 pmol / L); folic acid levels were slightly higher in aborters than in controls (mean: 20.4 *vs.* 19.0 nmol / L). As the mothers who volunteered for this study took folic acid tablets (5 mg / d), our calculations may most likely be attributed to the increased awareness of the importance of folate supplementation. In a German parallel study[100] (*cf.* Table 27 and Chapter 6.2.C), tHcy concentrations were not significantly increased in SGA preterms. Folate concentrations in the German SGA preterms were lower than in the Shri Lankan controls, but were still within the normal range; the higher folate status in the Shri Lankan preterms could be attributed to the periconceptional folic acid supplementation.

4.3.4 Pregnancy-Induced Hypertension, Homocysteine, Folate and Vitamin B_{12} Status and Their Association with Intrauterine Growth Retardation

PIH and further pregnancy hypertensive disorders in general continue to be one of the unresolved problems of modern obstetrics, with important implications for maternal and perinatal morbidity and mortality. Placental vascular insufficiency, associated with spiral

artery atherosclerosis and placental thrombosis, is the unifying pathophysiological feature found among obstetric complications such as IUGR, unexplained intrauterine foetal death, placental abruption and PIH[142]. As a consequence, uteroplacental blood flow, renal plasma clearance, and cerebral blood flow are decreased, whereas peripheral vascular resistance is increased, intravascular fluids are leaked into the interstitial space, and injuries are caused to various visceral organs producing hypoproteinaemia, increased transaminase levels, and clotting alterations. As a result, the incidence of preterm deliveries and especially of IUGR are increased as well as the incidence of depression in the newborns, the necessity for neonatal hospital admission, general morbidity in the newborns, and foetal and neonatal mortality. It has morever been suggested that there is an ethnic variation in the response of vessels to hypertension and possibly to placental function[26].

Although IUGR is a major cause of neonatal mortality and long-term morbidity, the frequency of such retardation in pregnancies complicated by PIH alone is a matter of controversy. There are only few studies available addressing the issue of IUGR in association with folate, vitamin B_{12} and tHcy levels as well as with pregnancy complications in pregnant women in developing countries. In our study, SGA infants from mothers with PIH had significantly higher tHcy ($p < 0.001$) and vitamin B_{12} ($p = 0.013$) concentrations and a strong tendency towards lower folate levels compared to SGA babies from normotensive mothers. Possible mechanisms could be:

- Renal metabolism of filtration of tHcy may be reduced in the mother and the infant.
- Oxidative stress in PIH may inflict effects on the transsulphuration pathway to produce more glutathione. More vitamin B_6 is required in this case; however, we did not test vitamin B_6 concentrations.
- In PIH, oxidative stress might cause a *shift in Hcy metabolism* from the remethylation to the transsulphuration pathway resulting in lower folate consume for Hcy remethylation (folate retention); in this case, supplementation with 5 mg / d folic acid which the mothers receive in Shri Lanka is not sufficient to remethylate Hcy to methionine, and we hypothesise that supplementation with vitamin B_6 could be important to prevent HHcy in case of PIH.
- Hypertension may reduce the excretion of Hcy via the mother's kidney or hypertension may reduce the removal of tHcy from the foetus to the mother via arterial blood and hyperfiltration.

Our observations are in line with previously reported results. In a prospective cohort study in an area known to have high levels of micronutrient deficiencies, pregnancy

complications, and IUGR (Lahore, Pakistan), LINDBLAD *et al.*[99] found significantly higher tHcy concentrations in South Asian women from a low socio-economic strata who developed hypertension in pregnancy, they were at an increased risk for giving birth to a SGA and / or preterm neonate. It is also known from previous studies that cord blood and tHcy status of the mother correlate strongly. According to the authors, one explanation for the differing results could be that the causes behind IUGR could be substantially different in the various socio-economic and geographic groups.

Another research finding of our analyses was that in the second trimester, SGA preterm singletons of mothers with PIH exhibited increased tHcy concentrations, compared to their AGA counterparts (5.74 *vs.* 4.65 µmol / L). In the literature, studies investigating the association between midtrimester maternal plasma concentrations of tHcy in asyptomatic women and subsequent development of pre-eclampsia or IUGR have reported variable results. For comparison, contradictory results were published by HOGG *et al.*[66]. According to the authors second-trimester plasma tHcy concentrations do not predict subsequent development of PIH and IUGR. This supports our suggestion that tHcy is a result of PIH and not causally related to PIH. Although plasma tHcy level may be elevated when a women has PIH or pre-eclampsia diagnosed or immediately before development of such a condition, they inferred from their evidence that elevated plasma tHcy concentration is not a useful marker for the subsequent development of PIH, pre-eclampsia, or IUGR. ONALAN *et al.*[121] obtained significant values for plasma tHcy within a study population with IUGR or pre-eclampsia, no significance was found for vitamin B_{12} or folate status in the same group. PIH was associated with an significantly increased risk for IUGR (adjusted OR 1.49 [1.14 – 1.93]) in a study by XIONG *et al.*[190], the risk for LBW was not increased, perhaps because of a slightly longer gestation and a lower rate of preterm birth in their patients. By contrast, pre-eclampsia and severe pre-eclampsia significantly increased the risk for IUGR and LBW. Our results demonstrated a significant difference in tHcy between SGA babies born to mothers with PIH compared to SGA infants from mothers without PIH ($p < 0.001$). Recent data from Spain[2] indicate that perinatal mortality in preterm and term neonates is increased by the factor of 3 – 5 in patients with PIH compared with pregnancy outcomes of age-matched women with otherwise comparable risks. In our study group, there were no stillbirths. Similarly, in a study from France[131], which included important covariates such as parity, maternal age, smoking habits, and prior LBW and considered timing of the insult, the onset of PIH between the 27th and 36th completed week of pregnancy was found to be associated with the highest SGA rate ($p < 0.005$), although the definition used for SGA birth weight was somewhat unusual (*i.e.*, value less than the fifth percentile of expected weight, according to the distribution of BW in relation to GA and adjusted for maternal weight) and SGA almost certainly represented severe growth retardation; the

rate was stronger if there was an early onset of the insult. CHAKRAVORTY *et al.*[26] in a study of Malaysian women suggested no effect of PIH on foetal growth, as measured by BW, *i.e.* PIH does not decrease BW: infants born to hypertensive cases (mean: BW = 2,724 g) compared with those born to controls (mean: BW = 2,805 g). Pre-eclampsia was found to be associated with a significant reduction of mean BW in the same study (more than twice standard error of the difference). The results may, however, reflect a brief period of compensation, because this effect may be mediated through uteroplacental blood flow.

Some studies in this context have suggested that chronic intrauterine hypoxia occurs and that the cause of growth retardation in some foetuses may be inadequate placental transfer. In a study of growth-retarded foetuses (n = 38) by SOOTHILL *et al.*[160], the oxygen tension was below the normal mean for GA in 33 cases. Oxygen tension measurements in all growth-retarded foetuses were significantly lower compared to normal foetuses (n = 150). There were significant positive correlations with hypercapnia, hyperlacticaemia and nucleated red cell count. The lack of a correlation between hypoxia and haematocrit and reticulocyte concentration was surprising, as a positive correlation between hypoxia and red cell count had earlier been demonstrated in normal human foetuses indicating an erythropietic response, which would tend to maintain blood oxygen content. Foetal hypoxaemia is a trigger for erythropoietin release and stimulation of red blood cell production resulting in polycythaemia[11]. Elevated nucleated red blood cell counts correlate with metabolic and cardiovascular status and are independent markers for poor perinatal outcome.

Thus, PIH represents a major threat to pregnant women and foetuses as well as neonates. Treatment options are limited, as many antihypertensives may negatively affect the foetus. As the occurrence of PIH early in the third trimester is particularly dangerous to the foetus, it is advisable to screen for hypertension during that period as it would permit a closer supervision of subsequent foetal growth.

4.3.5 Multiple Births

In developed countries, the rate of multiple births has generally increased since 1975. This has been attributed to both the higher proportion of mothers treated with ovulation-inducing hormones and the increasing use of *in vitro* fertilisation[122]. Overall, only little information is available, partly because the lack of population-based twin registries makes it difficult to collect growth data on twins. Some studies on the physical growth of twins in childhood have been published for Western countries including WILSON's detailed summary of the features of twin growth[28, 187]. In twin pregnancies, 15 -30 % are associated with IUGR and premature delivery[58].

Preliminary data on 11 pairs of twin or triplet preterms (SGA – AGA, SGA – SGA, AGA – AGA) in our study show that pairs had very similar concentrations of tHcy, folate and vitamin B_{12}. Although tHcy values seemed to be higher when at least one of the infants was growth-retarded, we were not able to establish a causal relationship between elevated tHcy status and SGA. This was a surprising observation because we have demonstrated a significant difference of the tHcy, folate and vitamin B_{12} status in singleton preterms (SGA – AGA).

4.4 Strengths and Limitations of This Study

The principal strengths of the present study are its prospective design and enrolment of a non-selected cohort of pregnant women who received supplementation with folic acid tablets and who refrained from prenatal smoking and alcohol abuse, also the inclusion of preterm neonates with different patterns of intrauterine growth. To the best of our knowledge, this is the first study to investigate SGA in relation to tHcy, folate, and vitamin B_{12} concentrations as well as to frequencies of MTHFR C677T and A1298C polymorphisms in venous umbilical cord blood of Shri Lankan preterm neonates; furthermore, the influence of PIH on these variables was elucidated for the first time. Another strength of the present study is the high number of individuals included as there were 205 preterm neonates with a GA < 37 weeks, also the inclusion of preterm neonates with different patterns of intrauterine growth. This allowed us to examine AGA and SGA infants of the same GA and to detect possible differences of tHcy concentrations in cord blood. In comparison with the parallel German study already mentioned above[100] (*cf.* Table 27, Chapters 4.3.3 and 6.2) as well as with further reports in the literature, our study overall confirms a different pattern of MTHFR genotypes in different ethnic groups.

Some limitations may apply to this study: We have not determined all possible confounding factors such as vitamin B_6 levels and have not addressed the effect of vitamin B_6 status on tHcy. Also, we did not measure other likely important variables such as maternal tHcy as the primary predictor of blood tHcy in the developing foetus, maternal folate and vitamin B_{12} levels. It has been demonstrated previously that pregnant women with low vitamin B_{12} levels were unable to provide the necessary amount of vitamin B_{12} to their foetuses[57]. It is known from previous studies that cord blood and tHcy status of the mother correlate strongly[99]. Hyperhomocysteinaemia at the time of delivery has also been demonstrated in a high percentage of pregnancies complicated by growth impairness[33, 75], however previous studies reported differences in plasma tHcy levels at 26 compared to 37 weeks of gestation. The study by JIANG *et al.*[78] further indicated that tHcy levels in SGA *vs.* AGA infants were not different until development of PIH. It was, too, reported that

status of B-vitamins in women varied significantly depending on the season in which blood was sampled[78].

As there were only a small number of Tamil and Moorish subjects (20 Tamil and 18 Moorish neonates), our observations should be validated by future research.

4.5 Conclusions

One novel finding of our study was that we were able to demonstrate higher concentrations of tHcy only in IUGR preterm neonates. The tHcy levels were elevated and there also was a relatively high vitamin B_{12} status, although all mothers received folic acid supplements during pregnancy. Therefore, these differences seem to be related to premature activities of enzymes that catabolise tHcy or to the inability of not fully developed organs to utilise the available cofactors folate and vitamin B_{12}. The differences between SGA and AGA preterms disappeared after 36 weeks of gestation. This fact suggests that folic acid might induce the expression or stability of the metabolising enzymes (methionine synthase, MTHFR), compensating for the premature enzymes in SGA. We thus infer that elevated tHcy concentrations are probably not causally related to SGA.

Previous studies have demonstrated an increased tHcy status in women with PIH compared to normotensive pregnancies. Total Hcy concentrations in cord blood and maternal blood correlated strongly in previous reports. It was, therefore, a further novel observation in our study that SGA preterms from mothers with PIH exhibited higher tHcy, folate, and vitamin B_{12} concentrations compared to SGA infants from mothers without PIH. Moreover, PIH had no effect on tHcy levels in the AGA group. Potential mechanisms could include:

- Hypertension may reduce tHcy elimination via the kidney in the mother or may reduce the removal of tHcy via transplacental transport.
- Utilisation of Hcy may be impaired secondary to immaturity.

4.6 Implications for Further Studies

Further research is needed to determine the potential nutritional role of tHcy during intrauterine life and the mechanisms responsible for placental-foetal transport and use of tHcy in normal and pathologic conditions.

The effect of vitamin B_6 status on tHcy is not known in this population. In future studies, it would be worthwhile to concurrently investigate the role of vitamin B_6 on Hcy catabolism in PIH in this population.

This study did not assess pre-gravid levels of vitamin B_{12} and it may be important to ensure vitamin B_{12} repletion in the periconceptional phase. This is particularly important, since vitamin B_{12} stores can last for several years, allowing for considerable flexibility in the timing of the periconceptional supplementation.

5 *Excursus*: Health Care in Shri Lanka

5.1 General Information on Shri Lanka

Shri Lanka is a small island in the Indian Ocean, her position lies midway between the eastern and western halves of the Indian Ocean as intersection point on the crossroads of trade. The main island of Shri Lanka has a maximum width of about 224 km (140 miles) in the east-west direction and a maximum length of 435 km (272 miles) in the north-south direction. The southernmost tip of the island is situated only about 600 km (375 miles) away from the equator. The surface extension may be compared in size with Tasmania or Tawain. The island lies just off the coast of the outermost south of the Indian subcontinent. The southern and eastern coastal sectors open up towards the Indian Ocean, whereas the island is separated from the Indian mainland in the northwest only by the narrow and shallow Palk Strait and the Gulf of Mannar. Both Shri Lanka and India remain connected as they rise on the same small continental shelf which ends abruptly in the south and east - an indication of the geologic-tectonic link of the island to the Indian subcontinent. Thus, Shri Lanka is a detached portion of the Indian mainland and is part of its vast southern plateau, the Deccan. In historical times, there existed a landbridge between the two nations.

In surface configuration, the country has a central highland massif with peaks as high as 2,524 metres 8,281 feet) situated in the south-centre. It functions as climatic divide, and it is surrounded more or less by an intermediate zone of upland ridges and valleys at a lower elevation. The intermediate zone is in turn surrounded by an outer and lower zone of lowlands and plains. A coastal fringe consisting of sandbars, lagoons and small islands skirt the main island. The population was 19.7 million as recorded in 2005.

In relation to other developing countries, Shri Lanka has a favourable per capita gross domestic product (2002: US-$ 870), a high rate of literacy (1994: males 92.5 %, females 87.5 %), a life expectancy comparable with developed countries (1996 - 2001: male 70.7 years, female 75.4 years, and free national health service with resonable access.

5.2 The Three Main Ethnic Groups in Shri Lanka

Shri Lanka shows a striking individuality as to the ethnic groups, this is closely bound up with the island's historical development. Its interesting genetic diversity reveals both European and Asian origins. Three ethnic groups determine the picture: Singhalese, Tamils, and Moors. Not many traces of the prehistoric inhabitants and hunters have yet been discovered, but it is evident that stone age men inhabited the island several

thousand years before Christ. Ethnic and religious affiliation still are of great social, political and cultural importance. In the following remarks, we will briefly look at the ethnic groups relevant to our study.

Singhalese: The ethnic group of the Singhalese represents the majority of the population. Particularly in the ancient rice cultivation territories of the historical kingdoms of Anuradhapura and Polonnaruwa, in the hill country around Kandy and in southwestern part of the country, the Singhalese make up the predominant group. The Singhalese trace back their origin to North Indians of Aryan origin and language (North Indian script), they were of an early Indian Vedic religion. There is evidence that from about the 5th century before Christ onwards immigrants from North India founded settlements in Shri Lanka. The original home of these Indo-Aryan settlers is considered to be the Indus region of the north-western part of the Indian subcontinent. Subsequently, a second immigration took place from the Ganges valley in Bengal and Orissa. They were farmers and they maintained close relations with India for centuries.

Today's ethnic group developed from the fusion with the native population, but also from marriage with inhabitants of the neighbouring Indian kingdoms which led to a substantial miscegenation of Indo-Aryan elements with those of Dravidian South India. Later, influences by other ethnic groups, particularly by Arabian seamen, Malayan soldiers, and European colonial rulers became noticeable, too. Continous migration of the Singhalese within the island in the past and the formation of settlement centres with typical specific cultural characteristics led to a historic-geographically induced differentiation, which gave rise to the distinction between *lowland Singhalese* and *up-country* or *Kandyan Singhalese*. These subgroups have been clearly distinguished in domestic population statistics as well as in everyday usage. However, not ethnic but rather historic-geographical reasons account for this distinction. The low-country Singhalese constitute the part of this ethnic group that has settled in the coastal provinces on the west and south coast where they have been exposed to Arabian and western-colonial cultural influences. In comparison, the up-country Singhalese settled down in the territories of the former Kandyan kingdom and of the ancient Singhalese kingdoms of Anuradhapura and Polonnaruwa, where they put up a sturdy resistance for a long period; in spite of the final conquest in 1815, they refused to give up their ancient customs and bargained with the British invaders for the retention of their own code of laws. They were able to resist colonial tutelage so that the Kandyan Singhalese are regarded as preservers of the traditional Singhalese customs. Because of their long political sovereignty, they have been able to assert their cultural independence much longer than the lowland Singhalese.

Tamils: In ethnic and cultural-religious contrast to the Singhalese, the Tamils follow (about 20 % in 1985; 1946: 826,000 - 12.3 %; 1981: 1,886,864 - 12.7 %). They came to the island in large numbers only in the 11th century and they founded an independent kingdom in the north of the island. Their home country was the South Indian Tamil Nadu. Thus, the Tamils originate from the Dravidian cultural and traditional context; their language, Tamil, is considered to be the oldest language of the Dravidian family of languages. The origin of this northern-melanid race have to date been inadequately clarified. It has been assumed that the the Dravidians may have been pushed away southwards by Indo-Aryan immigration; recent linguistic evidence suggests that the population reached the subcontinent from Iran by sea well after the Aryan colonisation of North India, then subdued the native inhabitants of South India and assimilated them. The Shri Lankan Tamil population and settlement centres in Shri Lanka are located in the north, especially on the Jaffna peninsula and on the east coast. They maintained almost all of their Hindu culture from South India. In 1981, 42 % of the Shri Lankan Tamils lived in the Jaffna district, 21 % on the east coast and 9 % in Colombo. In the central hill country, the spatial extent of the tea plantation area corresponds with the distribution of the [Indian] Tamils (73 %).

Moors: Among the ethnic groups, the Moors represent a contingent of 1,046,927 - 7.1 % (1981; 1946: 455,000 - 7.0 %) of the island's population. They are descendants of Arabian seamen and merchants in the Middle Ages. As they never used to travel with women, they married to Tamil women in Shri Lanka. For the most part, they adopted the Tamil language from them and some of their customs, *e.g.* for marriage. Their contacts with Shri Lanka took place during two epochs and, accordingly again, two subgroups are distinguished: Shri Lankan Moors and Indian Moors. The settlement of the Shri Lankan Moors resulted from the trade relations with the island during the pre-colonial era, presumably in the 14th century. Centres of the Arabian merchants and seamen were concentrated around coastal areas, the Shri Lankan Moors have been permanent settlers. Like the Tamils, the Moors have always lived in separation from the Singhalese, adhering to the code of life of the Muslim religion and displaying several culturally exclusive features.

5.3 Public Health Care Provisions

The modern health care system was set up in the late phase of British colonialism with a network of medical facilities. The medical attendance was carried out free of charge for every citizen. The British colonial administration had undertaken initial steps already in the second half of the 19th century, this interest concentrated on the plantation area in the

southwest due to economic considerations. Their earliest hospitals were located in Colombo, Jaffna, Trincomalee, and Galle. Vaccination against infectious diseases commenced in Shri Lanka in 1802. The first general hospital was opened in Pettah, Colombo, in 1819 for paupers. In 1858, an independent Civil Medical Department emerged from the Native Medical Establishment, which had been operated unter the aegis of the British military, and it gradually took over almost all health facilities. The General (now: National) Hospital, Colombo, was established at its present location in 1864. The hospital provides for a number of specialities, including neurology, cardio-thoracic surgery, accident service, and several intensive care units. A renal transplant service is also provided by a collaborative project of the university surgical and medical units of the Hospital. Two maternity hospitals, also Children's Hospital, Eye Hospital, and the Dental Institute are located in close proximity.

Primary health care through a network of dispensaries in towns became functional in 1877; this service was later extended to rural and estate areas. After the opening of the Colombo Medical School in 1870, district hospitals were established. In 1926, the establishment of the first 'health unit' on an experimental basis followed in Kalutara. A National Health Service was drafted in 1931 in connection with the Donoughmore Commission. The Medical College became the Medical Faculty of the University of Ceylon in 1942. British 'Western' allopathic medicine gradually became increasingly acceptable to the population from the second quarter of the 20th century. Following independence, the Ceylon Medical Department became the Department of Medical and Sanitary Services in 1948. The National Institute of Health Sciences was established in 1979 in Kalutara. It is the centre of the Department of Health Sciences in training health manpower required for the Primary Health Care programme. The National Institute of Health Sciences carries out basic training for assistant medical officers, public health inspectors, public health midwives, medical laboratory technologists and pharmacists. In addition, post-basic training for public health nursing sisters and ward sisters is also organised. The public health care sector comprises only the Western and Ayurvedic systems.

Government-sponsored health services are free and are delivered through a dense and extensive network of hygienic institutions located throughout the island, from primary level up to tertiary level. The primary care institutions include peripheral units, health centres, medical officers of health, maternity and nursing homes, district hospitals and rural hospitals. These primary care health facilities have maternity wards and offer basic medical care. A network of smaller facilities such as visiting stations (outpatient treatment), registered medical practitioners, nurses, other paramedical staff (public health nursing sisters, public health inspectors, public health midwives) and central dispensaries (*i.e.*,

pharmacy including outpatient care such as treatment of minor injuries); as smallest medical unit, however, these dispensaries are not attended to by a physician but by an assistant medical practioner. Base and provincial hospitals, located mainly in large towns, provide secondary level care. Teaching hospitals with specialised consultative services and few special hospitals provide tertiary care including treatment of cancer, tuberculosis, leprosy, mental illness, cancer, and other chronic, rheumatological, and infectious diseases. This is a very remarkable achievement for rural areas. The Shri Lankan health system can be considered as a role model for tropical and developing countries. After the tsunami of December 2004, *e.g.*, there was not a single disease outbreak because the system worked so well. According to the Ministry of Health (2002), on average, the public can freely access a health-care unit not further than 1.4 km from any home while the Western type of health-care services are available within 4.8 km of any patient's home. Specialised campaigns and programmes permanently cover malaria control, respiratory diseases, filariasis control, sexually transmitted diseases and health education.

The government sector provides health care for nearly 60 % of the population, encompassing the entire range of preventive, curative, and rehabilitative health-care provision. In 2002 alone, these health-care institutions provided facilities for 45 million outpatient visits and 4 million inpatient admissions, indicating that each person accesses government western type health care at least twice per year (1971 – 1973: 3 visits annually). 95 % of inpatient care is provided by the public sector. Expenditure on modern health services was 4.3 % of total government spending (1995). The curative service entails greater expenditure than preventive health. Medical officers and nurses are employed by statutory institutions and corporations (armed forces, police, insurances, petroleum corporation, tyre, plantations, etc.) in clinics serving their establishments. The Air Lanka (now: ShriLankan) Medical Centre served 4,800 employees and had a daily attendance of over 110 patients in 1995.

The government health system originally did not include health facilities on estates. These facilities on privately owned estates are managed by Regional Plantation Companies. Facilities on nationalised estates are managed by the Janatha Estates Development Board and the State Plantations Corporation. In 2001, the government took over 15 estate hospitals (Annual Health Bulletin, 2002).

5.4 Maternal and Neonatal Health Care

Development of specific services for mothers in Shri Lanka can be traced back to ancient and mediaeval times. According to ancient chronicles, the first maternity home was probably established between 522 and 524 *anno Domini*. The earliest indication of a

health service aimed specifically at mothers and children in modern times was the establishment of a Maternity Hospital in 1897. Today, the Family Health Bureau is the central organisation responsible for the planning coordination, direction, monitoring and evaluation of maternal and child health and family planning programmes in the country. It conducts in-service training programmes to update the knowledge and technical skills of the health staff as well as health services research and it implements special projects funded by international agencies to support and strengthen service delivery throughout the island. The Bureau has also to procure and distribute contraceptives and some of the essential equipment and supplies needed for family planning and maternal and child health activities. The Medical Information System was established in 1980 for the monitoring and evaluation of all family health activities. After a revision in 2000, it was in place from January 2001. Registration of live births, still births, deaths, and marriages was made compulsory with the enactment of civil registration laws in 1897, but compliance was less than 100 % even in 1995. Every live birth or death has to be registered before 42 days for a live birth and 5 days for a death, from the date of occurrence. According to the Department of Census and Statistics, state sector health institutions in the country attended approximately 92 % of deliveries during 1995 - 2000; the Demographic and Health Survey of 2000 reports that in the year 2000, 5.5% of all deliveries took place in private hospitals. As essential characteristic of the comparatively exemplary good medical care of the Shri Lankan population should be mentioned:

- the low maternal mortality
- the retrogressive, although still high neonate mortality.

Several intervention programmes are available to pregnant women in Shri Lanka. The Ministry of Health provides the following free of charge as a component of routine antenatal care to all pregnant women after the first trimester of pregnancy when they attend antenatal clinics:

1. Thriposha [Sinhala] (a vitamin-mineral fortified food supplement based on maize [66 %], soya [30 %], full cream milk powder [3 %], as well as vitamins and minerals [1 %]);
2. oral iron-folate supplements (ferrous sulphate containing 60 mg elemental Fe and 0.25 mg folic acid);
3. one course of mebendazole (an oral anthelminthic agent).

The “Thriposha” supplementary food programme for pregnant mothers, infants, and preschoolers has been the ongoing programme during the past four decades in order to improve the nutritional status. The programme commenced in 1973 with the co-operation of CARE Sri Lanka. Since 1991, the programme has been completely maintained with

government funds. The annual production target is approximately 10,440 metric tons which should be sufficient for 580,000 beneficiaries.

Infant and child care provides for immunization against six common childhood diseases, monitors growth and psychosocial development of the child, controls diarrhoeal diseases and acute respiratory infections. Immunization by age five years with bacillus Calmette-Guérin, whooping-cough-diphteria-tetanus vaccine as well as oral polio and measle vaccines covered 90 % in 1995. Compared the the infant mortality rate, which has declined remarkably, the efforts to reduce neonatal deaths seem to be insufficient, since more than 80 % of infant deaths occur in the neonatal period. Morbidity data from state sector hospitals indicate a high incidence of neonatal sepsis mostly caused by nosocomial pathogens.

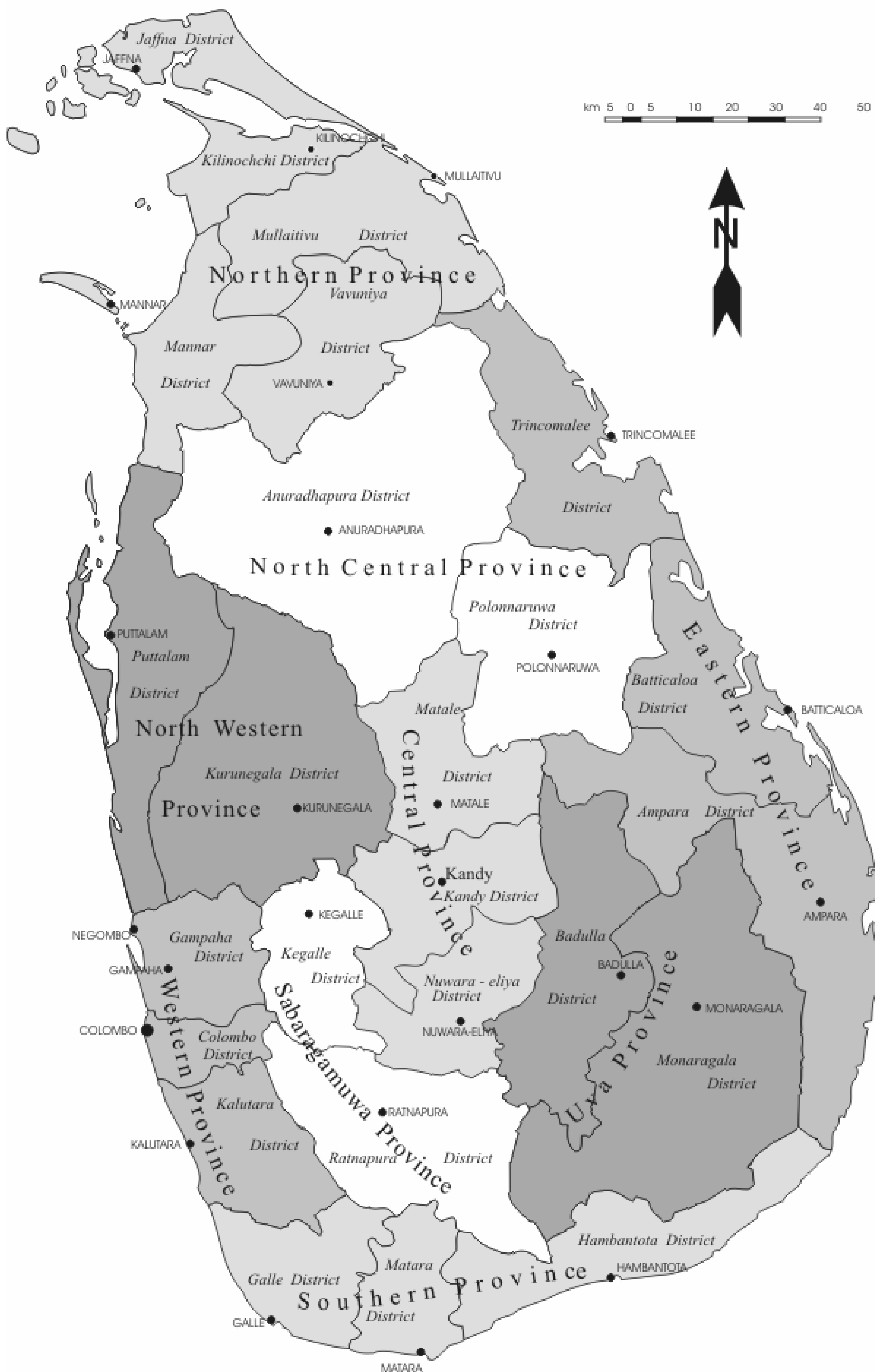
5.5 Castle Street Hospital for Women (Teaching)

Castle Street Hospital for Women (CSHW) is a large tertiary care referral centre affiliated to the University of Colombo and one of the two important and highly specialised teaching hospitals in the island; it also functions as study centre in hospital management, the focal point for the National Quality Assurance Programme of the Ministry of Health.

The institution was started as a Maternity Home to take over the surplus patients from the De Soysa Hospital, Colombo, where in 1948, there had been a major outbreak of puerperal sepsis and where in 1949 there were over 13,000 deliveries. CSHW was declared open in December 1950. The hospital was at that time located somewhat remote and on the outskirts of Colombo. The first patient was admitted on the 11th of December, and the first baby was born in the new hospital on the 13th of December. Statistics for the first 3 years of CSHW indicate that there were 23 deliveries in 1950, 1,653 in 1951, 4,765 in 1952, and 17,781 in 2007. Because of the population increase in the suburbs, the hospital was developed to a women's hospital consisting of five obstetrics and gynaecology units and a neonatology unit.

Up to 1972, the babies were all kept in a special baby room. Then after a severe diarrhoea epidemic, the present system was organised where babies are kept with their mother after delivery. The Premature Baby Unit at the CSHW, which caters to all deliveries of the hospital, also functions as a referral centre for complicated pregnancies needing special management of neonatal babies; it was refurbished between 1997 and 2000. In 2003, there were (only) fifteen cots with incubators and four cots with ventilators in the Neonatal Intensive Care Unit caring for more than 15,000 babies born there annually. It should be mentioned that the first HIV positive mother delivered at Castle Street Hospital in 1990.

Figure 21: Map of provinces, districts and main towns of Shri Lanka



Source: International Centre for Ethnic Studies, Kandy, Shri Lanka [http://www.ices.lk/sl_database/maps/towns.shtml]

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6.2 Original Papers and Conference Contributions Arising from This Thesis

A. *2009*
GOMES T, LINDNER U, TENNEKOON KH; KARANDAGODA KKW, GORTNER L, OBEID R
"Homocysteine in Small for Gestational Age and Appropriate for Gestational Age Preterm Neonates from Mothers Receiving Folic Acid Supplementation"
submitted for publication in: AMERICAN JOURNAL OF CLINICAL NUTRITION

B. *2009*
GOMES T, LINDNER U, KARANDAGODA KKW, TENNEKOON KH, GORTNER L, OBEID R
"Folate, Vitamin B_{12}, and Homocysteine Concentrations in Small for Gestational Age and Appropriate for Gestational Age Preterm Neonates Born to Mothers with Pregnancy-Induced Hypertension"
submitted for publication in: NEW ENGLAND JOURNAL OF MEDICINE

C. *05.2007*
Poster (collaboration):
LINDNER U, GOMES T, WOERNER J, TUTDIBI E, MONZ D, TENNEKOON KH, GORTNER L
"Plasma Homocysteine Levels and MTHFR-Polymorphisms in Preterm Infants in Two Ethnic Groups"
Pediatric Academic Societies' Annual Meeting. May 2007, Toronto, Canada
[*http://www.pas-meeting.org/2007Toronto/default.htm*]
[*http://www.abstracts2view.com/pasall/view.php?nu=PAS07L1_979*]
(Section 7929: Vitamins & Minerals, No. 354, Publication 7927.9; E-PAS2007:617927.9)

D. *08.05.2009*
Poster and oral presentation (*Poster session Nursing care / Neonatology*):
GOMES T, LINDNER U, KARANDAGODA KKW, TENNEKOON KH, TUTDIBI E, GORTNER L, OBEID R
"Folat-, Vitamin B_{12}- und Homocystein-Status bei Frühgeborenen von Müttern mit schwangerschaftsinduzierter Hypertonie aus Sri Lanka" ['Folate, Vitamin B_{12} and Homocysteine Status in Premature Newborns Born to Mothers with Pregnancy-Induced Hypertension from Sri Lanka']
24. Deutscher Kongress für Perinatale Medizin / 35. Jahrestagung der Gesellschaft für Neonatologie und Pädiatrische Intensiv-medizin. 06th – 09th May 2009, Berlin, Germany
[*http://www.dgpm-gnpi-2009.de*]
[*http://www.medizinkongress.com/perinatale-medizin2008/DGPM_GNPI_2009_PRO_Z3_M.pdf*]
(*PO-N 04.12*)

E. *18.09.2009* *Poster and oral presentation*:
GOMES T, LINDNER U, KARANDAGODA KKW, TENNEKOON KH, TUTDIBI E, GORTNER L, OBEID R
"Folate, Vitamin B_{12}, and Homocysteine Status in Premature Neonates Born to Mothers with Pregnancy-Induced Hypertension from Sri Lanka"
Sailing Towards New Horizons: International Conference on Frontiers in Molecular Life Sciences.17th – 18th September 2009, Colombo, Shri Lanka.
[*http://www.ibmbb.lk/conference2009/index.htm*]

6.3 Conference Contributions and Oral Presentations

A. *05.05.2007* *Poster and oral presentation* (*Posterwalk II - Cardiology, Pneumology, Intensive Care*):
GOMES T, Tutdibi E, LINDNER U, WURM D, GORTNER L
"Bleiingestion im Kindesalter" ['Lead Ingestion in Childhood']
56. Jahrestagung der Süddeutschen Gesellschaft für Kinder- und Jugendmedizin (SGKJ). 04th -06th May 2007, Würzburg, Germany
[*http://www.sgkj2007.de/flyer.pdf*] (*P22*)
[*www.thieme.de/klinpaed: Klin Pädiatr 219:108; Abstract*]
*[*cf. also: *Internist Prax 2008;48:685-691]*

B. *12.04.2008* *Poster and oral presentation* (*Posterwalk Molecular Medicine*):
GOMES T, GORTNER L, DOCKTER G, ROHRER R, DELB W, KÄSMANN-KELLNER B, THAKKER RV
"HDR-Syndrom: Erstbeschreibung einer neuen Genmutation" ['HDR Syndrome: First Description of a *de novo* Gene Mutation']
57. Jahrestagung der Süddeutschen Gesellschaft für Kinder- und Jugendmedizin (SGKJ). 11th – 13th April 2008, Ulm, Germany
[*http://www.sgkj2008.de/media/Hauptprogramm_07_03_2.pdf*]
[*www.thieme.de/klinpaed: Klin Pädiatr 220:119; Abstract*] (*PS-30*)

C. *31.05.2008* *Poster and oral presentation (Poster Infektionen II)*:
GOMES T, ENDERS G, GÄRTNER B, LÖFFLER G, HENTSCHEL J, LINDNER U, GORTNER L
"Therapie bei zwei extrem unreifen Frühgeborenen mit konnataler Cytomegalievirus-Infektion" ['Therapy for Two Extremely Immature Preterm Infants with Connatal Cytomegalovirus Infection']
34. Jahrestagung der Gesellschaft für Neonatologie und Pädiatrische Intensivmedizin. 29th – 31th May 2008, Zürich, Switzerland (P119)
[*www.thieme.de/fz/zgn: Z Geburtshilfe Neonatol 212:S83-S84; Abstract]*

Appendix

Abbreviations and Acronyms

Term	Explanation
µl	microliter
µmol	micromole
AGA	appropriate-for-gestational age
ANOVA	analysis of variance (statistical analysis)
ATP	adenosine-3'-phosphate, adenosine triphosphate
BHMT	betaine-homocysteine (Hcy)-methyltransferase
BMI	body mass index
bp	base pair(s)
BPD	bronchopulmonary dysplasia
BW	birth weight
C	celsius
CBS	cystathionine β-synthase, cystathione β-synthetase
cf.	confer
CI	confidence interval (statistical analysis)
contr.	controls
CSHW	Castle Street Hospital for Women (Teaching), Colombo 8, Shri Lanka
CVD	cardiovascular disease
d	day
DNA	deoxyribonucleic acid
dNTP	de(s)oxynucleotide triphosphate(s)
dUTP	deoxyuridylate triphosphate
ETDA	ethylene diaminetetraacetate (acid)
e.g.	exempli gratia; for example
FAM™	6-carboxy-fluorescein
FBP	folic acid binding protein(s); folate binding protein(s)
FPIA	fluorenscence polarisation immunoassay
GA	gestational age
GCE (A/L)	General Certificate of Education: Advanced Level [13th grade passed - school-leaving certificate; (in Germany:) Abitur]
GCE (O/L)	General Certificate of Education: Ordinary Level [10th grade passed]
Hcy	homocysteine
HHcy	hyperhomocysteinaemia
i.e.	id est; that is to say
IUGR	intrauterine growth retardation
km	kilometre(s)

Term	Explanation
kPa	kilopascal
LBW	low birth weight
MGB	minor groove binder(s); minor groove binder group(s)
ml	millilitre
MTHF	methylenetetrahydrofolate
MTHFR	methylenetetrahydrofolate reductase
n	number
n.d.	no date
ng	nanogram
nm	nanometer
nmol	nanomol
NTD	neural tube defect(s)
OL	Ordinary Level [10th grade: exam not passed]
OR	odds ratio (statistical analysis)
p	probability of exeeding (statistical analysis)
P	percentile(s)
PCR	polymerase chain reaction
pg	picogram
PI	(ROHRER's) ponderal index
PIH	pregnancy-induced hypertension
pmol	picomol
r	coefficient of correlation, sample (statistical analysis)
RLU	relative light unit(s)
rpm	revolutions per minute
SAH	S-adenosyl-homocysteine
SAM	S-adenosyl-methionine
SD	standard deviation (statistical analysis)
SGA	small-for-gestational age
SNP	single nucleotide polymorphism
Taq	Thermus aquaticus
T. G.	Thushari Gomes
tHcy	total homocysteine
THF	tetrahydrofolate
UNG	uracil-N-Glycosylase
UNICEF	United Nations Children's Fund [*formerly*: United Nations International Children's Emergency Fund]
VIC®	PCR dye (property of Applied Biosystems; chemical structure unpublished)
vs.	versus; against
WHO	World Health Organization

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Questionnaire

Proband Master Data (Data Entry Form)

DATE:	☐☐ ☐☐ ☐☐ (Year) (Month) (Day)	PROBAND-NO.:

1. DATA OF THE CHILD

Name (Child):		Prename:
Date of Birth:	☐☐ ☐☐ ☐☐ (Year) (Month) (Day)	Time: ☐☐ ☐☐ (Hours) (Minutes)
Gender of the child:	*male* ☐	*female* ☐
Multiple birth:	*yes* ☐	*no* ☐
Mode of delivery:	*vaginal* ☐	*Caesarean* ☐
Gestational age:	☐☐ ☐ / ☐ *wks*	Weight at birth: ☐☐☐☐ *g*
Birth length:	☐☐☐ *cm*	Head circumfer.: ☐☐☐ *cm*
APGAR:	☐☐ ☐☐ ☐☐ (1 min) (5 min) (10 min)	Umbilical cord blood: ☐ , ☐☐ *pH*
Distinctive features, malformations:	*no:* ☐	*yes, as follows:*
Postnatal complications:	*no:* ☐	*yes, as follows:*
Premature rupture of the amnion:	*yes* ☐	*no* ☐
Pulmonary maturity:	*yes* ☐	*no* ☐
Connatal sepsis:	*yes* ☐	*no* ☐
max. CrP:	☐☐☐ , ☐	max. IT-Ratio: ☐☐
Aortic isthmus stenosis (AIS):	*yes* ☐	*no* ☐
Contamination test haemoculture:	*yes* ☐	*no* ☐
Connatal haemoculture:	*yes* ☐	*no* ☐

	PROBAND-NO.:

1. DATA OF THE CHILD (*Cont.*)

Mechanical ventilation:		*yes* ☐	*no* ☐
Duration of mechanical ventilation:	☐☐☐ days		
CPAP:		*yes* ☐	*no* ☐
if so: Duration of CPAP:	☐☐☐ days		
O_2-demand:		*yes* ☐	*yes* ☐
if so: Duration of oxygenation:	☐☐☐ days		
Respiratory distress syndrome (RDS):		*yes* ☐	*no* ☐
if so: Level of RDS:	☐		
IVH:		*yes* ☐	*no* ☐
if so: Level of IVH:	☐		
ROP:		*yes* ☐	*no* ☐
if so: Stage of ROP:	☐		
Antibiosis:		*yes* ☐	*no* ☐
if so: which?			
SF:		*yes* ☐	*no* ☐
if so: No. of administrations:	☐		
Other therapies:			
Length of stay in hospital:	☐☐☐ days		
Duration of intensive care (therapy):	☐☐☐ days		
Name of parents:			
Residence:			
Phone number of parents:			

PROBAND-NO.:

2. DATA OF THE MOTHER

Name (Mother): Prename:

Date of birth: ☐☐ ☐☐ ☐☐ (Year) (Month) (Day)

Ethnicity: (Type) *Asian:* ☐ *Caucasian:* ☐

(Group) *Sinhalese:* ☐ *Tamil:* ☐

Moor: ☐ *other:*

Nationality: *Shri Lanka:* ☐ *other:*

School qualific.: Job:

Height (Mother): ☐☐☐ *cm*

Body weight (Mother): *before pregnancy* ☐☐ , ☐ *(kg, g)*

before birth ☐☐ , ☐ *(kg, g)*

Smoker: *yes* ☐ *no* ☐

Smoking during pregnancy: *yes* ☐ *no* ☐

Previous pregnancies: (*Nos.*) ☐☐

thereof: with „normal" outcome (*Nos.*) ☐☐

thereof: premature births (normal) (*Nos.*) ☐☐

thereof: premature births with growth retardation: (*Nos.*) ☐☐

ICSI: *no* ☐ *yes* ☐

IVF: *no* ☐ *yes* ☐

Diabetes mellitus: *no* ☐ *yes* ☐ *Type 1* ☐ *Type 2* ☐

- *Therapy:* *no* ☐ *yes, as follows*

OGTT during pregnancy: *yes* ☐ *no* ☐

- if OGTT yes, then pathological diagnosis:

PROBAND-NO.:

2. DATA OF THE MOTHER (*Cont.*)

Arterial hypertension: *yes* ☐ *no* ☐

- if hypertension yes, then which therapy:

Other diseases: *no* ☐ *yes, as follows:*

Nutrition during pregnancy:

(additional) Taking of vitamin preparations:

Screening examinations: *no* ☐ *yes* ☐ *Nos.* ☐☐

Infectious diseases *yes* ☐ *no* ☐

- *if so, which?*:

Taking of drugs during pregnancy: *yes* ☐ *no* ☐

- *if so, which drugs?*:

Vaccinations during pregnancy: *yes* ☐ *no* ☐

- *if so, which?*:

PROBAND-NO.:

3. DATA OF THE FATHER

Name (Father): Prename:

Date of birth: (Year) (Month) (Day)

Ethnicity: (Type) *Asian:* ☐ *Caucasian:* ☐

(Group) *Sinhalese:* ☐ *Tamil:* ☐

Moor: ☐ *other:*

Nationality: *Shri Lanka:* ☐ *other:*

School qualific.: Job:

Height: cm *anamnestic* ☐ *self-reported* ☐

Weight: , *(kg, g)* *anamnestic* ☐ *self-reported* ☐

Diseases: *yes* ☐ *no* ☐

- if so, which?:

Other:

Samples centrifugated on: (Year) (Month) (Day)

Entry in database: ______

Information Sheet and Declaration of Consent (*English*)

Retardation of intrauterine growth among premature infants in Sri Lanka (Clinical and experimental molecular as well as endocrine aspects. Molecular and hormonal regulation in case of intrauterine disturbance of growth.)

Information Sheet and Declaration of Consent

Complete title of the research work

Molecular and hormonal regulation in case of intrauterine growth retardation

Head responsible of the research work

Prof. Dr. L. Gortner / Dr. W. Karandagoda

Name of the participating child (Surname, First Name)

___________________________________, Date of birth: ______ Number: __

Dear Parents,

our study group is most interested in gaining new scientific findings regarding the above mentioned research study. Therefore, we would appreciate you consenting to the participation of your child. This participation is, of course, voluntary. This means your child will be part of the study only if you agree. To inform you about the purpose and about any possible advantages or risks when taking part, the responsible doctor or his/her substitute will have an in-depth conversation with you. Before this we would like you to read the following explanations. This will enable you to get a detailed insight into the study.

The Research Study

What is this study about?

In recent years we have learnt that children with low birth weight have a tendency to get overweight, high blood pressure and so-called adult diabetes (Diabetes mellitus Type 2).

We still don’t know yet whether the low birth weight or the increase in weight during the first few months of life increases the risk for these diseases.

We still don't know yet whether the low birth weight or the increase in weight during the first few months of life increases the risk for these diseases.

Within the scope of this study various hormones and genetic factors are to be analysed that might affect the birth weight and the increase in weight in the first few months of life. The hormones concerned here are cortisol, leptin and ghrelin as well as the insulin-like growth factors IGF-I and IGF-II and their binding proteins -1, -2 and -3.

The aim of our study is to gain more knowledge of the interplay between the hormones and genetic factors to be analysed, the birth weight and the further increase in weight. This would enable us to diagnose high-risk children in advance and to give the appropriate and necessary advice.

How will the research be carried out?

After cutting off the umbilical cord a blood sample will be taken from the child's side of the separated cord; this sample is going to be analysed later on.

This is the important aspect of the procedure as it is **non-invasive** and **non-painful**, thus preventing pain and harm to the infants.

Furthermore, both we and you together will take down data regarding pregnancy and birth from your mother pass and from your file as patient.

Do I or my child have an advantage personally when taking part?

Your participation offers **no personal advantages at all** for you or for your child. The participation of your child, however, enables us to gain new scientific findings for the benefit of patients in the future. With your participation you make an unselfish contribution to the progress in medicine.

Which risks and strains are to be expected?

In no way there are any risks and strains to be expected.

Are there any other things to pay attention to?

The personal data of you and your child will be protected.

The realization of the project requires us to ascertain, record and process your and your child's personal data. These data will only be used for the **scientific evaluation** of the study, for the **monitoring** of the study by the supervising authorities as well as for **archiving** the scientific results. In addition, these data may be used for the **publication** of the scientific results (for example in medical specialist journals).

The ascertainment, recording and processing of these data are subject to **very strict specific legal regulations** which are obeyed restrictively. This means passing on and inspection of personal data is only permitted to the appropriate supervising authorities and to persons of the funding institution who are under legal obligation to discretion. Moreover, a publication of data in scientific journals is only allowed if any reference to you and your child has been made unrecognizable either by establishing anonymity or by using another name, i.e. a pseudonym.

You can end your participation any time.

If you decide to end the participation of your child in the study, you can cancel your consent any time and **without giving any reasons**. Your withdrawal will bring you and your child **no disadvantages at all**. The **personal data** already ascertained at the point of your withdrawal will immediately be deleted.

Declaration of Consent

I have got a general idea of the scientific research by reading the information sheet handed out to me.

Afterwards, ________________________ has had an in-depth conservation with me,

on _____________ at ______ o'clock. Topic of the conservation was in particular

- the detailed subject matter and the practical course of the study, above all

  ____

- the question to what extent advantages, risks or strains are are to be expected,

  above all ____

- questions regarding data protection as well as my right of withdrawal at any given time.

I had the opportunity to ask questions, and have received a copy of the documents at issue. Afterwards, I was given enough time to think about the participation of my child. At the moment I do not have any further questions.

I agree to the participation of my child in the research project.

My consent also includes the use of the personal data as described, especially the ascertainment and processing of information about my health and the health of my child.

____________________________ ____________________________________

(Place, Date) (Signature of the parent)

Many thanks for your help - of course, we will immediately inform you, if we get to know any information in the course of the study that might influence your willingness to continue your participation.

____________________________ ____________________________________

(Place, Date) (Signature Head of Study / Substitute)

Information Sheet and Declaration of Consent (*Sinhala*)

ශ්‍රී ලංකාවේ ආපරිනිත බිළිඳුන් අතර ගර්භාෂ උන වර්ධනය

(ශායනික හා පරීක්ෂණාත්මක අනුක මෙන්ම අන්තරාගර්භාෂ අංශ - අන්තර්ගර්භාෂ වර්ධනයට භාජනය වන අවස්ථාවල අනුක හෝ හෝමෝනීය පාලනය)

තොරතුරු සටහන හා කැමැත්ත ප්‍රකාශ කිරීම.

පර්යේෂණ කාර්යයේ සම්පූර්ණ නාමය :-

අන්තර් ගර්භාෂ ඌණ සංවර්ධන අවස්ථාවේ අනුක හා හෝමෝනීය පාලනය

පර්යේෂණ කාර්යට වගකියයුතු ප්‍රධානියා :-

මහාචාර්ය (වෛද්‍ය) ගොටිනර් / වෛද්‍ය ඩබ්ලිව්. කරදගොඩ.

සහභාගීවන දරුවාගේ නම (වාසගම, පුද්ගලික නාමය)

……

උපන් දිනය :- ……………………………………………… අංකය:- …………………………

හිතවත් දෙමාපියනි,

අපගේ අධ්‍යන කණ්ඩායම ඉහත සඳහන් පර්යේෂණ අධ්‍යනය ගැන නව විද්‍යාත්මක තීරණ ලබාගැනීමට ඉතාමත් උනන්දුවෙනුයි සිටින්නේ, එම නිසා ඔබගේ දරුවා මෙම ප්‍රයත්නයට සහභාගීවීමට කැමැත්ත පළකිරීම අප ඉතා අගය කොට සලකනවා. සහභාගීවීම අන්ත වශයෙන්ම ස්වේච්ඡාවෙනුයි සිදුවන්නේ. එහි තේරුම, ඔබගේ දරුවා මෙම අධ්‍යනයේ කොටසක් වන්නේ ඔබ එකඟ වෙනවා නම් පමණයි. මෙම කාර්යයේ අරමුණ ගැනත්, එම සහභාගීවීම නිසා ඔබට අත්වන්නට ඉඩ තිබෙන යහපත ගැන මෙන්ම එහි යම් අනතුක් තිබෙනවා නම් ඒ ගැනත් ඔබ දැනුවත් කරන්නට මේ ගැන වගකිවයුතු වෛද්‍ය වරයා ගේ ඔහු / අය වෙනුවට ක්‍රියාකරන්නා හෝ එම කටයුත්ත ගැන ඔබ සමඟ ගැඹුරින් සිදුකෙරෙන සාකච්ඡාවක් පවත්වනවා ඇත. ඊට කලින් ඔබ මෙහි පහත දැක්වෙන විස්තර කියවා බලනු යෙහෙකි. එයින් ඔබට මෙම අධ්‍යනය ගැන විස්තරාත්මක වූත්, ගැඹුරු වූත්, අවබෝධයක් ලබාගන්නට හැකිවෙනවා නිසාකෙයි.

පර්යේෂණ අධ්‍යනය

මෙම අධ්‍යනය කුමක් ගැනද ?

අඩුබරින් යුතුව උපන්දින දරුවන් සුළු කලකදී පමණට වඩා වැඩි බරකින් යුත්නවීම. අධික රුධිර පීඩනය, වැඩිහිටි දියවැඩියා (Diabetes Mellitus - 2 වර්ගය) යන යන අසනීප වර්ග ඇතිවීමටත් ප්‍රවණතාවක් තිබෙන බව අපට මෑතකදී දැනගන්නට ලැබිණි.

අඩු බරින් ඉපදීම හෝ ජීවිතයේ පළමු මාස කීපය තුළදී බරින් වැඩිවීම යන මේවා පෙරකී අසනීප වැළදීමට තිබෙන අවදානම වැඩි කරනවාදැයි අප තවම දන්නේ නැහැ.

මෙම අධ්‍යනයේ විෂය සීමාව තුළ උත්පත්තියේ තිබෙනස බරට හෝ ජීවිතයේ පළමු මාස කීපය තුළ ශරීරයේ බර වැඩිවීම හෝ ඉඩපෑම් තිබෙන ඉඩකඩ නොයෙක් හෝමෝන හා ප්‍රභාවාත්මක සාධක අධ්‍යනයට ලක් කිරීමට නියමිත. මෙහැනෙට සම්බන්ධය ඇති හෝමෝන නම් (Cortison, Leptin and Ghrlein මෙන්ම IGF - 1 හා IGF - 2) ඉන්සුලින් වැනි වර්ධක සාධක සහ ඒවායේ සම්බන්ධන ප්‍රෝටීන් 1 - 2 හා 3 ය.

අපගේ අධ්‍යනයේ අරමුණු වන්නේ අධ්‍යනයට ලක් කෙරෙන හෝමෝන හා ප්‍රභවාත්මක සාධක උප්පත්ති බර හා තවදුරටත් ඔබ් ර බර වැඩිවීම යන ාමේවා අතර අන්තර් ක්‍රියාකාරීත්වය ගැන වැඩිදු දැනුම ලබාගැනීමය. මෙම අධ්‍යනය නිසා අපට මෙම අබාධවලට ලක්වීම වැඩි අවධානමකින් සිටින දරුවන් හැඳිනගෙන ඔවුන්ට උචිත හා අවශ්‍ය උපදෙස් දීමට හැකිවනු ඇත.

පර්යේෂණ සිදුකරන්නේ කෙසේද ?

පෙකණිවැල කපාදැමීමෙන් පසු වම වෙන්කළ රජ්ජුවෙහි දරුවාගේ පැත්තෙන් ලේ සාම්පලයක් ලබගනු ලැබේ. මෙම ලේ සාම්ලය පසුව විශ්ලේෂණයට ලක් කෙරේ.

මෙම ආකුමණික සේවානාවකින් තොර නිසාත්, වේදනාවක් නොදෙන ක්‍රියාවක් නිසාත්,කර්ය පරිපාටියේ වැදගත් පැතිකඩක් වයයි. මෙම තත්වය නිසා දිළිඳුන්ට වේදනාවක් හා පීඩාවක් නොවන බැවිනි.

තවද, අප හා ඔබ වක්වම බගර්භනී භව හා ප්‍රසූතිය ගැන ඔබගේ මවගෙන් හා රෝගියා ලෙසට ඔබගේ ලිපිගොනුවෙන් දත්ත ලබාගන්නෙමි.

මට සහභාගිවීම නිසා මාහට හෝ මාගේ දරුවාට පෞද්ගලිකව අත්වන වාසියක් තිබේද ?

ඔබ පර්යේෂණයට සහභාගිවීමෙන් ඔබට හෝ දරුවාට කිසිත් පෞද්ගලි වාසියක්අත් නොවේ. නමුත් ඔබගේ දරුවා සහභාගිවීම නිසා මතුවට ඇතිවන රෝගීන්ගේ ප්‍රයෝජනය සඳහා නව විද්‍යාත්මක නිගමන ලබාගැනීමට හැකිවේ. මෙම පර්යේෂණයට සහභාගිවීමෙන් ඔබ වෛද්‍ය විද්‍යාවේ දියුණුවට නිර්ද්දරණාධහාෂයෙන් සිදුකරන සේවයක් සිදුකරන්නෙහිය.

මුහුණපාන්නට සිදුවන අනතුරු හෝ පීඩා වන් මොනවාද ?

අනතුරක් හෝ පීඩාවක් ඇති වන්නට කිසිදු ඉඩක් නැත.

<u>අවධානය යොමු විය යුතු තවත්ල කරුණු කිසිවක් තිබේද ?</u>

ඔබ හා ඔබේ දරුවා විසින් සපයනු ලබන පෞද්ගලික දත්ත සුරක්ෂිතව තබාගනු ලැබේ.

මෙම ව්‍යාපෘතිය සාර්ථක කර ගැනීමට ඔබ හා ඔබේ දරුවා විසින් සපයන දත්ත විමසා දැන ගැනීමට, වාර්ථගත කර ගැනීමට හා පිරිසැකසුම් කිරීමට සිදුවේ. මෙම දත්ත අධ්‍යනයේ විද්‍යාත්මක ඇගයුමටත්, අධීක්ෂණයටත් බලධාරීන් විසින් අධ්‍යනයේ ප්‍රගතිය සමීක්ෂණය කිරීමට මෙන් විද්‍යාත්මක ප්‍රතිඵල ලබා ගැනීමටත් භාවිතා කෙරේ. ඊට අමතරව, විද්‍යාත්මක ප්‍රතිඵල පළකිරීමටත් (උදාහරණයක් වශයෙන් කිවහොත්, විශේෂඥ වෛද්‍ය විද්‍යා සඟරාවල්) පාවිච්චි කරනු ලැබේ.

මෙම තොරතුරු විමසා ලබාගැනීම, වාර්තා ගත කර ගැනීම හා පිරිසැකසුම් කිරීම විධිමත්, නිශ්චිත හා සීමිත භාවිතා වන නෛතික රෙගුලාසිවලට යටත්ව සිදුකෙරේ. මෙහි තේරුම, පෞද්ගලික තොරතුරු තැනකින් තැනකට ලැබෙන්න සැලැස්වීම හා පරීක්ෂාවට ලක්කිරීමට කළහැක්කේ උචිත අධීක්ෂණ බලධාරීන්ට සහ අභිමත අනුව ක්‍රියා කිරීමට නෛතිකව බැඳී සිටින මුදල් යොදවන ආයතනවලට පමණකි. තවද, විද්‍යාත්මක සඟරාවල දත්ත පළකිරීමට ඉඩදෙනුයේ ඔබ හෝ ඔබගේ දරුවා ගැන තිබෙන සඳහන් නිර්නාමික බව තහවුරුකිරීමෙන් හෝ වෙනත් නමක් (ව්‍යාජ නමක්) භාවිතා කොට නොහෙළිදිය හැකි තත්වයට පත්කොට තිබුනොත් පමණකි.

<u>ඔබගේ සහභාගීත්වය ඔබට ඕනෑම විටක නවතා දැමිය හැක.</u>

මෙම අධ්‍යනයට ඔබගේ දරුවා සහභාගීවීම නැවැත්වීමට ඔබ තීරණය කරන්නේ නම් ඕනෑම විටක හා හේතුවක් නොදක්වාම ඔබට එසේ කල හැකියග ඔබගේ දරුවා ව්‍යාපෘතිය හැරයාමෙන් ඔබට කිසිත් අවාසියක් සිදුනොවේ. ඔබ ඉවත් වන මොමොත වන විට ලබාගෙන තිබෙන පෞද්ගලික දත්ත නොපමාව මකා දමනු ලැබේ.

…………………… ……………………………………

දිනය අත්සන මව / පියා

Information Sheet and Declaration of Consent (*Tamil*)

தகவல் பத்திரமும், உடன்பாட்டைத் தெரிவித்தலும்

ஆய்வுப் பணியின் முழுமையான பெயர்:

கருப்பையுட் குறை வளர்ச்சியின்போது மூலக்கூறு சார்ந்த மற்றும் ஹோர்மோன் சார்ந்த கட்டுப்பாடு.

ஆய்வுப் பணிக்கு பொறுப்பு வகிக்கும் தலைவர்:

பேராசிரியர் (மருத்துவர்) எல். கொட்னர் / மருத்துவர் டபிள்யு. கரந்தகொட

பங்குபற்றும் குழந்தையின் பெயர் (குடும்பப் பெயர், முதற் பெயர்):

.... பிறந்த திகதி: .... இலக்கம்: ....

அன்பார்ந்த பெற்றோர்களே,

எமது ஆய்வுக் குழுவினர் மேற்போன்ற ஆய்வுக் கற்கை சார்ந்த புதிய விஞ்ஞான ரீதியான முடிவுகளைப் பெற்றுக் கொள்வது தொடர்பில் மிகவும் கரிசணையுடன் செயலாற்றுகின்றனர். எனவே, உங்கள் குழந்தைகள் இந்த முயற்சியில் பங்குபற்றுவதற்காக உங்கள் உடன்பாட்டைத் தெரிவித்ததையிட்டு நாம் பெரிதும் போற்றுகின்றோம். உண்மையில் இந்த பங்கேற்பு சுய விருப்பின் பேரிலேயே இடம்பெற வேண்டும். நீங்கள் அதற்கு உடன்பட்டால் மாத்திரமே உங்கள் குழந்தை இந்தக் கற்கை ஆய்வில் பங்குபற்றலாம் என்பதே அதன் பொருளாகும். இந்தப் பணியின் நோக்கம் குறித்தும், இத்தகைய பங்குபற்றல் காரணமாக உங்களுக்கு கிட்டக்கூடிய நன்மைகள் குறித்தும், அதில் பங்குபற்றுவதால் யாதேனுமொரு ஆபத்து நேரிடுமாயின் அது குறித்தும் உங்களை அறிவுறுத்துவதற்காக இது விடயத்தில் பொறுப்பு வகிக்கக் கூடிய மருத்துவர் அல்லது அவர் சார்பாகச் செயலாற்றுபவர் அந்த விடயம் குறித்து உம்முடன் ஆழமான கலந்துரையாடல் ஒன்றில் ஈடுபடுவார். அதற்கு முன்னர், நீங்கள் இங்கு கீழ்க் காணப்படும் விளக்கங்களை வாசித்துப் பார்க்கலாம். அதன் மூலம், இந்தக் கற்கை ஆய்வு குறித்த பரந்த அறிவை நீங்கள் பெற்றுக் கொள்ளக் கூடியதாக அமையும்.

ஆய்வுக் கற்கை

இந்தக் கற்கை எதனைப் பற்றியது?

நிறையிற் குறைந்ததாகப் பிறக்கின்ற குழந்தைகள் ஒரு குறுகிய காலத்துள் அளவுக்கு மீறிய நிறை உடையதாக இருத்தல், அதிக குருதி அழுத்தம், வயது வந்தவர்களிடையிலான நீரிழிவு என்ற பெயரால் அறியப்படும் *Diabetes Mellitus - Type 2* போன்ற நோய்களுக்கு உள்ளாகும் ஒரு போக்கு காணப்படுவதை அண்மைக் காலத்தில் எம்மால் அவதானிக்க முடிந்தது.

நிறையில் குறைந்ததாகப் பிறத்தல் அல்லது வாழ்நாளில் முதல் ஒரு சில மாத காலத்துள் நிறை அதிகரித்தல் ஆகியன மேற்கூறிய நோய்நொடிகளின் தொற்று ஆபத்தைத் தீவிரப்படுத்துகின்றதா என்பது இதுகாறும் அறியப்படவில்லை.

இந்தக் கற்கையின் விடயப்பரப்பு எல்லைக்குள், பிறப்பின்போது காணப்படும் நிறை அல்லது வாழ்நாளில் முதல் ஒரு சில மாத காலத்துள் உடல் நிறை அதிகரித்தல் என்பவற்றின் மீது தாக்கம் செலுத்தும் பல்வேறு ஹோர்மோன்கள், பிறப்பியல் காரணிகள் என்பன பகுப்பாய்வுக்கு உட்படுத்தப்படவுள்ளது. இது விடயத்தில் தொடர்புடைய ஹோர்மோன்களாவன: *Cortisol, Leptin, Ghrlein* மற்றும் *IGF-I, IGF-II* இன்சுலின் போன்ற வளர்ச்சிக் காரணிகளும் அவற்றுடன் இணைந்த 1-2-3 ஆகிய புரதங்களும்.

ஆய்வுக்கு உட்படுத்தப்படும் ஹோர்மோன்கள் மற்றும் பிறப்பியல் காரணிகள், பிறப்பின்போது காணப்படும் நிறை மற்றும் தொடர்ச்சியான உடல் நிலை அதிகரிப்பு ஆகியவற்றுக்கு இடையிலான இடைச் செயற்பாடு பற்றிய மேலதிக அறிவைப் பெற்றுக் கொள்வது எமது கற்கை ஆய்வின் நோக்காகும். இந்தக் கற்கையின் வாயிலாக எமக்கு இத்தகு உபாதைகளுக்கு உட்படுவதற்கு அதிக ஆபத்தான அறிகுறிகள் தென்படும் குழந்தைகளை இனங்கண்டு, அவர்களுக்கு உசிதமானதும் அவசியமானதுமான ஆலோசனைகளை வழங்கக் கூடியதாக அமையும்.

ஆய்வை எவ்வாறு மேற்கொள்வது?

கொப்பூழ் நாணை (*Umbillical Cord*) அறுத்த பின்னர், அவ்வாறு அறுத்து அப்புறப்படுத்தப்பட்ட நாணில் குழந்தையின் பாகத்திலிருந்து குருதி மாதிரியொன்று (*Blood Sample*) பெற்றுக் கொள்ளப்படும். இந்தக் குருதி மாதிரியானது பின்னர் பகுப்பாய்வுக்கு உட்படுத்தப்படும்.

இது ஆக்கிரமிப்புத் தன்மை கொண்டதாக இல்லாதிருப்பதன் காரணமாகவும், வலியொன்றை ஏற்படுத்தாததாக இருப்பதனாலும் இதுவே நடவடிக்கை முறையின் முக்கிய நோக்காக உள்ளது. இந்த நிலையானது குழந்தைகளுக்கு வலியையோ, துன்பத்தையோ ஏற்படுத்தாது.

இதற்குப் புறம்பாக, நாமும் நீங்களும் இன்றிணைந்து கர்ப்பம் தரித்தல் மற்றும் உமது தாயிடமிருந்து பிரசவம் பற்றியும் நோயாளி என்ற வகையில் உங்கள் கோப்பிலிருந்தும் தரவுகளைப் பெற்றுக் கொள்வோம்.

இதில் பங்குபற்றுவதன் மூலம் எனக்கு / எனது குழந்தைக்கு தனிப்பட்ட முறையில் கிட்டக்கூடிய நன்மைகள் ஏதும் உள்ளனவா? நீங்கள் ஆய்வில் கலந்து கொள்வதன் மூலம் உங்களுக்கோ, உங்கள் குழந்தைகளுக்கோ தனிப்பட்ட முறையில் ஏதும் நன்மைகள் கிட்டாது. எனினும், உங்கள் குழந்தை பங்குபற்றுவதன் காரணமாக எதிர்காலத்தில் நோயாளிகளின் நன்மைக்கான புதிய விஞ்ஞான ரீதியான முடிவுகளைப் பெற்றுக் கொள்ளக் கூடியதாக அமையும். இந்த ஆய்வில் பங்குபற்றுவதன் மூலம் நீங்கள் மருத்துவத் துறையின் முன்னேற்றத்துக்கு பரோபகாரத்துடன் அளப்பரிய சேவையாற்றியவராவீர்கள்.

எதிர்நோக்க நேரிடும் ஆபத்துக்கள் / நலி சோர்வுகள் எவை?

ஆபத்துக்கள் / நலி சோர்வுகள் ஏற்படுவதற்கான எவ்வித வாய்ப்பும் இல்லை.

கவனம் செலுத்தப்பட வேண்டிய வேறு ஏதேனும் காரணிகள் உள்ளனவா?

நீங்களும், உங்கள் குழந்தையும் வழங்கும் தனிப்பட்ட தரவுகள் பாதுகாப்பானவையாகப் பேணப்படும்.

இந்தக் கருத்திட்டத்தை வெற்றி கொள்ளச் செய்வதற்கு உங்களாலும், உங்கள் குழந்தையினாலும் முன்வைக்கப்படும் தரவுகள் குறித்து விசாரிப்பதற்கும், பதிவு செய்து கொள்வதற்கும், பரீட்சிப்பதற்கும் நேரிடும். ஆய்வின் விஞ்ஞான ரீதியான மதிப்பீட்டுக்கும், கண்காணிப்பு அதிகாரிகளால் ஆய்வின் முன்னேற்றத்தைக் கண்காணிப்பதற்கும் விஞ்ஞான ரீதியான பெறுபேறுகளைப் பெற்றுக் கொள்வதற்கும் இத்தகு தரவுகள் பயன்படுத்தப்படும். அதற்குப் புறம்பாக, விஞ்ஞான ரீதியான பெறுபேறுகளை வெளியிடுவதற்கும் (எடுத்துக்காட்டாக, விசேட மருத்துவவியல் சஞ்சிகைகளில்) இத்தகு தரவுகளைப் பயன்படுத்தலாம்.

இந்தத் தரவுகள் குறித்து ஆராய்தல், பதிவு செய்தல், பரீட்சித்துச் செயற்படுத்தல் ஆகியன முறையாகவும், குறிப்பாகவும், வரையறையுடனும் பயன்படுத்தப்படும் சட்ட ரீதியான ஒழுங்கு விதிகளுக்கு உட்பட்டு மேற்கொள்ளப்படும். இதன் பொருள் யாதெனில், பொருத்தமான கண்காணிப்பு அதிகாரிகளுக்கும், தற்றுணியின்படி செயலாற்றுவதற்கு சட்ட ரீதியான கடப்பாடுடைய நிதியளிக்கும் நிறுவனத்திற்கும் மாத்திரமே தனிப்பட்ட தரவுகளை இடத்திற்கு இடம் கிடைக்கச் செய்வதற்கும், பரீட்சிப்பதற்கும் அனுமதியளிக்க முடியும் என்பதாகும். மேலும், நீங்கள் / உங்கள் குழந்தை பற்றிய குறிப்புகள் அநாமதேயமாக இருப்பதை உறுதி செய்வதன் மூலம் / பிறிதொரு பெயரைப் (புனை பெயர்) பயன்படுத்தி அவர் இன்னார் என்பதை இனங்காண மடியாத நிலையில் காணப்படின் மாத்திரமே, விஞ்ஞான சஞ்சிகைகளில் தரவுகள் பிரசுரிக்கப்பட அனுமதியளிக்கப்படும்.

நீங்கள் எந்தவொரு வேளையிலும் உங்கள் பங்கேற்பைத் துண்டித்துக் கொள்ள முடியும்.

இந்தக் கற்கை ஆய்வில் உள்ள குழந்தையின் பங்குபற்றுகையைத் துண்டித்துக் கொள்ள நீங்கள் தீர்மானிப்பின், எந்தவொரு வேளையிலும், எந்தவொரு காரணத்தையும் முன்வைக்காத நிலையில் நீங்கள் அவ்வாறு துண்டித்துக் கொள்ளலாம். உங்கள் குழந்தை இந்தக் கருத்திட்டத்திலிருந்து ஒதுங்கி விடுவதன் மூலம் உங்களுக்கு எவ்வித பாதகமும் ஏற்படப் போவதில்லை. நீங்கள் இதிலிருந்து ஒதுங்கிக் கொள்ளுமிடத்து, பெற்றுக் கொள்ளப்பட்ட தனிப்பட்ட தரவுகள் உடனடியாக பதிவழிக்கப்படும்.

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திகதி பெற்றோர் ஒப்பம்

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Curriculum Vitae

Personal Data

Name:	D. G. Thushari Sandamali Gomes
Date of birth	18.05.1972
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Parents	*Father:* D. G. Irantha Gomes, Superintendent, Janatha Estate Development Board *Mother:* Shirani Gomes, *née* Wattege, Teacher, Civil Service

School Education

1979 – 1983	Primary school: *Musæus College*, Colombo, Shri Lanka
1984 – 1993	Grammar (secondary) school: *Musæus College* and *Anula College*, Colombo, Shri Lanka, with exam passes *Passes*: - General Certificate of Education: O-Level - General Certificate of Education: A-Level (Abitur)
08.1996	General Certificate of Education: Advanced-Level, exam subject: German (*additional voluntary school-leaving certificate*), Department of Examinations, Colombo, Shri Lanka

Continuing Education

1993 – 1996	German language courses, German Cultural Institute ("Goethe-Institut"), Colombo, Shri Lanka
1996 – 1997	Scholarship by the German Cultural Institute, Colombo, Shri Lanka: Preparatory Course "Zentrale Mittelstufenprüfung Deutsch" at the Goethe-Institut in Bonn-Bad Godesberg, Germany; final exam at the Goethe-Institut in Schwäbisch-Hall, Germany
1997 (since April)	Attendance of additional German courses at the German Cultural Institute, Colombo, Shri Lanka, as preparation for the higher grade examination; exam passed
03.1998 - 05.1998	Nursing traineeship at Medicity Hospital (Pvt.), Panadura, Shri Lanka

Course of the Study of Human Medicine

08.1998 – 06.1999	"Studienkolleg" (*Study College*), Field: Medicine, Bonn, Germany
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Practical Clinical Training during Studies ("*Famulatur*")

27.07.2002 - 27.09.2002	*Gynaecology:* Colombo South Teaching Hospital, Kalubowila, Shri Lanka
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Extracurricular Activities at the University

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Occupation as Physician

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Graduate Medical Education and Vocational / Advanced Training

2003	English language course, field: medicine, at the University of The Saarland, sponsored by "Deutscher Famulantenaustausch, Bonn"
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Declaration

I hereby certify to have written this paper independently. The material presented in this study has previously not been submitted for the award of a degree or diploma in any university or any other tertiary institution, and, to the best of my knowledge, contains no material previously published elsewhere or written by another person except where due reference and acknowledgement is made in the thesis itself. Also, the text has not been published or is not extracted in whole or in part from a thesis by which I have qualified for or have been awarded another degree or diploma. I wish to emphasise that I alone am responsible for any shortcomings in this study and for any errors that a reader may detect.

All research procedures reported in this thesis were approved by the appropriate Ethic Committee in Germany and the Ethical Review Committee in Shri Lanka.