Aus Klinische Chemie und Laboratoriumsmedizin/Zentrallabor
Des Universitätsklinikums des Saarlandes, Homburg/Saar
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The role of poor nutritional status and hyperhomocysteinemia in complicated pregnancy in Syria

Dissertation for awarding the degree of

DOCTOR OF THEORETICAL MEDICINE

Faculty of medicine

UNIVERSITY OF SAARLAND

2006

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Born on 18.02.1975

In Homs-Syria

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Abbreviations

- Aa : acetic acid
- ADMA : Asymmetric dimethylarginine
- An : acetonitril
- ATP : Adenosine triphosphate
- BHMT : Betaine:homocysteine methyltransferase
- BLB : blood lysis buffer
- CbI : Methylcobalamine
- CBS : Cystathionine β-synthase
- CH_2-THF : 5,10-methylene-tetrahydrofolate
- CH_3-THF : Methyletetrahydrofolate
- Cho : Cholesterol
- Con : Control group
- CV : coefficient of variation
- Cys : Cystathionine
- DTT : 1,4-dithiothreitol
- EC : Eclampsia
- EC-SOD : Extracellular superoxide dismutas
- EDTA : Ethylenediamine tetraacetic acid
- eNOS : NO synthase
- g : gram
- GC-MS : Gas chromatography-mass spectrometery
- h : hours
- Hcy : Homocysteine
- HELLP : Hemolysis, Elevated Liver enzymes, and Low Platelet count
- HHcy : Hyperhomocysteinemia
- HLD : High density lipoprotein
- HPLC : High Performance Liquid Chromatography
- ISSHP : International Society for the Study of Hypertension in Pregnancy
- IUGR : Intrauterin growth retardation
- LDH : Lactate dehydrogenase
- MAT : Methionine adenosyltransferase

- meth : methanol
- Meth : Methionine
- min : minutes
- MMA : Methylmalonic acid
- MS : Methionine synthase
- MTBDSFA: N-methyl-N (tert-butyldimethylsilyl) rifluoracetamide
- MTHFR : Methylenetetrahydrofolate reductase
- NF-κB : Nuclear factor-κB
- NHBPEP : National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy
- NO : Nitric oxide
- NOS : Nitric oxide synthesis
- NP : non-pregnant women
- $ONOO^-$: Peroxynitrite
- PARP : Poly (ADP-ribose) polymerase
- PCR : Polymerase Chain Reaction
- PE : Preeclampsia
- PGL_2 : Prostacyclin
- PLP : pyridoxal-5- phosphate
- PN.HCL : pyridoxine.HCl
- RFLP : Restriction Fragments Length Polymorphism method
- ROS : Reactive Oxygen Species
- s : second
- SAH : S-adenosylhomocysteine
- SAM : S-adenosylmethionine
- SDS : sodium dodecyl sulphate
- SGOT : serum glutamic-oxaloacetic transaminase
- SGPT : serum glutamic-pyruvic transaminase
- SH : sulfuhydryle group
- SHMT : Serine hydroxymethyltransferase
- -S-S- : disulfide groups
- TG : triglyceride
- TH : transient hypertension
- tHcy : total homocysteine

- THF : Tetrahydrofolate
- TNF- α : Tumor necrosis factor-α
- Vit B12 : vitamin B12
- Vit B6 : vitamin B6
- wk : week
- WLB : white lysis buffer

SUMMARY

Low maternal B-vitamins status and hyperhomocysteinemia have been related to several pregnancy complications and adverse outcomes. Several prospective, retrospective, and case-control studies provided evidences that indicate the involvement of hyperhomocysteinemia in the etiology of preeclampsia, since endothelial dysfunction is a major complication in this disease. So far, low B-vitamin status is the most common cause of hyperhomocysteinemia. In Syria, a high prevalence of hyperhomocysteinemia and B-vitamin deficiency were found, which were mostly attributed to the Syrian lifestyle. Furthermore, low B-vitamin status and hyperhomocysteinemia have been described as independent risk factors for coronary heart diseases and venous thrombosis in this population.

Aiming to investigate the role of low maternal B-vitamin status and hyperhomocysteinemia in complicated pregnancies in Syria, maternal B-vitamin (folate, vitamin B12, vitamin B6) and related metabolic markers, including homocysteine, cystathionine, and methylmalonic acid were measured in a group of Syrian normotensive pregnant women and those whose pregnancy was complicated with preeclampsia.

Twelve-hour fasting blood samples were obtained from normotensive pregnant women (n = 98; 29 to 40 gestational weeks; 19 to 36 years old), and preeclamptic women (n = 177; 30 to 40 gestational weeks; 18 to 38 years old) of the same socio-economic status. Serum concentrations of homocysteine, cystathionine, and methylmalonic acid were assessed by gas chromatography-mass spectrometry. Vitamin B12 and folate in serum were measured by chemiluminescence immunoassay. The concentration of vitamin B6 was determined in plasma using high-performance liquid chromatography methods. Plasma concentration of holotranscobalamin Π was measured using a radioimmunoassay kit. The C677T methylenetetrahydrofolate reductase (MTHFR) gene mutation was investigated using a polymerase chain reaction/restriction fragment length polymorphism method. Other parameters were measured in Syria using Hitachi 917 automated analyser.

Higher concentrations of homocysteine, cystathionine, and methylmalonic acid were closely linked to a lower status of the B vitamins. In healthy pregnant women, homocysteine concentrations increased significantly with increasing gestation (from 5.6 to 8.0 μmol/L). Increased tHcy concentrations was associated with decreased serum folate concentrations by about 46 % (from 18.6 to 10.1 ng/ml), whereas vitamin B12 concentration displayed a small

decrease, only about 17 %. Serum homocysteine and cystathionine concentrations were significantly higher (median homocysteine 9.3 versus 6.0 μmol/L; median cystathionine 284 versus 232 nmol/L) and serum folate concentrations were significantly lower (median folate 7.3 versus 15.9 ng/ml) in preeclamptic women as compared to controls. These differences between patients and controls were seen in each tertile of gestation age. Preeclamptic women were more likely to have folate deficiency compared to healthy pregnant women (19 % of patients versus 5 % of controls). A very high prevalence of vit B12 deficiency was found in both groups, indicated by elevated methylmalonic acid (58.6 % in controls and 68.0 % in patients) and low holotranscobalamin Π concentrations (76.2 % in controls and 78.6 % in patients). Maternal vitamin B6 concentrations were abnormal low and correlated inversely and significantly to cystathionine in both groups. The frequency of the homozygous genotype of methylenetetrahydrofolate reductase (MTHFR TT) in preeclamptic women was not significantly different from that in healthy pregnant women (6.9 % compared with 12.4 %). The influence of MTHFR TT genotype on homocysteine concentrations was found to be dependent on folate status. Pregnant women with homozygous genotype had significantly higher homocysteine concentration compared to those with wild-type genotype (CC) only when serum folate concentrations were below 8.9 ng/ml. An increase in the risk of hyperhomocysteinemia was associated with folate levels ≤ 8.9 ng/ml and methylmalonic acid ≥ 339 nmol/L, and this risk was increased progressively when low folate status accompanied with elevated methylmalonic levels. Furthermore, there was statistically no significant association between the maternal MTHFR genotype or decreased vitamin B6 levels and the risk of hyperhomocysteinemia. There was an association between maternal homocysteine or folate concentrations and risk of preeclampsia. Pregnant women with serum homocysteine concentration > 7.8 μmol/L or folate concentrations < 8.7 ng/ml experienced a 21.6-fold and 9.9-fold, respectively, increase in the risk of preeclampsia. There was statistically no significant association between the maternal MTHFR genotype or decreased vitamin B12 levels and the risk of preeclampsia.

Elevated serum concentrations of homocysteine, cystathionine, and methylmalonic acid in preeclamptic women suggest disturbed homocysteine metabolism due to poor status of the B vitamins. Higher homocysteine concentrations in preeclamptic women are due to lower folate status. In preeclamptic women lower vit B12 concentration causes folate trap resulting in increased folate requirement for efficient remethylation of homocysteine to methionine. Higher cystathionine concentration in Syrian preeclamptic women is due to insufficient vitamin B6 concentration associated with increased activation of transsulfuration pathway due

to oxidative stress. Increased the risk of preeclampsia with increased homocysteine levels confirms the aetiological role of homocysteine in preeclampsia by inducing endothelial dysfunction.

Finally, the poor nutritional status in Syrian women, which is attributed to Syrian lifestyle, and associated hyperhomocysteinemia seem to be important factors in preeclampsia. Therefore, the improvement of B-vitamin status by supplementation is necessary to prevent pregnancy complications in women of childbearing age in this population. However, in populations with a high prevalence of vitamin B12 deficiency, like our population, vitamin B12 supplementation, in addition to folate supplementation, should be considered in order to improve vitamin status and lower homocysteine levels.

ZUSAMMENFASSUNG

Erniedrigte B-Vitamine und die Hyperhomocysteinämie wurden mit verschiedenen Schwangerschaftskomplikationen und einem ungünstigen Verlauf in Zusammenhang gebracht. Verschiedene prospektive, retrospektive und Fall-Kontroll Studien weisen auf eine Beteiligung der Hyperhomocystenämie bei der Ätiologie der Präeklampsie hin, zumal die endotheliale Dysfunktion für die Pathophysiologie dieser Erkankung eine zentrale Rolle spielt. In Syrien wurde eine hohe Prävalenz der Hyperhomocysteinämie und des Vitamin B Mangels gefunden, was im wesentlichen auf die syrische Lebensführung zurückzuführen ist. Außerdem wurden der Vitamin B-Mangel und die Hyperhomocysteinämie als unabhängige Risikofaktoren für kardiovaskuläre Erkrankungen und für venöse Thrombosen in dieser Bevölkerung beschrieben.

Um die Bedeutung des niedrigen mütterlichen Vitamin B-Status und der Hyperhomocysteinämie bei Schwangerschaftskomplikationen in Syrien zu untersuchen, wurden die mütterlichen B-Vitamine (Folat, Vitamin B12 und Vitamin B6) und die Metaboliten Homocystein, Cystathionin und Methylmalonsäure in syrischen normotensiven Schwangeren und Präeklampsie Patientinnen gemessen.

Nach zwölfstündigem Fasten wurden Blutproben von normotensiven Schwangeren (n = 98; 29. bis 40. Schwangerschaftswoche, Alter: 19 – 36 Jahre) und präeklamptischen Schwangeren (n = 177; 30. – 40. Schwangerschaftswoche; Alter: 18 – 38 Jahre) mit gleichem sozioökonomischen Status entnommen. Die Serumkonzentrationen des Homocysteins, Cystathionins, und der Methylmalonsäure wurden mit Hilfe einer Gaschromatographie-Massenspektrometrie-Methode bestimmt. Vitamin B12 und Folat im Serum wurden mit einem Chemilumineszenz-Immunoassay gemessen. Die Vitamin B6 Konzentration wurde mit einer Hochleistungs-Flüssigkeitschromatographie-Methode bestimmt. Die Plasmakonzentrationen des Holotranscobalamin II wurden mit einem Radioimmunoassay gemessen. Die C677T Methylentetrahydrofolat-Reduktase (MTHFR) Genmutation wurde mit der Polymerasekettenreaktion und einem Restriktionsenzym-Fragmentlängen-Polymorphismus untersucht. Weitere Parameter wurden in Syrien mit einem Hitachi 917 Analyseautomaten gemessen.

Erhöhte Konzentrationen des Homocysteins, Cystathionins und der Methylmalonsäure waren eng mit einem niedrigen Vitamin B-Status assoziiert. In gesunden schwangeren Frauen

stiegen die Homocysteinkonzentrationen signifikant mit zunehmender Dauer der Schwangerschaft an (5.6 bis 8.0 µmol/l).

Erhöhte Homocysteinkonzentrationen waren mit erniedrigten Serumfolatkonzentrationen von etwa 46 % (18.6 – 10.1 ng/ml) assoziiert, wohingegen die Vitamin B12 Konzentrationen nur einen kleinen Abfall von etwa 17 % zeigten. Die Homocystein und Cystathionin Konzentrationen waren signifikant höher in den Präeklampsie-Patientinnen im Vergleich zu den Kontrollen (mediane Homocysteinkonzentrationen 9.3 gegenüber 6.0 µmol/l; mediane Cystathioninkonzentrationen 284 gegenüber 232 nmol/l), während die Serumfolat Konzentrationen in den Präeklampsie Patientinnen im Vergleich zu den Kontrollen signifikant niedriger waren (mediane Folatkonzentrationen 7.3 gegenüber 15.9 ng/ml). Diese Unterschiede zwischen Patienten und Kontrollen wurden in allen Terzilen der Schwangerschaftsdauer beobachtet. Ein Folatdefizit wurde häufiger bei Präeklampsie Patientinnen als bei gesunden Schwangeren gefunden (19 % gegenüber 5 %). Eine sehr hohe Prävalenz eines Vitamin B12 Defizits, das durch erhöhte Methylmalonsäurekonzentrationen (58.6 % in Kontrollen und 78.6 % bei Patientinnen) und niedrige Holotranscobalamin II Konzentrationen (76.2 % in Kontrollen und 78.6 % in Patientinnen) angezeigt wurde, konnte in beiden Gruppen gefunden werden. Die mütterlichen Vitamin B6 Konzentrationen waren ungewöhnlich niedrig und korrelierten invers und signifikant mit der Cystathioninkonzentration in beiden Gruppen. Die Prävalenz für das homozygote Vorliegen der Mutation der Methylentetrahydrofolatreduktase (MTHFR 677TT) unterschied sich nicht signifikant zwischen den Präeklampsie-Patientinnen und den Kontrollen (6.9 % gegenüber 12.4 %). Der Einfluss des MTHFR Genotyps auf die Homocysteinkonzentration war vom Folat Status abhängig. Schwangere Frauen mit homozygotem Genotyp hatten nur dann eine signifikant höhere Homocysteinkonzentration im Vergleich zu denen mit dem Wildtyp-Genotyp (CC), wenn die Serumfolatkonzentration unter 8.9 ng/ml lag. Eine Zunahme des Risikos für eine Hyperhomocysteinämie war mit Folatspiegeln ≤ 8.9 ng/ml und Methylmalonsäurekonzentrationen ≥ 339 nmol/l assoziiert. Außerdem wurde das Risiko für eine Hyperhomocysteinämie besonders stark erhöht, wenn bei niedrigem Folat Status gleichzeitig die Methylmalonsäure erhöht war. Es war bestand keine signifikante Assoziation zwischen dem mütterlichen MTHFR Genotyp bzw. dem erniedrigtem Vitamin B6 Spiegel und dem Risiko der Hyperhomocysteinämie. Es bestand eine Assoziation zwischen dem Risiko für die Präeklampsie und dem mütterlichen Homocysteinspiegel sowie dem Folatspiegel. Schwangere Frauen mit Serumhomocysteinkonzentrationen > 7.8 µmol/l oder Folatkonzentrationen < 8.7 ng/ml hatten ein 21.6 fach bzw. 9.9 fach erhöhtes Risiko für die Präeklampsie.

Der mütterliche MTHFR Genotyp und erniedrigte Vitamin B12-Spiegel waren nicht signifikant mit der Präeklampsie assoziiert.

Die erhöhten Serumkonzentrationen des Homocysteins, Cystathionins und der Methylmalonsäure bei Präeklampsie Patientinnen legen einen aufgrund eines defizitären Vitamin B-Status gestörten Homocystein Metabolismus nahe. Die höheren Homocysteinkonzentrationen in Präeklampsie Patientinnen sind auf einen erniedrigten Folat Status zurückzuführen. In Präeklampsie Patientinnen verursacht erniedrigtes Vitamin B12 eine Folat Falle, die einen erhöhten Folat Bedarf für eine effiziente Remethylierung des Homocysteins zum Methionin zur Folge hat. Die erhöhte Cystathioninkonzentration in syrischen Präeklampsie Patientinnen ist auf eine inadäquate Vitamin B6 Konzentration und eine Aktivierung des Transsulfurierungsweges aufgrund von oxidativen Stress zurückzuführen. Das mit steigenden Homocysteinspiegeln zunehmene Präeklampsierisiko bestätigt die ätiologische Bedeutung des Homocysteins für die endotheliale Dysfunktion bei der Präeklampsie.

Der unzureichende Ernährungszustand der syrischen Frauen, der auf die syrische Lebensführung zurückzuführen ist, und die damit einhergehende Hyperhomocysteinämie sind wichtige Faktoren für die Präeklampsie. Daher ist eine Verbesserung des Vitamin B-Status durch Supplementation notwendig, um Schwangerschaftskomplikationen bei Frauen im gebärfähigen Alter in dieser Bevölkerung zu verhindern. Jedoch sollte in einer Bevölkerung mit hoher Prävalenz des Vitamin B12 Mangels zusätzlich eine Vitamin B12 Supplementation zur Folat Supplementation in Betracht gezogen werden, um den Vitamin Status zu verbessern und den Homocysteinspiegel zu senken.

Pregnancy is a physiological process comprising fundamental changes in the female organism. In most women these pregnancies associated changes are well tolerated. However in more than 40 % of all pregnant women complications occur. Pregnancy associated complications range from marginal pigmentations of the skin to the death of mother and fetus. Hypertensive disorders are very frequent complications during pregnancy and may cause severe fetal and maternal consequences. Low maternal B-vitamins status and hyperhomocysteinemia have been related to several pregnancy complications and adverse outcomes. Several prospective, retrospective, and case-control studies provided evidences that indicate the involvement of hyperhomocysteinemia in the etiology of preeclampsia, since endothelial dysfunction is a major complication in this disease. So far, low B-vitamin status is the most common cause of hyperhomocysteinemia. In Syria, a high prevalence of hyperhomocysteinemia and B-vitamin deficiency were found, which were mostly attributed to the Syrian lifestyle.

1. 1. Homocysteine story

In 1962, Carson and Neill, (1962) suggested for the first time an association between elevated homocysteine (Hcy) levels and diseases. In mentally retarded children they observed elevated Hcy levels in plasma and urine. Two years later, Mudd et al. and Gibson et al. noted that the homozygous defect of the cystathionine β-synthase is associated with an increased risk for death at very young age. However, that time there was no logical explanation for this observation. Based on findings obtained from infants with homocystinuria and methylmalonic aciduria, who died at 7 weeks of age, McCully hypothesized that elevated Hcy levels causes vascular changes and subsequent thrombosis (McCully et al., 1969). The potential role of Hcy in atherothrombotic disease has drawn the attention of scientists from many fields. In the meantime numerous studies considering Hcy and various diseases have been published. Recently, hyperhomocysteinemia is known as a risk factor for cardiovascular disease (Wald et al., 2002; Herrmann et al., 2001; McCully KS., 1996; Boushey et al., 1995), adverse pregnancy complications (Nelen et al., 2001; 2000; Aubard et al., 2000; Vollset et al., 2000), and neuropsychiatric disorders such as Alzheimer's disease (Schroecksnadel et al., 2004; Morris MS., 2003; Nilsson et al., 2002), and immune activation (Schroecksnadel et al., 2004 a).

1. 2. Homocysteine: its forms and related thiols

The non-proteine forming amino acid Hcy is a byproduct of the degradation of methionine (Meth) into the nonessential aminothiol cysteine. Normally, Hcy is metabolized via two pathways: the remethylation and transsulfuration pathways (figure 1. 3). Meth is an essential protein forming amino acid, which is mainly obtained by food intake or remethylation of Hcy (Mudd et al., 2001). Structurally, Hcy closely resembles Meth and cysteine (figure 1. 1).

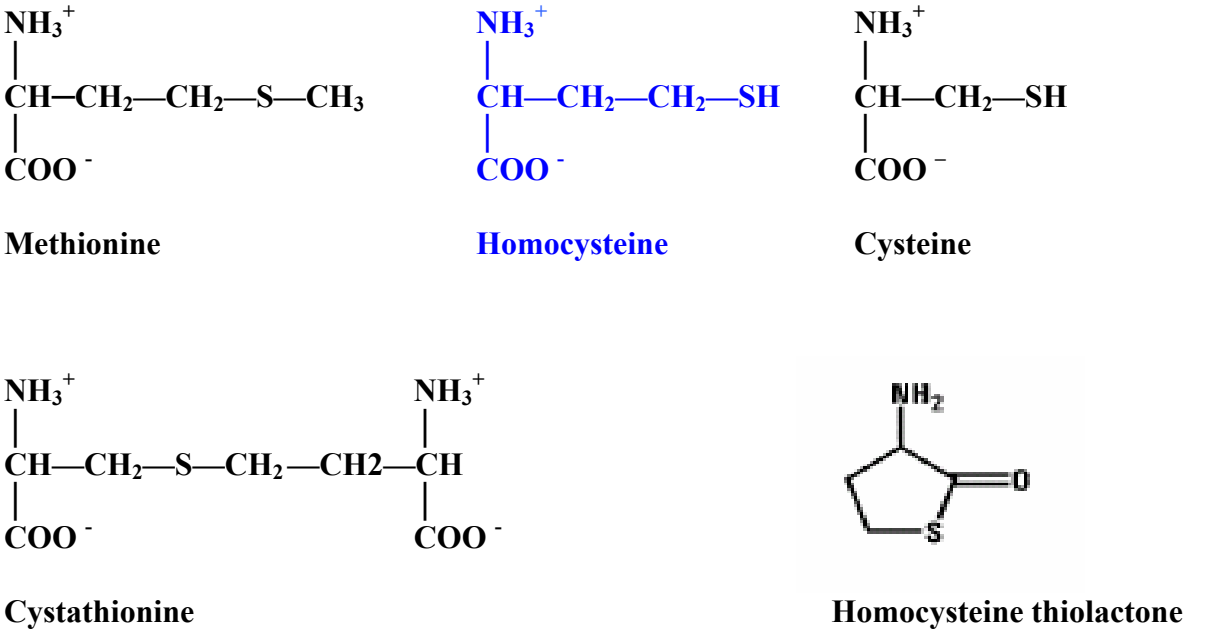


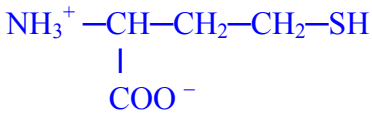
Figure 1. 1. The formulas of methionine, homocysteine, cysteine, and cystathionine

Hcy is synthesized within the cell. Due to its toxicity, the intracellular Hcy concentration is precisely regulated, < 1 µmol/L, and any excess is exported into the extracellular compartments. Because of the sensibility of the sulfuhydryle groups (-SH) to oxidation, only 1 % to 2 % of total plasma Hcy is found in the reduced form L-homocysteine. The remaining, 98 %, occur in the oxidized disulfide form (Hcy and mix disulfide) (Ueland PM., 1995; Jacobsen et al., 2001; 1998; Mudd et al., 2000). The disulfide form of Hcy is either formed by autooxidation or reaction with other thiol-containing compounds, (-SH) or disulfide (-S-S-)

groups (Bourdon and Blache, 2001). However, the oxidized forms of Hcy are divided into free and protein-bound forms. The free oxidized forms refer to the non-protein bound disulfides which include either the homocystine, symmetrical disulfide of two Hcy molecules, or the mixed disulfide of Hcy with free cysteine. In contrast, protein-bound Hcy includes mixed disulfides of Hcy with plasma proteins containing free cysteine (Mansoor et al., 1992) (figure1. 2). The total Hcy (tHcy) concentration refers to all Hcy species existing in plasma. Another Hcy derived thiol compound is Hcy-thiolactone. It is a highly reactive intra-molecular thioester of Hcy. It occurs in all cells and causes homocysteinylation of cellular and extracellular proteins that lead to impaired function (Jakubowski et al., 2004; 2000). Increased Hcy levels cause a great activation of Hcy-thiolacton production. The detoxification of Hcy-thiolactone is mediated by the thiolactonase enzyme, a constituent of high density lipoproteins (Jakubowski H., 2000 a; 2000 b).

I REDUCED FORM

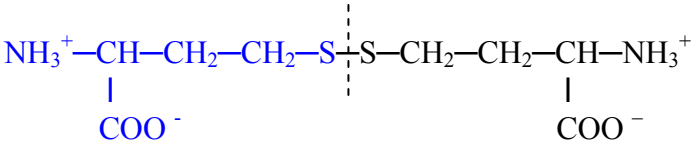
- **Homocysteine** (1-2 %)



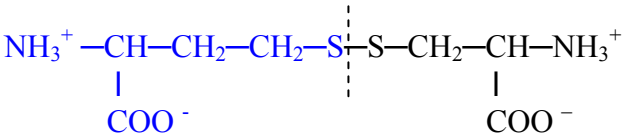
II OXIDIZED FORMS

- **Free forms**

- **Homocystine** (5-10 %)



- **Homocysteine-cysteine** (5-10 %)



- **Protein-bound form**

- **Protein-homocysteine** (80-90 %)

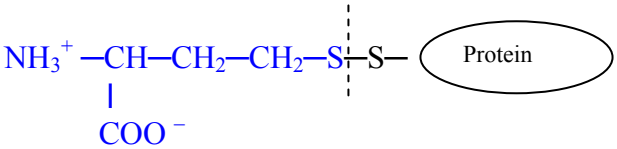


Figure1. 2. Illustration of all forms of Hcy present in plasma. The percentage of each form in plasma is given in brackets

1. 3. Homocysteine metabolism

Hcy is the final product of the Meth metabolism, and can be metabolized by two biochemical pathways: remethylation and transsulfuration. The remethylation converts Hcy back to Meth and the transsulfuration degrades Hcy into cystathionine (Cys) and further more to cysteine and taurine. Although Hcy is not a protein forming amino acid, it is involved in many important processes: cysteine and glutathione synthesis (Mosharov et al., 2000); catabolism of choline and betaine; and recycling of intercellular folates. Cellular Meth can be used for protein synthesis or be converted to S-adenosylmethionine (SAM). This reaction is catalyzed by the Meth adenosyltransferase (MAT) (figure 1. 3) (Storch et al., 1990), and requires the presence of adenosine triphosphate (ATP) (Markham et al., 1980). MAT activity is significantly regulated by the intracellular SAM level. SAM has a vital role throughout the body (Clarke and Banfield, 2001), particularly in central nerves system: It donates a methyl group for a lot of different reactions e.g. synthesis of creatine, phosphatidylcholine, and polyamines (for cell growth, gene expression, etc.); methylation of the CpG island of DNA (Katz et al., 2003), and acts as a precursor for the synthesise of cysteine and glutathione (Bottiglieri T., 2002) (figure 1. 3). The loss of this methyl group converts SAM into S-adenosylhomocysteine (SAH). SAH is then hydrolysed into adenosine and Hcy. This reversible reaction is catalysed by the SAH-hydrolase. The regeneration of Meth from Hcy is catalysed by two different methyltransferase enzymes. The first one is betaine:homocysteine methyltransferase (BHMT), which is located in liver and kidney. This enzyme utilizes betaine (trimethyl-glycine) as a donor of methyl groups. In cases of folate and/or cobalamin deficiency, this pathway maintains the tissue concentration of Meth. The second enzyme is methionine synthase (MS), also known as 5-Methyltetrahydrofolate:homocysteine methyltransferase. MS is present in almost all the cells throughout the body and catalyses the transfer of a methyl group from methyletetrahydrofolate (CH_3-THF). The remethylation by MS needs methylcobalamine as a cofactor (Banerjee et al., 2003; 1990: Matthews RG., 2001) (figure 1. 3). The cobalamin-dependent remethylation links the vitamin B12 (vit B12) metabolism with the folate cycle. Genetic or acquired inhibition of this enzyme will block the incorporation of CH_3-THF into the Meth cycle and cause mild hyperhomocysteinemia.

The transsulfuration pathway occurs only in liver, kidney, small intestine, and pancreas tissue. Cysteine and taurine are essential products of the transsulfuration which are centrally involved in cardiac and hepatic metabolism as well as in glutathione production. The transsulfuration is catalyzed by two pyridoxal phosphate-dependent enzymes (Mudd et al., 1989): cystathionine β-synthase (CBS) and cystathionine γ-lyase. CBS catalyses the

irreversible condensation of serine and Hcy to form Cys, and cystathionine γ-lyase hydrolysis Cys to cysteine and α-ketobutyrate (figure 1. 3). Cysteine undergoes further degradation to taurine, glutathione, and inorganic sulfur, which is excreted in the urine. CBS contains heme as a prosthetic group that is necessary to bind the active form of vitamin B6 (vit B6) (Meier et al., 2001; Kery et al., 1994).

In healthy individuals, the balance between transsulfuration and remethylation pathways is highly regulated and mainly employed to insure sufficient amounts of intracellular SAM (Finkelstein JD., 2000). In the case of decreased intracellular Meth (e.g. fasting state) remethylation is activated and transsulfuration activity becomes down regulated. In such a situation only 10 % of Hcy is catalyzed by CBS. The cellular folate cycle is shifted towards the formation of CH_3-THF. Thereby, utilization of the Meth for purine and pyrimidine biosynthesis is reduced (Scott et al., 1983). Contrary a Meth-rich diet will increase SAM levels within the cells. SAM then upregulates the CBS activity driving Hcy into the transsulfuration pathway (Finkelstein JD., 2000a; 2000b; 1984; Mato et al., 2002; Janosik et al., 2001). Additionally, SAM acts as an allosteric inhibitor for methylenetetrahydrofolate reductase (MTHFR) and BHMT causing aberration in the remethylation pathway (Jencks and Matthews, 1987). Moreover, an increased SAM level causes an increased cellular SAH concentration (figure 1. 3), which is a strong inhibitor of the adenosyl methionine-dependent methyltransferases. However, it is estimated that Hcy is recycled to Meth several times before it becomes irreversibly degraded by the transslfuration pathway (Mudd et al., 1980).

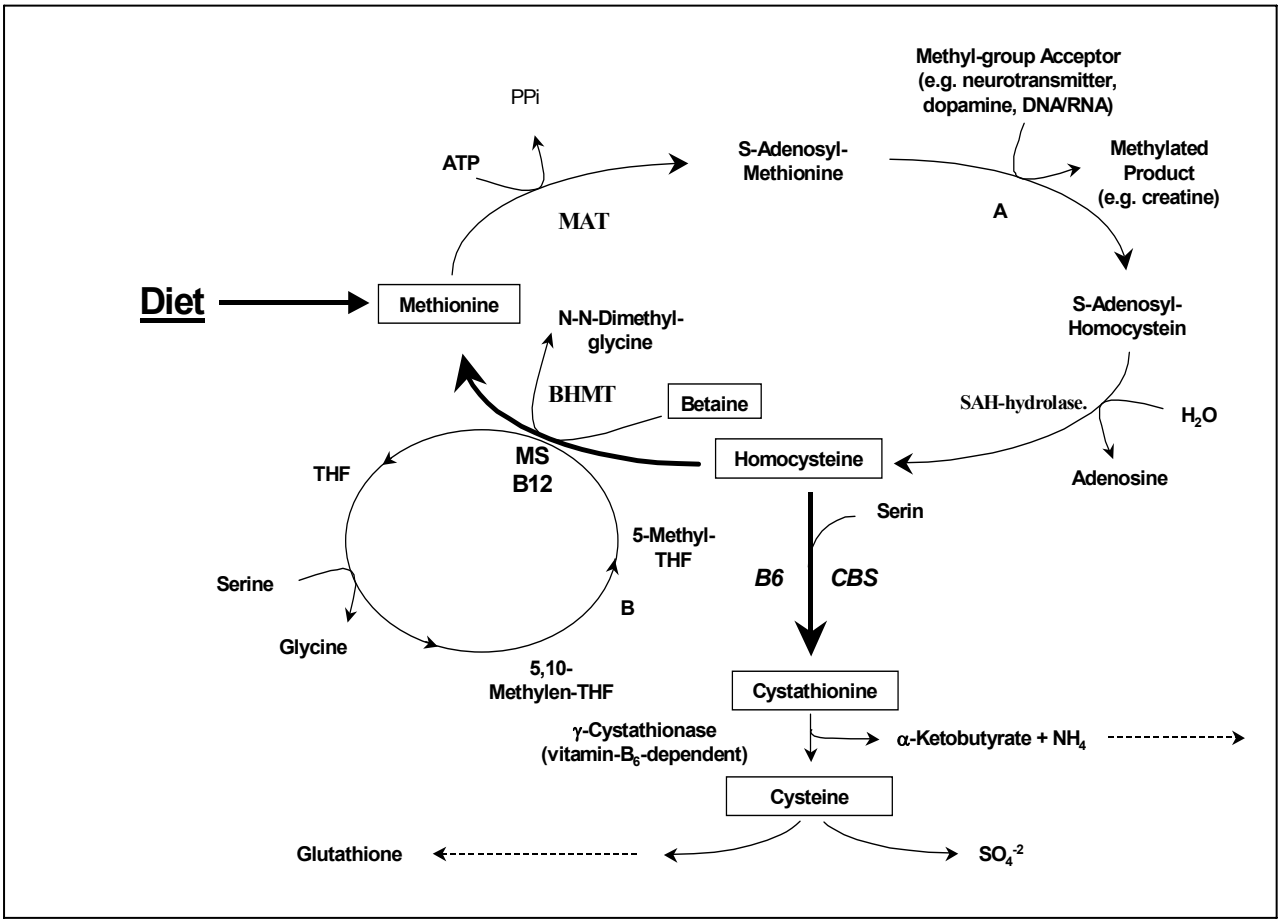


Figure 1. 3. Methionine metabolism. BHMT: Betaine:homocysteine methyltransferase, CBS: cystathionine B-synthase (vit B6-dependent), MAT: Methionine adenosyltransferase, MS: Methionine synthase (vit B12-dependent), THF: Tetrahydrofolate, A: Methyl-Transferases B: 5,10-Methylen-THF-Reductase

A main regulator of Hcy degradation is the folate cycle. Folate, a water-soluble B vitamin, acts as a coenzyme to accept or donate one carbon units needed in several metabolic pathways: remethylation of Hcy to generate Meth, the synthesis of thymidylate and purines, and the formation of methyl group. The first step in the folate cycle is the conversion of tetrahydrofolate (THF) to 5,10-methylene-THF (CH_2-THF) using serine as a source of carbon units and vit B6-dependent serine hydroxymethyltransferase (SHMT) enzyme. A portion of the produced CH_2-THF undergoes irreversible reduction to CH_3-THF via MTHFR. CH_3-THF is the only circulating form of folate, and is used for the remethylation of Hcy to Meth. As shown, folate, vit B12, and MS work together within the cells and their work is tightly regulated. Fasting plasma Hcy is markedly increased in patients with folate (Stabler et al., 1985; Kang et al., 1987) or cobalamin deficiency (Stabler et al.,1985) but is usually normal in vit B6-deficient subjects (Miller et al., 1992). Defects in one or more of them cause serious

problem. For instance, the genetic defect of MS or the deficiency of vit B12 leads to a trap of CH_3-THF within the cells. This makes CH_3-THF unable to be recycled again into the pool of active folates, i.e. "biologically dead". The consequence are abnormal levels of the intracellular folate in the presence of normal or elevated circulating folate levels. Moreover, the blocked THF regeneration leads to a reduced thymidylate synthesis causing megaloplastic anemia (Hoffbrand and Jackson, 1993).

1. 4. Hyperhomocysteinemia (HHcy)

HHcy is a terminology suggested to describe the presence of abnormal elevation in tHcy levels. Normal range for tHcy concentration is not totally specified although others tend to consider values between (5-12 µmol/L) as normal. However, according to the D.A.CH.-Liga Homocysteine (German, Austrian, and Swiss Homocysteine Society) fasting tHcy (< 12 µmol/L) is considered safe and should be the target level during homocysteine-lowering treatment. D.A.CH.-Liga Homocysteine classified several types of HHcy according to fasting tHcy levels:

- Moderate HHcy is defined as tHcy concentrations between 12-30 µmol/L and has a prevalence of 5-10 % in total population. Unhealthy lifestyle, vegetarian diet, impaired renal function, mild folate or vit B12 deficiency, and MTHFR 677 C→T polymorphism are common causes for moderate HHcy.
- Intermediate HHcy is defined as tHcy concentrations between 30-100 µmol/L and its prevalence in general population is ~ 1 %. Moderate to severe deficiency of vit B12 or folic acid and renal failure can cause intermediate HHcy.
- Severe HHcy is defined as tHcy concentrations higher than 100 µmol/L and has a prevalence of 0.02 %. This form is seen in individuals with homocystinuria or severe vit B12 deficiency .

It is important to mention that the above reported values identifying different types of HHcy are commonly used in a non-pregnant population. Normal pregnancy is associated with lower tHcy levels compared to non-pregnant state. Therefore, applying these values on pregnant women leads to misleading interpretation. HHcy, however, is a controversial term, and the cut-off value differs according to the population. Elevated tHcy levels is a proxy measure for the deficiency of the B vitamins. Therefore, identifying the cut-off value of HHcy should be achieved in the light of B-vitamin status. Up to date, no cut-off value identifying HHcy in pregnant women is available. In the current study, the cut-off value (Hcy > 8.2 µmol/L)

representing the 95th percentile of Hcy distribution in normotensive pregnant women who had adequate status of folate and vit B12 was used.

1. 5. Factors influencing homocysteine concentration

The regulation of the Meth cycle and the Hcy pathway is tightly associated with the availability of folate, vit B6, and vit B12. While folate donates its methyl for the remethylation of Hcy, vit B 12 and B6 are important co-factors for MS and CBS, respectively. Additionally, there are many other factors influencing Hcy which can be classified as:

● **Physiologic determinants:** such as sex (Selhub et al., 1999), age (Selhub et al., 1999; Andersson et al., 1992), race (Ubbink et al., 1995), and pregnancy (Kang et al., 1986).

● **Lifestyle factors:** such as coffee consumption (Husemoen et al., 2004), alcohol drinking (de Bree et al., 2001 a), and physical activity (Herrmann et al., 2003; Nygard et al., 1995).

● **Genetic factors:** such as CBS enzyme (Kraus et al., 1999), MTHFR enzyme (Rozen R., 1997), MS enzyme (Leclerc et al., 1996), and methionine synthase reductase enzyme (Matthews et al., 1998).

● **Drugs:** Such as lipid lowering drugs, hormons, antiepileptic drugs. For more details the reader is referred to the paper of Stanger et al. (2003).

● **Diseases:** For details see table (1. 1).

Table 1. 1. Diseases affecting Hcy metabolism

Disease	Effect on Hcy	Mechanism	Reference
Autoimmune diseases (rheumatoid arthritis)	↑	- Drug use - Vitamin deficiency - Gastrointestinal dysfunction	Roubenoff et al., 1997 Van Ede et al., 2001.
Endocrine disorders			
- Early stage of diabetes	↓	- Glomerular hyperfiltration. - The effects of insulin.	Wollesen et al., 1999 Schneede et al., 2000.
- Late stage of diabetes	↑	-Nephropathy and impaired renal clearance.	Audelin et al., 2001.
- Hypothyroidism - Hyperthyroidism	↑ ↓	- Thyroid hormones influence the synthesis of flavin mononucleide(FAD).	Nedrebo et al., 1998. Diekman et al., 2001. Hustad et al., 2000.
Gastrointestinal disorders (ulcerative colitis, Crohn`s disease,…..)	↑	- Malabsorption of vit B12 and folate	Gregory et al., 2001. Schneede et al., 2000.
Gout	↑	-Altered tubular excretion -decreased glomerular filtration	Istok et al., 1999.
Hyperproliferating diseases (cancer, psoriasis)	↑	-The rapidly dividing cells use the the methyl group of Meth and the one carbon unite of THF at the expense of increased tHcy levels.	Refsum and Ueland, 1990.
Renal disease	↑	Decreased the remethylation of Hcy in the kidney	Van Guldener et al., 1999

↑ increase; ↓ decrease

1. 6. Pathogenetic mechanism of homocysteine

Since McCully`s observation in 1969, scientists tried to find the mechanism by which HHcy adversly affects the vessels. In recent years numerous in vitro and in vivo studies have gained new insights in the pathomechanisms of Hcy. One of these effects of Hcy is a reduction in the endothelial function. Endothelial cells synthesize several agents that are centrally involved in the regulation of vasoconstriction and vasodilation (Cooke JP., 2000). Endothelium-derived

vasoconstrictors are thromboxane A_2, prostaglandin H_2, and endothelin 1. The endothelium-derived vasodilators are nitric oxide (NO) and prostacyclin (PGL_2) (Shimokawa H., 1999). HHcy mediates the endothelial dysfunction by several mechanisms (figure 1. 4):

- HHcy may reduce the bioactivity of endothelium-derived nitric oxide (NO):

During HHcy, the reaction of NO with superoxide produces peroxynitrite ($ONOO^-$), which is a potent oxidant. $ONOO^-$ causes activation of poly (ADP-ribose) polymerase (PARP) which is an important mediator of vascular dysfunction in disease (Mujumdar et al., 2001). Also, $ONOO^-$ can oxidize tetrahydrobiopterin, a critical cofactor for NO synthase (eNOS), leading to a reduced activity of eNOS or an eNOS-uncoupling where the electrons are transported to molecular oxygen forming $O_2^{\cdot -}$ rather than to L-arginine forming $NO^{\cdot}$ (Laursen et al., 2001). More that, HHcy inhibits the activity of eNOS by increasing the levels of asymmetric dimethylarginine (ADMA), which is an endogenous inhibitor of NO synthases, leading to reduce the bioavilability of No (Stuhlinger et al., 2001; Boger et al., 2000).

- Hcy increases oxidative stress and levels of reactive oxygen species (ROS):

Elevated tHcy levels inhibit the expression or function of antioxidant enzymes such as extracellular superoxide dismutas (EC-SOD) by stimulating the degradation of endothelial heparan sulfate proteoglycan (Yamamoto et al., 2000). More that, Hcy increases the activity of vascular sources of $O_2^{\cdot -}$ including xantine oxidase, cyclooxygenase, nitric oxide syntesis (NOS), and NAD(P)H oxidase (Bagi et al., 2002; Hanna et al., 2002; ungvari et al., 2002; Mohazzab et al., 1994).

- HHcy can upregulate components of the inflammatory cascade:

Hcy activates nuclear factor-κB (NF-κB) and causes overexpression of cytokines (e.g. tumor necrosis factor-α (TNF- α) (Hunt and Tyagi, 2002; Wang and Siow, 2000) leading to inhibition of vasoconstriction and thereby impairment of endothelial function. Also, TNF- α increases the activity of NAD(P)H oxidase causing, consequently, increased superoxides levels seen in HHcy (Frey et al., 2002; Fichtlschere et al., 2001).

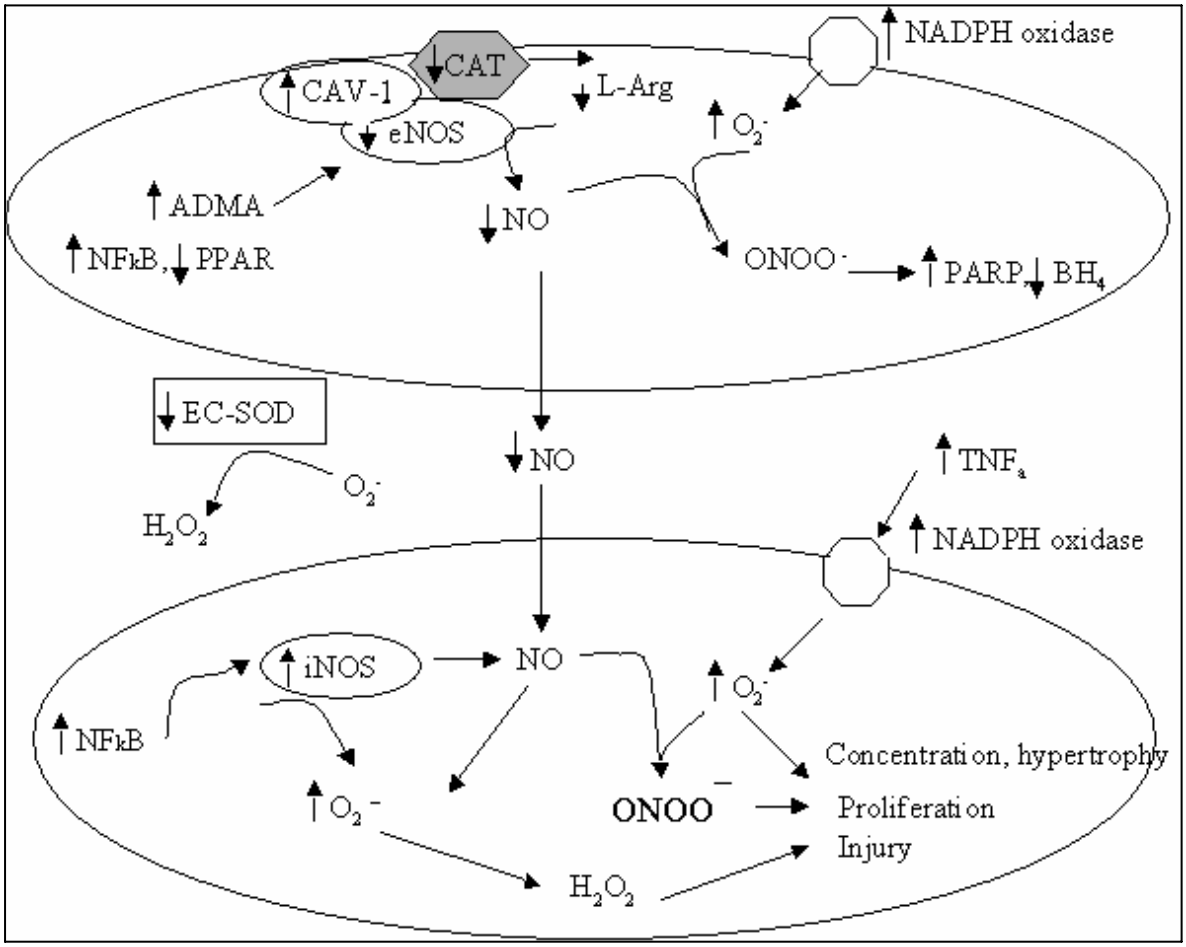


figure 1. 4. Changes within the vessel wall in response to HHcy. HHcy causes decreased activity of the transporter for L-arginine (CAT-1), increased expression of caveolin-1, reduced expression of eNOS that can be also inhibited by ADMA

1. 7. Classification of pregnancy hypertension

Hypertension in pregnancy is defined according to the International Society for the Study of Hypertension in Pregnancy (ISSHP) as a diastolic blood pressure of ≥ 90 mmHg measured on two consecutive occasions 4 hour apart, or a single reading of diastolic blood pressure of 110 mmHg or above. Frequently, hypertension in pregnancy is accompanied by proteinuria, defined as an urinary protein loss of ≥ 300 mg in a 24 hour specimen (Higgins and de Swiet, 2001).

The American National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy (NHBPEP) suggested diagnostic criteria to discriminate among the different types of hypertension in pregnancy (Roberts et al., 2003; report of the NHBPEP

working group on high blood pressure in pregnancy, 2000). According to the NHBPEP criteria, women with increased blood pressure are classified as a follows:

- *Chronic hypertension*: the hypertension that is present before pregnancy or before the 20th week of gestation, or that is diagnosed for the first time during the pregnancy and persists postpartum.
- *Gestational hypertension*: onset of hypertension after the 20th week of gestation. The combination of gestational hypertension and proteinuria is named preeclampsia syndrome. Women who do not manifest proteinuria or other related findings are retrospectively divided into two subgroups:
    - Transient hypertension: hypertension resolves by 12 weeks postpartum.
    - Chronic hypertension: hypertension does not resolve by 12 weeks postpartum.
- *Preeclampsia superimposed upon chronic hypertension*: the occurrence of preeclampsia in a woman with preexisting hypertension.
- *Preeclampsia*: onset of hypertension in combination with proteinuria after the 20th week of gestation and the remission of these signs after the delivery (Lindheimer et al., 1999). Preeclampsia can be classified into a mild, a moderate and a severe form according to the associated symptoms (table 1. 2). *Eclampsia* is the most severe form of preeclampsia, and is characterized by the occurrence of generalized convulsions during pregnancy, labour, or within 7 days after the delivery in the absence of preexisting epilepsy or convulsive disorders. Postpartum seizures account for about 44 % of all seizures (Munro PT., 2000). *The HELLP-syndrome* (**H**emolysis, **E**levated **L**iver enzymes, and **L**ow **P**latelet count) is the most lifethreatining complication of preeclampsia and eclampsia. Nearly 10 % of severe preeclamptic women and 30-50 % of eclamptic women sustain a HELLP-syndrome.

Table 1. 2. Classification of preeclampsia according to the ISSHP guidelines

	Mild	**Moderate**	**Severe**
Diastolic blood pressure	90-100 mmHg	100-110 mmHg	> 110 mmHg
Headaches	minimal	mild	marked, persistent
Visual symptoms	minimal	mild	marked, persistent
Blindness	absent	absent	present
Convulsions	absent	absent	present
Upper abdominal pain	absent	absent	present
Fetal growth retardation	absent	absent	present
Intravascular hemolysis	absent	absent	present
Thrombocytopenia (< 10^5)	absent	absent	present
Oliguria < 400 dL/24 hour	absent	absent	present
creatinine, uric acid levels	normal	mildly elevated	markedly elevated
SGOT, SGPT, LDH	normal	mildly elevated	markedly elevated

SGOT: serum glutamic-oxaloacetic transaminase; SGPT: serum glutamic-pyruvic transaminase; LDH: lactate dehydrogenase

1. 8. Incidence and Risk factors

Hypertensive disorders occur in 12-22 % of all pregnancies. Preeclampsia is the most frequent hypertensive disorder during pregnancy. Worldwide, 3-14 % of all pregnancies are complicated by preeclampsia. In industrialized western country the frequency ranges between 5-8 % (Sibai et al., 1995; Cunningham and Lindheime, 1992; Saftlas et al., 1990). Three to five percent of all cases of preeclampsia occur during the first pregnancy. Between 5-10 % of these cases develop severe preeclampsia according to the ISSHP criteria. The maternal morbidity and mortality because of preeclampsia account for about 16 % of all maternal deaths in the UK. More than 40 % of iatrogenic premature deliveries are attributed to preeclampsia. The incidence of eclampsia is 0.2 % of all pregnancies and causes the termination of 1 in 1000 pregnancies.

The developing of preeclampsia is related to several risk factors such as:

- Nulliparity, primiparity (Skjaerven et al., 2002; Eskenaziet et al., 1991).
- Previous preeclampsia and positive family history of preeclampsia (Nilsson et al., 2004; Saftlas et al., 1990).
- Multiple (twin, triplet) pregnancies (Mastrobattista et al., 1997; Coonrod et al., 1995).

- Black race and age < 19 or > 35 years (Chesley LC., 1984).
- Work-related factors (Klonoff et al., 1996).
- Diseases such as hypertension (Sibai et al., 1995), renal disease (Rey and Couturier, 1994), diabetes mellitus (Nilsson et al., 2004), and thrombophilic disorders are also associated with an increased risk for preeclampsia.

Additionally, several studies suggested elevated tHcy levels as a relevant risk factor for preeclampsia.

1. 9. Hcy in normal and pregnancy complicated with preeclampsia

Preeclampsia is a leading cause of maternal mortality. Recently, it was proposed that preeclampsia is a two-stage disease. The first stage is characterized by reduction of placental perfusion. The second stage is dominated by the maternal syndrome: hypertension accompanied with proteinuria (Roberts and Cooper, 2001). Oxidative stress has been suggested as a major factor for the progression of the disease. Together with other maternal factors such as age, nulliparity, multiple pregnancies, etc., oxidative stress causes endothelial dysfunction, which is supposed as the underlying pathomechanism of preeclampsia (Var et al., 2003; Sikkema et al., 2001). Up to date, The etiology of preeclampsia is still not fully understood. However, its occurrence and progression depend on a complex pattern of interactions between genetic make-up and acquired factors (Roberts and Cooper, 2001).

Pregnancy is associated with higher B vitamins requirements to respond well to the increased demands of maternal and growing infants. B vitamins (folate, vit B12, and B6) play as cofactors in numerous of metabolic reaction such as one carbon metabolism required for DNA and RNA synthase and cell division. Serum concentrations of these vitamins are commonly decline throughout pregnancy (Cikot et al., 2001). It is thought that this decline is related to higher metabolic rate and active transport of the vitamins into the placental tissues and the fetus. Maternal B-vitamins status from preconception throughout pregnancy strongly affects the infant status of these vitamins at birth (Murphy et al., 2004; Monsen et al., 2001). The influence of maternal B-vitamins status on the nutritional status of infants is even extended into the lactation (Allen LH., 2005; Black et al., 1994). For instance, in a breast milk sample collected from lactating Guatemalan women and their infants at 3 month, breast milk vitamin B-12 was low in 31 %, and 62 % of infants had low or deficient vit B12 concentration at age 7 to 12 months (Casterline et al., 1997).

Maternal nutritional status has received increasing attention as an important risk factor that influences the outcome and progress of pregnancy (Vollset et al., 2000; Ray et al., 1999). Low

maternal folate status has been associated with increased the risk of preterm delivery, low birth weight, and NTD (Scholl et al., 2000; Hibbard BM., 1964). Likewise, vit B12 deficiency has been associated with maternal megaloblastic anemia, and increased the risk of very early recurrent abortion, and NTD (Groenen et al., 2004; Savage et al., 1994). Therefore, prenatal vitamin supplementation has been recommended (Rolschau et al., 1999; Czeizel AE., 1993). In US, folic acid-enriched products improved the maternal folate status and led to a 15–30 % decrease in neural tube defects. Additionally, a decrease in the incidence of preeclampsia and gestational hypertension in women with folate supplementation has been found (Hernandez-Diaz et al., 2002; Sanchez et al., 2001).

Elevated tHcy concentration is a proxy measure for deficiency of B-vitamins (folate, vit B12, and vit B6). Maternal HHcy was associated with serious pregnancy complication affecting adversely the mothers as well as their offsprings (Vollest et al., 2000). In a study included 93 women and their offspring, Murphy et al. found that the fetal tHcy concentration and birth weight were significantly correlated to maternal tHcy from preconception throughout pregnancy. Additionally, mothers in the highest tHcy tertile at 8 wk gestation were three time more likely to give birth to a neonate in the lowest weight tertile. Neonates of mothers in the highest tHcy tertile at labor weighed 228 g less than those born to mothers in the two lowest tertile (Murphy et al., 2004). Several studies concerning the association between maternal HHcy and adverse outcome were reported (Cotter et al., 2003; Nelen et al., 2000; Van der Molen et al., 2000; Vollest et al., 2000; Goddijn et al., 1996; Rajkovic et al., 1997; Powers et al., 1998; Dekker et al., 1995; Steegers-Theunissen et al., 1995).

Serum tHcy concentrations fall in normal pregnancy as early as 8-10 weeks' gestation (Murphy et al., 2002; Andersson et al., 1992). The lowest values of Hcy, approximately 50-60 % of that found in non-pregnant women, have been found in the second trimester (Andersson et al., 1992; Kang et al., 1986). In the third trimester, Hcy increases towards its preconception values (Holmes et al., 2005; Lopez-Quesada et al., 2003). Nevertheless, Hcy concentration before delivery remains lower than that at preconception (Murphy et al., 2004). Several mechanisms have been proposed to explain the decrease in maternal tHcy, including the normal increase in the glomerular filtration rate that accompanies pregnancy, the increase in plasma volume and associated haemodilution, increased the uptake of maternal Hcy by the fetus, increased maternal B-vitamins intake, and the hormonal effect on Hcy metabolism (Murphy et al., 2002; walker et al., 1999; Bonnette et al., 1998; Malinow et al., 1998). However, the exact mechanism is still unclear, but one possible benefit outcome of lower Hcy

in pregnancy may be the protection of the mother and fetus from Hcy-dependent pregnancy complications (Holmes VA., 2003).

HHcy adversely affects the vessels causing endothelial dysfunction and vascular damage (Geisel et al., 2003; Herrmann and Knapp, 2002; Stanger et al., 2001). Recently, HHcy has been closely related to preeclampsia, since endothelial dysfunction is one major complication in this disease (Powers et al., 2001; 1998; Roberts et al., 1989). A study in Netherland included women with a history of severe early-onset preeclampsia showed that the incidence of HHcy in these women were 18 % compared with an incidence of 2.5 % in normal population (Dekker et al., 1995). A study from African women showed an odds ratio for eclampsia of 6.03 among women in the highest quartile of the control Hcy distribution compared with women in the lowest quartile. The corresponding odds ratio for preeclampsia was 4.57 (Rajkovic et al., 1999). Another study included Peruvian women found that relative to women in the lower quartile of the control Hcy distribution, women who have tHcy concentration in the highest quartiles experienced a 2.9-fold increased risk of preeclampsia (Sanchez et al., 2001). The same group found that after adjustment for potential confounder, the relative risk of preeclampsia increased to 4-fold, suggesting that elevated maternal tHcy levels plays a significant role in the pathogenesis of preeclampsia. Several studies were initiated addressed Hcy as a biomarker with predictive value early in the pregnancy for identifying women at risk of subsequent development of preeclampsia (D'Anna et al., 2004; Cotter et al., 2001; Hietala et al., 2001; Hogg et al., 2000; Sorensen et al., 1999). Unfortunately, conflicting results were obtained.

In preeclamptic women, elevated Hcy concentrations have been found throughout all pregnancy stages and postpartum (Lopez-Quesada et al., 2003; Cotter et al., 2001; Sanchez et al., 2001; Wang et al., 2000; Rajkovic et al., 1999; Sorensen et al., 1999; powers et al., 1998; Dekker et al., 1995). Furthermore, women with a history of preeclampsia also have elevated Hcy levels (Raijmakers et al., 2004; vollset et al., 2000). The reason behind tHcy elevation during preeclampsia is still not clear. However, several explanations have been suggested, including renal insufficiency (Brattstrom L., 2003), decreased the reformation of Meth from Hcy for fetal demand (Malinow et al., 1998), disturbance of the Hcy metabolism by the liver (Oosterhof et al., 1994), decrease in the whole body remethylation (Powers et al., 2004), reduction of B-vitamins occurred during preeclampsia (Park et al., 2004). However, there are also studies that did not find difference in maternal Hcy concentration between preeclamptic and normal pregnant women (D'Anna et al., 2004; Herrmann et al., 2004; Mayerhofer et al., 2000) (table 1. 3). The discrepancy in the obtained results may be contributed, somewhat, to

the differences in the factors that determine Hcy concentration in the body and which were not measured together in most of these studies (i.e. vitamin status, genetic factors, lifestyle, renal function, diseases, drugs consumption, socioeconomic status, etc.). Folate was measured in only some studies. Although many publications reported no significant difference in folate levels between preeclamptic and control pregnants (Powers et al., 1998; Rajkovic et al., 1997), recent studies found that lower folate levels were associated with a higher risk of preeclampsia (Sanchez et al., 2001; Rajkovic et al., 2000). The two existing studies measuring vit B12 in preeclamptic women did not observe an association between the risk for preeclampsia and low serum vit B12 concentration (Powers et al., 1998; Rajkovic et al., 1997). The mutation of the MTHFR 677C→T has been postulated as a risk factor for preeclampsia. Many studies performed to investigate the impact of this mutation for the genesis of preeclampsia. Existing results are conflicting. Japanese and Italian pregnants with the C677T mutation have been found to be prone to preeclampsia (Grandone et al., 1997; Sohda et al., 1997), while Australian women are not (Kaiser et al., 2001; 2000). In a group of Americans, Powers et al. (2003) demonstrated that MTHFR mutation is not a risk factor for preeclampsia if prenatal folate is substituted.

Table 1. 3. Summary of the existing studies about preeclampsia and Hcy

Author/date	Study groups	Sampling	Result
D`Anna, 2004	PE= 27 IUGR= 36 Con=63	In the early second trimester, and at delivery	- No differences in Hcy between study groups in the early second trimester. - At delivery, preeclamptic women had significantly higher Hcy levels than controls.
Herrmann, 2004	PE= 24 HELLP=20 Con= 34	At 35 weeks of gestational age	- Elevated Hcy levels are seen only in HELLP group compared with control group. - Folic acid, vit B6, and MMA were not different between the study groups.
Patrick, 2004	Black PE= 26 Con=52 White PE= 34 Con=48	Third trimester	- Folic acid concentrations were lower in black women compared with white women. - Black women with PE had elevated Hcy levels compared with black women with normal pregnancy, white women with preeclampsia, and white women with normal pregnancy
Cotter, 2003	PE= 71 Con= 142	At 16 wk	Women who developed nonsevere PE Had higher Hcy levels in early pregnancy.
Lopez-Quesada, 2003	PE= 32 Con=64	Third trimester	Hcy and folate were significantly higher in PE compared with controls in the third trimester.
Tug, 2003	PE= 20 Con= 20	Third trimester	Preeclamptic women had elevated Hcy levels compared with normotensive control.
Cotter, 2001	PE=56, Con=112	Second trimester	PE have elevated Hcy in early pregnancy compared with normal pregnancy
Hietala, 2001	PE= 34 Con= 68	At 16 wk	No differences in Hcy levels between women who developed PE or who remained normotensive.
Power, 2001	PE= 17 TH= 16 Con= 34	At delivery	Hcy and cellular fibronectin were significantly higher in preeclamptic women compared to subjects from the other two group.
Raijmakers, 2001	PE= 20 Con= 10 NP= 10		Con had Hcy levels lower than NP PE had higher Hcy levels than con
Sanchez et al., 2001	PE= 125 Con= 179	Third trimester	Women in the highest quartile of Hcy and lowest quartile of folate experinced increased risk of PE, whereas no increased risk of PE associated with low plasma Vit B12 concentration.
Hogg, 2000	PE= 4 PIH= 12 IUGR= 22 Con=		- At 26 wk no significant differences in Hcy levels between PE, PIH, and Con - At 36, PE and PIH had higher Hcy compared with Con.
Mayerhofer, 2000	PE=45 Con=45	Second and third trimester	No difference in Hcy levels between prerclampsia group and control group

Wang, 2000	PE=43 Con=26	Within one week befor delivery	THcy was significantly higher in preeclampsia group compared with control group.
Rajkovic, 1999	EC=33 PE=138 Con=185	Postpartum	The mean Hcy levels was significantly higher in women with PE and EC than in control group (P<·001).
Sorensen, 1999	PE=52 Con=56	Second trimester	Second trimester elevation of Hcy was associated with a 3.2- fold increase risk of preeclampsia.
Powers, 1998	PE=21 Con=33	Antepartum	Hcy, malondialdehyd, TG. Fibronectin are higher in PE than in Control (P<·04, P<·001, P<·001, P<·006 respectively).
Rajkovic, 1997	PE=20 Con=20	At the delivery	- PE had significantly higher tHcy than control group (P<·001) - Folic acid and vit B12 were not significantly different between the two groups.
Dekker, 1995	PE=41 EC=7 HELLP=53	Postpartum	17,7 % of women with a history of severe PE had a positive methionine loading test

Con = Control group include normotensive pregnant women
EC= Eclampsia
HELLP= "Hemolysis, elevated liver enzyme, low platelet" syndrome
MMA= Methylmalonic acid
NP= Non-pregnant women
PE= Preeclampsia
TH= Transient hypertension

2. THE AIM OF THE STUDY

The overall maternal and prenatal mortalities in Syria was estimated to be 4.3 % and 2.6 %, respectively. Recently, a case-control study on Syrian patients with coronary heart disease has shown a high prevalence of HHcy (Hcy > 12 μmol/L) and functional vit B12 deficiency, indicated by elevated MMA and low holoTC, in patients and, more importantly, in healthy subjects. Additionally, a more recent case-control study on Syrian young patients with a history of thrombosis has shown that low levels of folate or vit B12 were independently and strongly associated with the risk of venous thrombosis, and this risk was stronger than that introduced by elevated Hcy levels. The high prevalence of HHcy and B-vitamin deficiency in Syrian population was attributed to Syrian lifestyle. Elevated maternal tHcy concentrations and low B-vitamins status have been recently related to several pregnancy complications and adverse outcomes. Therefore, the high prevalence of HHcy and B-vitamins deficiency in Syria is a serious problem in this region where a high birth-rate is present.

The current study was undertaken with the aim to investigate the role of low maternal B-vitamin status and HHcy in complicated pregnancies in Syria. For this purpose maternal B vitamins concentrations, homocysteine, and other associated metabolites, including Cys, MMA, and holoTC, were measured in a group of Syrian preeclamptic women and normotensive pregnant women of the same socio-economic status using modern laboratory analyser. The direct measurement of serum B-vitamin does not well represent the functional supply with these vitamins, and the parallel measurement of the metabolites provides a more sensitive and specific approach for identifying B vitamins status at the cellular levels.

MTHFR C677T mutation is associated with decreased enzyme activity and may therefore provoke HHcy in the presence of low folate status. Recently, MTHFR TT has been postulated as a risk factor for preeclampsia. Therefore, the MTHFR genotype was investigated in this study.

3. MATERIALS AND METHODS

Subjects and study design

Two hundred and seventy five nulliparous, uni and multi parous women at the second and the third trimester of their pregnancy were included in this study. Subjects were divided into two groups: women with a normal pregnancy (n = 98), women with pregnancy complicated with preeclampsia and eclampsia (n = 177). Subjects have been recruited between July 2002-2003 at the department of obstetrics and gynecology of the university of Damascus, Syria New Maternal Hospital. According to the ISSHP guidelines, preeclampsia (PE) was diagnosed when hypertension and proteinuria occurred after 20 weeks of gestation and disappeared spontaneously after the delivery, and eclampsia (EC) was defined as hypertension and proteinuria in combination with seizures not related to other diseases. Hypertension was defined as a diastolic blood pressure of ≥ 90 mmHg in two consecutive occasions 6 hour (h) apart. Proteinuria was defined when urine protein concentration exceeded 300 mg per 24 h or "+2" in a dipstick test in two random specimens collected at least 6 h apart. Eligible controls were subjects without hypertension and proteinuria throughout the entire pregnancy. Neither patients nor controls had chronic hypertension, renal or metabolic disease, platelet disorders, autoimmune disorders or epilepsy. All subjects filled in a questionnaire to register anthropometric, reproductive, and lifestyle characteristics (smoking, coffee consumption, diet, physical activity, vitamins use)(table 4. 1). Gestational age was calculated considering the first day of the last menstrual period as day 0. A major problem was the registration of maternal weight and blood pressure before the pregnancy since most women did not have routine medical pre-pregnancy care. Two formal approval were obtained to perform this study from the ministry of health and the board of hospital of the university of Damasccus. Informed consent was obtained from all participants.

Preanalytical sample handling

Fasting venous blood samples were drawn after 12 h of fasting from the antecubital vein using ethylenediamine tetraacetic acid (EDTA) containing Vacutainer® tubes and dry Vacutainer® tubes (BD, Germany). EDTA-sample were used for whole blood and plasma analyses. Blood samples collected into dry Vacutainer® tubes were used for serum preparation. Blood sampling was done immediately after the diagnosis of preeclampsia. Therefore, the day of blood sampling throughout the pregnancy was not standardized. However, in all cases one

blood sample was taken within 8 h before the delivery. The blood was allowed to clot on ice. Plasma and serum were separated within 40 minutes (min) after sample collection by centrifugation at 2000 × g for 20 min. Several aliquots of plasma and serum were prepared and stored at – 80 ºC for further analysis. After removal of EDTA-plasma the remaining cells of the patients group were used to extract genomic DNA by a manual isolation protocol (see below). Genomic DNA of the controls was extracted from frozen whole blood using a commercial kit (QIAamp® DNA Mini Kit; Qiagen, Germany).

Laboratory analysis

Serum concentrations of blood glucose, creatinine, uric acid, urea, total-bilirubin, alanin aminotransferase (GPT), aspartat aminotransferase (GOT), cholesterol (Cho), triglyceride (TG), and high-density lipoprotein (HLD) were measured on a Hitachi 917 automated analyzer using commercial assays from Roche diagnostic, Germany (table 3. 3). Moreover, tHcy, Cys, MMA, vit B6, vit B12, and folate have been determined. The C677T MTHFR polymorphism was genotyped using a polymerase chain reaction/restriction fragment length polymorphism method. The methods used are listed in (table 3. 1).

Table 3. 1. Overview about the main outcome measures

Parameter	Method	equipment,manufacturer	Reference range
Hcy	GC-MS	Agilent, USA	<12 µmol/L.
Cys	GC-MS	Agilent, USA	65-301 nmol/L.
MMA	GC-MS	Agilent, USA	73-271 nmol/L.
Vit-B6	HPLC	Bio-Rad, Germany	4.8-36.9 ng/ml.
Vit-B12	Chemiluminescence	Bayer, Germany	156-674 pmol/L.
Folate	Chemiluminescence	Bayer, Germany	5-14.6 ng/ml.
HoloTC	Radioimmunoassay	Axis-Shield, Norway	≥ 35 pmol/L.
Routin Parameters	Hitachi analyser	Roche, Germany	
MTHFR mutation	PCR-based RFLP	Qiagen, Germany	

3. 1. Analytes detected by GC-MS (Hcy, Cys, MMA)

Serum tHcy, Cys, and MMA were separated by gas chromatography (GC) and quantified by mass spectrometry (MS). In general, separation and quantification of a mixture of compounds by GC-MS is based on the relative affinity of each component to the stationary phase of the GC, over which the mobile phase continuously flows. Compounds with a low affinity will elute earlier from the column than those with a high affinity. Since gas chromatography requires analytes in a volatile form, the sample were converted into a gaseous form using the method described by Stabler et al. (1993) and Allen et al. (1993).

The mass spectrometer provides the mass spectrum representing the abundance of ions of a given mass (abundance, Y axis) versus the mass to charge ratio of these ions (m/z, X axis). The effluent of the GC enters the mass spectrometer system through the interface. In the ion source the mass is ionised by electrons and undergoes fragmentation. Then, a quadrupole or a mass filter separates the ions that appear at the same time according to their mass. Finally, the detector collects and measure the received ions. The displayed peaks are proportional to the total number of ions of each mass (figure 3. 1). Deuterated Hcy, Cys, and MMA were added to the samples as an internal standard. The usage of an internal standard represents an easy way to calculate the concentration of a distinct parameter independently from the recovery that may differ from one sample to the other.

The concentrations of Hcy, Cys, and MMA were determined by dividing the integrated area of the endogenous substances by the integrated area of the deuterated internal standard. The results were multiplied with a factor which is the equivalent amount of deuterated internal standard that was added to each sample. The formula is:

$$\text{Concentration} = \frac{\text{Area of the endogenous parameter} \times \text{concentration of the internal standard}}{\text{Area of the internal standard}}$$

As: factor = 39.2 µmol/L, 1000 nmol/L, 4087.5 nmol/L for Hcy, Cys, and MMA, respectively.

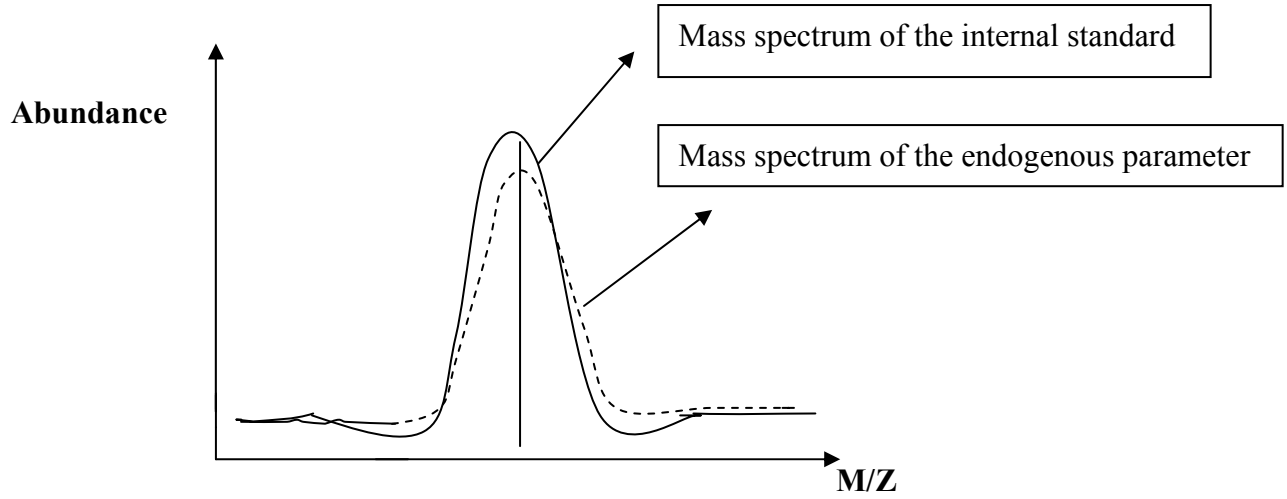


Figure 3. 1. Illustrate the mass spectrum which is a plot of the number of ions as a function of mass to charge ratio (m/z)

In this study, the mass selective detector operated in the ion monitoring mode in which the ions mass/charge (M/Z) 420.2, 362, 289 were monitored for endogenous Hcy, Cys, and MMA respectively. The ions M/Z 424.2, 366, 292 were monitored for deuterated Hcy, Cys, and MMA, respectively. The GC column operated with the following conditions:

Flow	1.0 ml/min	Initial temperature	80 ˚C
Film thickness	0.25 µm	Final temperature	310 ˚C
Length	30 m	Temperature limits	60 ˚C to 325 ˚C
Phase ratio	250	Rate of temperature increase	30 ˚C/min
Head pressure	53.3 psi		

3. 1. 1. Determination of tHcy and Cys

Hcy and Cys were quantified in the same (400 µl) sample. In one run, 20 to 22 samples and two pool sera were analysed. In the following, the detailed sample preparation is described:

1. In a 10 ml plastic tube, were added:

- 1 mL water for chromatography (Merck, Germany)
- 400 µL serum sample (patient or pool)
- 50 µL of deuterated Cys (concentration = 83×10^{-4} mol/L) (CDN Isotopes, Canada)
- 20 µl of deuterated Hcy (concentration = 784×10^{-3} mol/L) (CDN Isotopes, Canada)

- 30 µl of a freshly prepared Dithiothreitol (DTT) (Carl Roth GmbH, Germany) as a reducing agent (10 mg DTT in 1 ml NaOH (1 N))

2. This mixture was incubated at 42 ˚C for 30 min to allow reduction of disulfides of homocystine to Hcy

3. The mixture was applied to a disposable column (Bio-RAD, Germany) containing 100 mg (dry weight) of an anion exchange resin (Bio-RAD, Germany) that had previously been washed once with 1 ml of methanol and once with 3.3 ml water

4. The column was washed three times with 3 ml water and once with 3 ml of methanol

5. Hcy and Cys were eluted into a vial using 1.1 ml of 0.4 N acetic acid in methanol

6. The elutes were dried by vacuum centrifugation at 45 ˚C using a concentrator (Eppendorf, Germany)

7. The dried elutes were converted into a volatile derivative by adding 20 µl Acetonitril (Merck, Germany) and 10 µl N-methyl-N (tert-butyldimethylsilyl) rifluoracetamide (MTBDSFA) (Machery and Nagel, Germany). Subsequently, samples were incubated for 5 min in a microwave by 440 Watt and put into the GC-MS analyser (Hewlett-packard, USA). Finally, 1 µL was injected for analysis.

Deuterated and endogenous Hcy were eluted at approximately 13.3 min (retention time). The retention time of the deuterated and endogenous Cys was ≈ 17.1 min. In normal population, the cut-off value for Hcy is 12 µmol/L, and Cys has a reference interval of 65-301 nmol/L.

3. 1. 2. Determination of MMA

In one run, 20 to 22 samples and two pool sera were analysed. The sample preparation for the quantification of MMA was as follows:

1. In 10 ml plastic tube, were added:

- 1 mL water for chromatography
- 400 µL serum sample (patient or pool)
- 50 µL of deuterated MMA (concentration = 1635 × 10^{-7} mol/L) (CDN Isotopes, Canada)

2. This mixture was transferred to a previously activated anion exchange Resin

3. The column was washed once with 3 ml water, and three times with 3 ml of acetic acid in methanol (0.01 N)

4. The sample was eluted into a vial using 1.1 ml of 4 N acetic acid in (1 N) HCL. The elutes were dried and derivatized using the same protocol as for Hcy and Cys (see above)

The retention time of MMA and MMA internal standard was 17.1 min. The expected values of MMA in healthy people are between 73-271 nmol/L.

A serum pool was used for internal quality control. The within-day coefficients of variation (CV) for Hcy, Cys, and MMA were 4.9 %, 1.3 %, and 5.1 %, respectively. The interassy CV`s were 4.9 % for Hcy, 3.9 % for Cys, and 4.8 % for MMA.

Table 3. 2. Summarization of the sample preparation for the metabolites determined by GC-MS

	Hcy	**Cys**	**MMA**
Before applying the sample to the column			
Water	1 ml	1 ml	1 ml
Hcy internal standard	20 µl	-	-
Cys internal standard	-	50 µl	-
MMA internal standard	-	-	50 µl
Reducing agent (DTT)	30 µl	-	-
Incubation at 42 ºC	30 min	-	-
After applying the sample to the column			
First wash (water)	3 x with 3 ml	3 x with 3 ml	1 x with 3 ml
Second wash	1 x with 3 ml meth	1 x with 3 ml meth	3 x with (0.01N) Aa+meth
Elution from the column	0.4 N (Aa+meth)	0.4 N (Aa+meth)	4 N (Aa+HCl)
Drying of the elutes by vacuum centrifuge at 45 ºC			
Derivatization of elutes with derivatizing agent (An + MTBDSFA)			
Application of the samples to the GC-MS analyser.			

Abbreviations:
Aa: acetic acid, An: Acetonitril; Cys: cystathionine; DTT: 1,4-dithiothreitol; Hcy: homocysteine; meth: methanol; MMA: methylmalonic acide; MTBDSFA: N-methyl-N (tert-butyldimethylsilyl) trifluoracetamide.

Preparation of the anion exchange resin

The anionic exchange resin was washed by an equivalent amount of HCl (1 N). After removal of HCL, the resin was washed again by an equivalent amount of methanol. Then, the resin was left to dry in an oven at 60 ºC for three to five hours or over night at 37 ºC. Before sample application, 100 mg of dry ion exchange resin were put into a disposable column and washed with different solutions before application of the samples as described in the methods above.

Solution used in GC-MS methods:

- 4 N acetic acid stock solution:

  24 ml acetic acid + 76 ml methanol for chromatography

- 0.4 N acetic acid (used for elution Hcy and Cys)

  *10 ml acetic acid (4 N) + 90 ml methanol for chromatograp*hy

- 0.01 N acetic acid (the column`s washing solution for MMA measurement)

  12.5 ml acetic acid (0.4 N) + 487.5 ml methanol for chromatography

- Solution for MMA elution

  90 ml acetic acid (4 N) + 10 ml HCL (1 N)

Technical specifications of the GC-MS system

The GC-MS system was provided by Hewlett-Packard, USA.

The GC-column contained HP-5 MS (crosslinked 5 % phenyl methyl siloxane) as a stationary phase (Model No: Hp 19091S-433) was provided by Agilent technologies®, USA.

Capillary gas chromatograph	model 6890
Autosampler	model 7774
Mass-selective detector	model 5973

The system was controlled via the MS DOS chemstation (Agilent technologies®, USA).

3. 2. Determination of Vit B6

In this study, pyridoxal-5-phosphate (PLP), the active form of vit B6, was measured by reversed phase high performance liquid chromatography (HPLC) with fluorescence detection using a commercial kit from Immundiagnostik (Germany). In vivo, vit B6 can be found in three forms: pyridoxine, pyridoxal, and pyridoxamin.

HPLC is based on different affinities of the sample compounds for the mobile and stationary phase. Comparable to GC, the HPLC system consisted of a mobile phase reservoir, a pump for transporting the mobile phase through the system, an injector for sample application into the column, the chromatography column, a fluorescence detector, and a computer. Reversed-phase chromatography is characterized by elution of the sample compounds with a mobile phase that is significantly more polar than the stationary phase. Vit B6 eluted after ~3 min. For the calculation of vit B6 concentration a calibrator with a known vit B6 concentration was included in each run. Vit B6 concentration was then calculated by the following formular:

$$\text{Vitamin B6} = \frac{\text{Peak height of patient} \times \text{calibrator conc. (ng/ml)}}{\text{Peak height of calibrator}}$$

The detection limit was 0.2 ng/ml with persistent linearity up to 250 ng/ml. The detailed test protocol is listed below:

High molecular substances were precipitated by adding precipitating reagent to the samples. After removal of the supernatant, vit B6 was derivatized by incubation of the sample at 60 ˚C for 20 min with derivatizing reagent. Then, samples were placed onto the HPLC system. After injection, samples passed the column and were subsequently eluted by isocratic elution, in which the composition of the mobile phase remains constant during the elution process.

In each run one calibrator (one point calibration), two controls (high and low), and 21 samples were analysed. Sample preparation was as follows:

- In a micro centrifuge tube (Eppendorf, Germany) were added:
  - 200 µl serum sample, calibrator, or control
  - 50 µl precipitating reagent® (Immundiagnostik, Germany)
- The mixture was mixed vigorously to remove the high molecular substances and incubated at + 4 ˚C for 10 min

- After centrifugation for 5 min at 20000 g (Hettich centrifugator EBA 12, Germany), 100 µl of supernatant were transferred to a 1.5 ml plastic cup, and 250 µL derivatisation solution® (Immundiagnostik, Germany) were added
- The tube was incubated for 20 min in a water bath at 60 ˚C
- Prior to injection, this mixture was cooled at 2-8 ˚C for 10 min and centrifuged for 5 min at 20000 g
- 200 µl of the supernatant were transferred to a sealed auto sampler vial, and 20 µl were injected for analysis.

Chromatographic conditions and materials

Column material:	prontosil Eurobond C 185.0 µm (Immunodiagnostik,Germany)	
Column dimension:	125 mm × 4 mm	
Flow rate:	1-1.5 ml/min/temperature: 25	
Wavelength of detection	Excitation	320 nm
	Emission	415 nm
Injection volume:	20 µl	
Running time:	10 min	

The HPLC-system was provided by Agilent, (Bio-RAD, Germany).

Mobile phase, calibrators, and controls were provided by Immunodiagnostik, Germany.

The CV´s for high (23.45 ng/ml) and low (8.25 ng/ml) controls were 5.22 % and 5.68 %, respectively. The normal range of the Vit B6 concentration is 4.3 – 17.9 ng/ml.

3. 3. Determination of folic acid and vit B12 in serum

Folate and vit B12 were measured on an ADVIA centaur automated analyzer (Bayer Diagnostics, Germany) using commercial assays from Bayer Diagnostics. Both assays are competitive chemiluminescence immunoassay. The principle of these assays is a competition of endogenous folate and vit B12 with acridinium ester-labeled folate and vit B12, respectively, for a limited number of binding sites on a solid phase. The solid phase consists of biotin-labeled folate binding protein and purified intrinsic factor, respectively. Both proteins are covalently coupled to paramagnetic particles in the solid phase. Prior to the incubation with acridinium ester-folate and vit B12, samples are treated with DTT to release folate and vit B12 from endogenous binding proteins. After binding of endogenous folate and vit B12, the unbound folate and vit B12 are washed away. Then, the chemiluminescence reaction is initiated by adding acid and base reagents. The concentrations of folate and vit B12

are inversely related to the relative light units (RLUs) detected by the system. Low and high controls were used for quality control. The CV´s for vit B12 were 3.56 % and 4.47 % at 1201 and 613 pg/ml, and for folate were 8.24 % and 7.94 % at 8.87 and 4.63 ng/ml.

3. 4. Analysis of MTHFR-polymorphism

MTHFR-polymorphism was analyzed by a polymerase chain reaction/restriction fragment length polymorphism (PCR/RfLP) method as previously described by (Frosst et al., 1995). The PCR product was digested with the restriction enzyme Hinf I (MBI, Germany) and then plotted by gel electrophoresis.

3. 4. 1. DNA isolation

● Manual isolation

- 10 ml EDTA-blood and 40 ml blood lysis buffer (BLB) (1 x) were mixed in a 50 ml tube (BD, Germany), and placed on ice for 30 min
- The tube was centrifuged for 10 min at + 4 ˚C with 2500 g. Then, the supernatant was removed, and the remaining leucocytes (pellet) were washed with BLB three times
- The remaining white leucocyte layer were resuspended in 0.5 ml BLB
- Then, 4 ml white lysis buffer (WLB), 200 µl Proteinase K, and 200 µl of 20 % sodium dodecyl sulphate (SDS, 20 % g/v) were added and the all suspension were incubated in a water-bath at + 37 ˚C for, at least, 12 h
- The next day, 1.5 ml of 6 M sodium chlorid (NaCl) was added. After mixing for 15 seconds (s), the suspension was centrifuged for 15 min at + 4 ˚C with 3000 g
- The supernatant containing the soluble DNA was transferred into a sterile 50 ml tube and filled up with 2.5 times volume of absolute ethanol
- The tube was shaked gently until the DNA appeared
- Then, the DNA containing tube was centrifuged for 1-2 min with 6000 g, and the supernatant was discarded
- The obtained DNA was washed from salts using 1 ml of ethanol 70 %. The washing procedure was repeated five times to remove any trace of the salts
- Finally the DNA was resuspended in 0.5 ml tris-EDTA-buffer (TE-buffer) and incubated in a water-bath for one h at 60 ˚C

The DNA was stored at + 4 ˚C until analysis.

Composition of solutions used for manual isolation

BLB (20 x)

- 3.1 M ammonium chloride (Merck) MW = 53.49 g
- 0.2 M potassium bicarbonate (Merck) MW = 100.1 g
- 20 mM EDTA (pH = 8) (Merck) MW = 372.24 g/mol

Adjust PH to 7.4 and fill up to 1000 ml with sterile water. Prior to use dilute 20x with sterile water.

WLB (1 x)

- 10 mM Tris (hydroxymethyle)- aminomethan (Merck) MW = 121.14 g/mol.
- 400 mM Nacl (Merck) MW = 58.44 g/mol.
- 2 mM EDTA (pH = 8) MW = 372.24 g/mol

fill up to 1000 ml with sterile water.

TE- buffer .

- 10 mM Tris (hydroxymethyle)- aminomethan) MW = 121.14 g/mol
- 0.1 mM EDTA (PH = 8) MW = 372.24 g/mol

Adjust PH to 7.5 and fill up to 200 ml with sterile water

Proteinase K

20 mg Protinase K (Merck)

Add sterile water until 1 ml. Aliquot the solution and store it at -20 ºC.

SDS (20 % g/v)

20 g SDS (Merck) MW = 288.38 g/mol

Add sterile water until 100 ml. Leave the solution at room temperature.

NaCl (6 M)

175.2 g Nacl MW = 58.44 g/mol

Add sterile water until 500 ml

Ethanol 70 %

70 ml ethanol absolute (Merck)

30 ml sterile water

Ethanol absolute

The ready to be used solution is stored at – 20 ºC.

● Quick isolation

Quick isolation was done using the commercially available QIAamp® DNA Mini Kit, which is based on the adsorption of DNA onto a silica-gel membrane after lysis with "Qiagen agent" and Proteinase K in the presence of a high salt concentration and ethanol (96-100 %). The procedure was as follows:

- In a 1.5 ml tube, 200 µl blood, 200 µl AL® buffer, and 20 µl Proteinase K were added
- After well mixing, the suspension was incubated at 56 ºC for 10 min (for cells lysis and proteolysis)
- Two hundred microliter ethanol (96-100 %) were added
- The mixture was applied to a QIAamp spin column (provided with the kit) and centrifuged at 6000 g for 1 min. Then, the filtrate was removed
- The column was washed with 500 µl AW_1® buffer and centrifuged at 6000 g for 1 min. Then, the filtrate was removed again
- The column was washed again with 500 µl AW_2® buffer and centrifuged at 6000 g for 3 min
- The QIAamp spin column was placed in a clean 1.5 ml tube and 200 µl AE® buffer were added to elute the DNA. After incubation for 1 min at room temperature the column was centrifuged at 6000 g for 1 min. The filtrate contained the isolated DNA can be stored for long time at + 4 ºC.

3. 4. 2. PCR /RFLS

The principle of PCR is the synthesis of multiple replicates of a target DNA sequence. In this study, the replicates were then used to detect changes in the base sequence by RFLS. RFLS is a method using a cleavage enzyme (restriction enzyme) to fragment the PCR product at a defined point. If this point is mutated, the restriction enzyme can not cleave. The fragments are then analysed by gel electrophoresis. The PCR consists of 3 steps forming one cycle: denaturation, annealing, and elongation. To obtain sufficient amounts of the PCR product, multiple cycles have to be performed. Each step requires a different temperature and the instrument that takes samples through these cycles is known as thermocyclers. The MTHFR PCR was carried out in a total volume of 15 µl and contained the following ingredients:

- Nucleotides tri-phosphat (NTP- mix 2.5 mM) (Promega, Germany) 1.50 µl
- Exonic primer (10 pmol/µl) (GibcoBRL, Eggenstein) 1.20 µl
- Intronic primer (10 pmol/µl) (GibcoBRL, Eggenstein) 1.20 µl
- Tag polymerase (Roche, Mannheim) 0.45 µl
- PCR buffer (10 x + Mg Cl) (Roche, Mannheim) 1.50 µl
- PCR-water (Eppendorf, Hamburg) 7.65 µl
- DNA (sample) 1.50 µl

The primers for analysis of the A→V change generate a fragment of 198 bp. The primers are:

- Exonic primer 5´- TGA AGG AGA AGG TGT CTG CGG GA- 3´
- Intronic primer 5´- AGG ACG GTG CGG TGA GAG TG-3´

The PCR parameters were as follows:

1. Initial denaturation at 94 ºC for 1 min
2. 36 cycles denaturation at 94 ºC for 60 s, annealing at 60 ºC for 45 s, and extension at 72 ºC for 30 s.
3. Final extension for 10 min at 72 ºC to ensure complete extension of all PCR products.

The amplified fragments were digested with the restriction enzyme HinfI (MBI, Germany) for three hours at 37 ºC. The mix consisted of 12.5 µl of amplified DNA, 1 µl of restriction enzyme HinfI and 1.4 µl enzyme buffer. The restriction enzyme will recognize the sequence 5′ G↓ANTC 3′ in the two DNA strand and will divide the 198 bp PCR product into a 23 bp and a 175 bp fragments. The fragments were then detected using the horizontal slab gel electrophoresis.

3. 4. 3. Gel electrophoresis

DNA-fragments were applied on a 3 % (g/v) agaroase/NuSieve® GTG® Agarose gel (BMA, USA):

Three grams agarose powder were cooked with 100 ml 1 × Tris-Borate-EDTA (TBE buffer) (GibcoBRL, Eggenstein) until the agarose was totally dissolved

- The solution was cooled to 60 ºC. Then, 12 µl of ethidium bromide (Carl Roth GmbH, Germany) were added (from a stock solution of 10 mg/ml in water)
- This agarose solution was poured on a plastic plate that had previously been equipped with a comb to form the wells. The gel was left for 30 min at room temperature for hardening

- The comb was removed and the gel was transferred to an electrophoresis tank (Biotec Fischer, Reiskirchen) that was filled with sufficient amount of electrophoresis buffer (1 x TBE).
- Each well was filled with 16.5 µl of the DNA mix or DNA-standard (GibcoBRL, Eggenstein) of a known size (1 Kb). A voltage of 125 V for ~ 45 min was applied to allow the DNA fragments to migrate from the starting point into the body of the gel
- Finally, the gel was tested by ultraviolet light and a photo for the gel was taken.
  The marker was used to determine the sizes of unknown DNAs if any systematic change of the gel happens during electrophoresis.

The different genotypes are characterized by the following bands:

- MTHFR-677 CC (wildtype): one band with 198 bp
- MTHFR-677 CT (heterozygotes): one band with 198 bp + one band with 175 bp.
- MTHFR-677 TT (homozygotes): one band with 175 bp.

● The DNA-standard consisted of 1000 bp (1 kb), and was used at 66 ng/µL. The marker was prepared from a (1 µg/µL) stock solution as follows:
Sixty-six microliters of stock solution were mixed with 230 µL of loading dye solution, 10 µl of 1 M Tris buffer, and 10 µl of 2 M NaCl. Then, sterile water was added till 1 ml.

● Ten ml of stop mix contained 1.0 ml bromphenol blue (Merck, Darmstadt), 2.5 mL xylene cyanol (Merck, Germany), 2 ml 50 mM EDTA, 2.38 ml glycerine (> 99.5 % purity), and 2.1 ml sterile water.

3. 5. RIA-Methods (holotranscobalamin-Π (HoloTc- Π)

HoloTC- Π was assayed using a commercial RIA kit (Axis-Shield, Norway). This kit is based on the method described by Ueland et al. (2002). Briefly, total transcobalamin (TC) was first isolated from the serum sample or calibrator by incubating the serum with magnetic microspheres coated with monoclonal anti-human TC antibodies (capturing reagent). The vit B12 content of the sequestered holoTC was dissociated from TC by adding a DTT in phosphate buffer (reducing reagent) and denaturing reagent (extractant). At the same time, the released vit B12 was converted to the stable cyano form with potassium cyanide and quantified in a competitive binding assay. The ^{57}C labelled vit B12 (tracer) competed with the cyano form of vit B12 for a specific number of binding sites of immobilized Intrinsic factor

(IF). After 1 h incubation, the unbound tracer was removed by centrifugation and the pellet was counted in a gamma counter. The concentration of vit B12 in the sample was inversely correlated to the measured radioactivity and determined by interpolation from a calibration curve that was constructed using holoTC calibrators of known concentrations (0, 10, 20, 40, 80, and 160 pmol/L). Quality control sera were applied by the manufacturer of the kit. The CV´s for high and low controls were 9 % and 12 %, respectively.

The expected values in healthy individuals are 35-171 pmol/L.

3. 6. Clinical chemical parameter

The following analytes were measured on a Hitachi 911 automated analyser using commercial assays (Roche Diagnostic, Germany): ALT, AST, total-bilirubin, creatinine, cholesterol, glucose, HDL-C plus, urea, uric acid. The methods are shortly described in table 3. 3.

Table 3. 3. The routine chemistry of the study groups

Parameter	Principle	Reference range
ALT (GPT)	ALT catalyzes the transamination of L-alanine to α-ketoglutarate forming pyruvate and L-glutamate. The increase in pyruvate is determined in an reaction catalysed by lactate dehydrogenase accompanying with simultaneous oxidation of reduced NADH to NAD. The rate of photometrically determined NADH decrease is directly proportional to the rate of formation of pyruvate and thus the ALT activity	Men: up to 41 U/L Female: up to 31U/L
AST (GOT)	AST catalyzes the transamination of L-aspartate to 2-oxoglutarate forming L-glutamate and Oxalacetate. The Oxaloacetate formed is reduced to malate by malate dehydrogenase with simultaneous oxidation of reduced NADH to NAD. The change in absorbance with time (due to the conversion of NADH to NAD) is directly proportional to AST activity.	Men: up to 37 U/L Female: up to 31U/L
Bilirubin	In strong acid solution containing 2,5-dichlorophenol diazonium salt, total bilirubin couples to form azobilirubin(red azo dye) that is directly proportional to the total bilirubin and determined photometrically.	Adults: up to 1 mg/dl
Creatinine	In alkaline solution, creatinine forms a yellow orange complex with picrate. The color intensity is directly proportional to the creatinine concentration and can be measured photometrically.	Male: 0.70-1.20 mg/dl Female:0.50-0.90 mg/dl

Cholesterol	Cholesterol ester is hydrolyse to cholesterol by the action of cholesterol esterase. The cholesterol is oxidized to a keton (cholest-4-en-3-one) by cholesterol oxidase and forms, simultaneously, H_2O_2 that is yield a dye by reaction of peroxidase. The colour intensity, which measured photometrically, is proportional to the concentration of cholesterol.	Adults: < 200 mg/dl
Glucose	G-6-P dehydrogenase oxidizes G-6-P in the presence of NADP to gluconate-6-P. The amount of NADPH produced is directly proportional to the amount of glucose in the sample and is measured by absorbance at 340 nm.	Male: 0.70-1.20 mg/dl Female:0.50-0.90 mg/d
HDL-C plus	The cholesterol esterase linked to polyethylene glycol (PEG) breaks the cholesterol ester of HDL-cholesterol into free cholesterol and fatty acids. The cholesterol is then oxidized by PEG-linked cholesterol oxidase to Δ^4 -cholestenone and H_2O_2. In the presence of Peroxidase and other reagents, H_2O_2 forms a blue dye that is measured by photometer.	Male: 35-55 mg/dl Female: 45-65 mg/dl
Urea	Urea is hydrolysed by Urease to form CO_2 and ammonia. The ammonia formed then reacts with α-ketoglutarate and NADH in the presence of GLDH to yield glutamate and NAD^+ the decrease in absorbance due to consumption of NADH is measured kinetically.	10-50 mg/dl
Uric acid	The measurement depends on an enzymatic assay (uricase cleaves uric acid to form allantion). This enzymatic assay involves a Peroxidase system coupled with oxygen acceptors (4- aminophenazone) to produce a chromogen in the visible spectrum.	Male: 3.4-7 mg/dl Female: 2.4-5.7 mg/dl

3. 7. Material and instruments

Materials

- Acetic acid	Merck, Germany.
- Acetonitrile	Merck, Germany
- Agarose (NuSieve GTG)	BMA, USA.
- AG MP-1M Ion Exchange Resin Microporous Anion Resin (100 – 200 Mesh) chloride form.	Bio – RAD, Germany.
- Ammonium chloride	Merck, Germany.
- calibrator and controls of vit B6	Immunodiagnostik, Germany.
-1,4-Dithiothreitol ($C_4H_{10}O_2S_2$)	Merck, Germany.
- DL- (2-Amino-2-Carboxyethyl)- homocysteine 3,3,4,4-d_4 (cystathionine-d_4)	CDN isotopes, Canada.
- DL-Homocysteine-3,3,3', 4,4,4', 4, -d_8 (homocysteine-d_8).	CDN isotopes, Canada.
- DNA isolation kit	Qiagen, Germany.
- DNA-Standard 1 KB	Gibco BRL, Germany.
- d NTP- mix	Promega, Germany.
- Ethidium bromide	Karlsruhe, Germany.
- EDTA (triplex®-II)	Merck, Germany.
- Ethanol absolute	Merck, Germany.
- Folate reagent and calibrators	Bayer diagnostics, Germany.
- Methanol for chromatography	Merck, Gerrmany.
- Methyl-d_3-Malonic Acid	CDN isotopes, Canada.
- Mobile phase of vitamin B6	immunodiagnosik, Germany.
- MTBDSFA	Machery and Nagel, Germany.
- Potassium bicarbonato	Merck, Germany.
- Protenase K	Merck, Germany.

- PCR buffer	Roche, Germany.
- PCR- water	Eppendorf, Germany.
- Primers	Gibco BRL, Germany.
- Reagent of ALT (GPT)	Roche, Germany
- Reagent of AST (GOT)	Roche, Germany
- Reagent of Bilirubin	Roche, Germany.
- Reagent of cholesterol	Roche, Germany.
- Reagent of creatinine	Roche, Germany.
- Reagent of folic acid	Bayer, Germany.
- Reagent of HoloTc-П	Axis-Shield, Norway.
- Reagent of HDL-C plus	Roche, Germany.
- Reagent of triglycerides	Roche, Germany.
- Reagent of vitamin B12	Roche, Germany.
- Reagent of urea	Roche, Germany.
- Reagent of uric acid	Roche, Germany.
- Restriction enzyme Hinf I	MBI, Germany.
- Sodium Dodecyl Sulphate	Merck, Germany.
- Sodium chlorid	Merck, Germany.
- Ammonium chloride	Merck, Germany.
- Tag polymerase	Roche, Germany.
- TBE-buffer	GibcoBRL, Germany.
- Tris (hydroxymethyle)-Aminomethan.	Merck, Germany.
- Vit B6 HPLC-kit	immunodiagnosik, Germany.
- Vit B12 reagentss and calibrators	Bayer diagnostics, Germany.
- Water for chromatography	Merck, Germany.

Instruments

- ADVIA Centaur	Bayer diagnostics, Germany.
- Balance ME215P	Sartorius, Göttingen.
- Centrifugator EBA 12	Hettich, Tuttlingen.
- Electrophoresis tank	Biotec Fisher, Reiskirchen.
- Florescence detector G1321A	Agilent, Böblingen.
- Gas chromatography HP 6890	Hewlett Packard, USA
- Hitachi 911	Roche, Mannheim.
- HPLC Agilent 1100	Agilent, Böblingen.
- Mass-spectrometer HP 5973	Hewlett Packard, USA.
- Mixer	Scientific Industry, USA.
- Piptten	Eppendorf, Hamburg.
- Robocycler® Gradient 96	Stratagene, USA.

3. 8. Statistics

SPSS 11.0 for Windows 98 was used for all statistical analyses. Kolmogorov-Smirnov criterion was used to asses the normal distribution of the continuous variables. All variables were not normally distributed and, thus, data were log-transformed to normalize distribution due to their skewed distribution. Data are presented as medians (10^{th}-90^{th} percentile), or number of subjects and percentage. Medians in tow independent groups or several independent groups were compared using nonparametric Mann-Whitney test and Kruskal-Wallis test, respectively. The chi-square test was applied to assess differences in frequencies of measured variables. Spearmans's rank correlation was determined to identify significant correlations between continuous variables. Further data analysis was performed in a subgroup that consisted of 63 pairs of age and gestation-age-matched patients and controls. Differences in biochemical markers between the matched pairs were assessed using a paired t-test. All tests were two-sided, and probability values < 0.05 were considered significant. The risk of HHcy (Hcy > 8.2 μmol/L) and preeclampsia disorder were computed by a logistic regression analysis.

4. RESULTS

4. 1. Anthropometric and anamnestic data

The study samples consisted of 275 pregnant Syrian women. Ninety-eight subjects (35.6 %) were normotensive throughout their pregnancy and served as control group (Con), 177 (64.4 %) developed preeclampsia (PE). Of them 24 women developed eclampsia, the severe form of preeclampsia. Anthropometric and reproductive data are shown in table 4.1. Briefly, median maternal age did not differ between patient and control subjects. preeclampsia was found to be more prevalent in women at both extremes of reproductive age, ≤ 19 years and ≥ 35 years. Preeclamptic women delivered significantly earlier than normotensive women. Pre-term deliveries (gestational age < 37 weeks) were found in 45.2 % of preeclamptic women. Of them 22.5 % were affected by eclampsia. Low (< 2500 gram, g) and very low birth-weight (< 1500 g) were found in 34 % and 17 %, respectively, of newborns of the preeclamptic women. Fetal death occurred in 18 % of cases delivered between 24 and 40 weeks. Women with preeclampsia had a higher median body mass index (kg/m^2) than controls (29.3 vs. 27.1 kg/m^2, P = 0.006). Cesarean section was done in 35 % of cases and vaginal delivery was done in 47 % of cases. In preeclampsia, cesarean sections were significantly more frequent in subjects with a BMI ≥ 25 compared to those with a BMI < 25 (34.3 % vs. 13.3 %). The prevalence of hypertension was more in primigravida (55.2 %) than in multigravida (44.2 %). Smoking status in study groups did not differ. However, only a very low percent of women were considered smokers (2 % controls and 5 % patients).

Table 4. 1. Characterization of subjects

Characteristic	Controls (n = 98)	Preeclampsia (n = 177)
- Age, years	25 (19-36)	26 (18-38)
≤ 19 years, n (%)	14 (14.4 %)	33 (18.8 %)
≥ 35 years, n (%)	12 (12.4 %)	46 (26.1 %)‡
- Gestational age at recruitment, wk.	35 (29-40)	37 (30-40)*
- PTD[1]<37 wk.	NA	80 (45.2 %)
- Birth weight, (g)	NA	2400 (1100-3420)
VLBW[2] <1500 g, (%)	NA	17 %
LBW[3] <2500 g, (%)	NA	34 %
Still birth, (%)	NA	18 %
- Maternal weight, kg	71 (59-86)	75 (60-95)*
- Maternal height, cm	160 (153-166)	160 (152-167)
- BMI[4], Kg/m²	27.1 (24.4-33.5)	29.3 (24.6-37.0)*
BMI < 25, (%)	21 %	11 %
Overweight, (%)	51 %	48 %
Obsesity, (%)	28 %	42 %
- Delivery		
Normal, (%)	NA	47 %
Cesearian, (%)	NA	35 %
- Parity		
Nulliparity, n (%)	39 (39.8 %)	97 (54.8 %)
1 child, n (%)	25 (25.5 %)	16 (9.0 %)
> one child, n (%)	34 (34.7 %)	64 (36.2 %)
- Smoking		
No, (%)	98 %	95 %
Yes, (%)	2 %	5 %
- Vitamin use, n (%)	88 (91 %)‡	103 (63 %)
beginning of supplementation	9	9
Duration of supplementation, wk	19	8*

Data are presented as medians (10^{th}-90^{th} percentile), unless otherwise mentioned. * significant difference vs. controls. ‡ Chi-square test.

[1] PTD: preterm delivery
[2] VLBW: Very low birth weight
[3] LBW: Low birth weight
[4] BMI: Body mass index (kg/m^2)

4. 2. General medical examination

Serum concentrations of creatinine, urea, uric acid, liver enzymes, cholesterol (Chol), triglycerides (TG), and HDL-cholesterol (HDL-Chol) are summarized in table 4. 2. Median serum levels of creatinine, urea, and GOT were significantly higher in patients compared to controls. Similar result was observed for uric acid (6.6 vs. 4.0 mg/dl, $P < 0.001$). Of note, serum uric acid concentrations were significantly higher in women who developed eclampsia as compared to those who developed preeclampsia (7.3 vs. 6.5 mg/dl, $p = 0.008$). Serum Chol and TG levels were elevated in all groups, and were significantly higher in preeclampsia than in controls. No differences were seen in HDL-Chol between study groups. The same results were obtained after adjusting for the gestation age at inclusion.

Table 4. 2. The parameters of medical characteristic of the study groups

	Controls, (n = 98)	Preeclampsia, (n = 177)	Reference interval
Creatinine, mg/dl	0.57 (0.45-0.69)	0.71* (0.53-0.95)	$\leq$ 0.9
Uric acid, mg/dl	4.0 (3.1-5.7)	6.6* (4.7-9.1)	2.4-5.7
Urea, mg/dl	14 (9-21)	24* (15-38)	10-50
GPT, U/L	12 (8-20)	12 (7-62)	< 34
GOT, U/L	19 (14-26)	25* (16-92)	< 37
Chol, mg/dl	246 (194-330)	279* (192-409)	≤ 200
TG, mg/dl	252 (174-400)	335* (186-592)	≤ 200
LDL-Chol, mg/dl	133 (83-212)	142 (78-236)	60-140
HDL-Chol, mg/dl	58 (42-79)	61 (43-82)	33-55
RR sys	_____	160 (140-180)	
RR diast	_____	100 (90-120)	

Data are presented as medians (10^{th}-90^{th} percentile). * significant difference vs. controls

4. 3. Hcy and B-vitamin status

Median tHcy level was significantly higher in preeclamptic women compared to controls (table 4. 3). Since there is no established reference range for tHcy level during pregnancy, elevated tHcy was defined as a serum tHcy level > 8.2 µmol/L. This value represents the 95th percentile of Hcy concentration among normotensive women who had adequate status of vit B12 and folate. Accordingly, elevated tHcy levels (tHcy > 8.2 µmol/L) were more prevalent in preeclamptic women (65.2 %) than in controls (22 %) (figure 4. 1). Preeclamptic women had significantly higher Cys levels than controls. Fifty-eight percent of patients but only 23 % of controls had Cys levels above the upper reference limited (URL) (URL: Cys > 301 nmol/L). Impaired cobalamin status was seen in a high frequency in patients and controls. Nearly 60 % of all subjects had vit B12 deficiency (serum vit B12 < 211 pg/ml), and an even higher proportion (77.7 %) and (64.6 %) exhibited low holoTC and elevated MMA, respectively. Vit B6 deficiency (vit B6 < 4.3 ng/ml) was very frequent in both groups (82 % of contros and 88.3 % of patients). Figure 4. 1 illustrates the prevalence of abnormal Hcy, Cys, MMA, and B-vitamins in all groups.

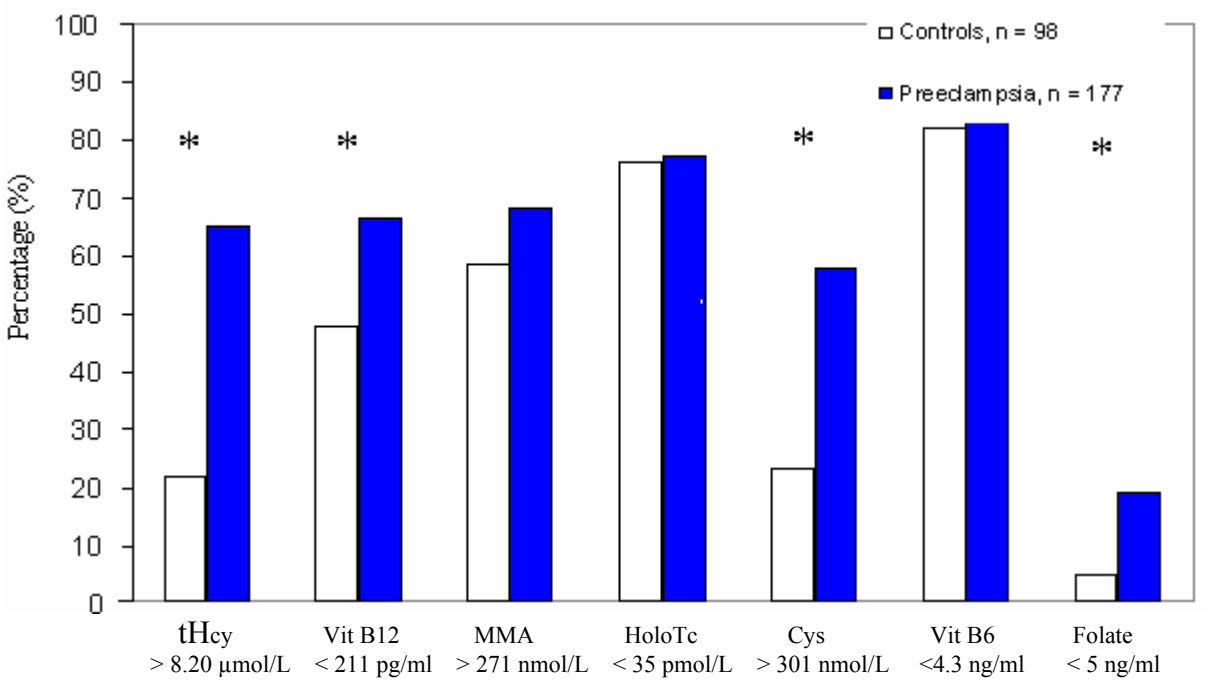


Figure 4. 1. The prevalence of abnormal metabolites and B-vitamins levels in controls and patients

Median folate concentrations was significantly lower in preeclamptic women (7.3 ng/ml) compared to controls (15.9 ng/ml). Folate deficiency (folic acid < 5 ng/ml) was observed in 5 % of controls versus in 19 % of preeclampsia (figure 4. 1). Furthermore, normotensive pregnant women used more frequently vitamin supplementation than patient women (91 % vs. 63 %, P < 0.001) (figure 4. 2). Vitamin supplementation mainly included folic acid at a daily dose of 0.5-5 mg. In folic acid-supplemented women, supplementation was initiated on average at 9 weeks of gestation in both groups, and continued for an average duration of 19 weeks in controls and 8 weeks in preeclamptic women (p < 0.001). As expected, folic acid supplementation during pregnancy improved the folate status. Supplemented women had significantly higher folate levels in both patients (8.5 vs. 5.7 ng/ml, P < 0.001) and controls (15.8 vs 8.8 ng/ml, P= 0.048). Additionally, supplemented patient women had significantly lower folate levels compared to supplemented normotensive women (8.5 and 15.8 ng/ml, respectively, P < 0.001) (figure 4. 3). Furthermore, supplementation associated with relatively lower levels of tHcy in both group (figure 4. 3). These differences remained significant after adjusting for the gestation age at inclusion.

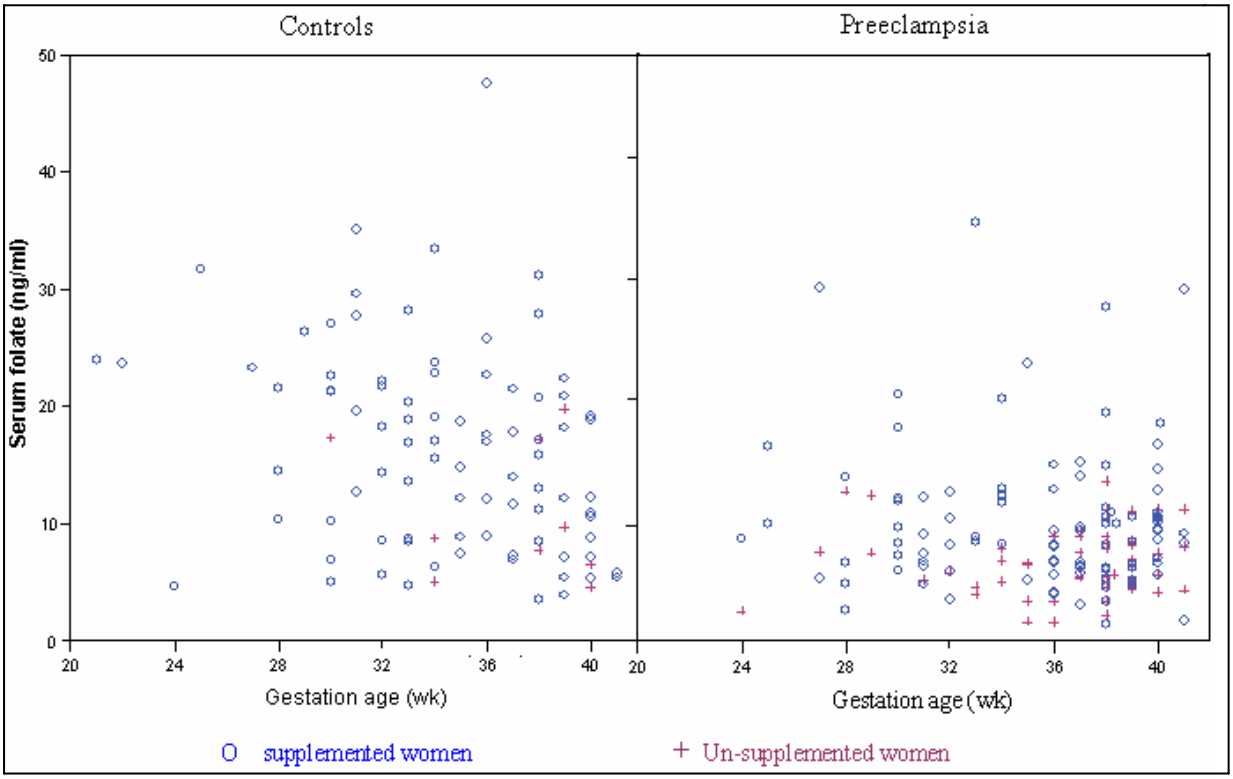


Figure 4. 2. Scatter plots of folic acid concentrations in supplemented and un-supplemented women. □ supplemented women, + un-supplemented women.

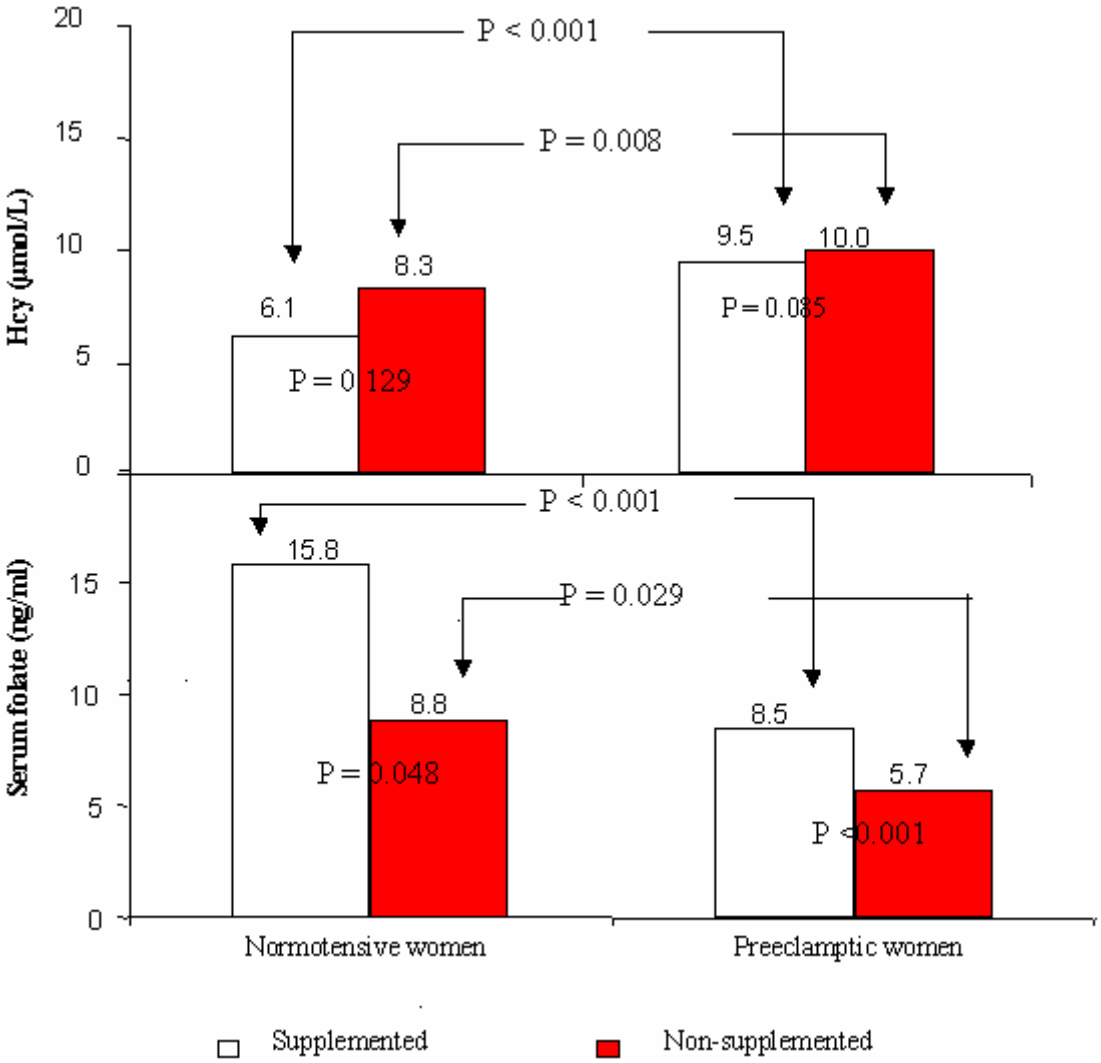


Figure 4. 3. serum tHcy and folate in supplemented and unsuplemented pregnant women

We conducted data analysis of 63 pairs of controls and patients who were matched for maternal age and gestational age (table 4. 3). Serum concentration of Hcy and Cys were significantly higher, and serum concentrations of folate and PLP were significantly lower in patients compared to healthy women. Vit B12 status, indicated either by measurement of serum vit B12 or by MMA and holoTC did not differ significantly between patients and controls. In patients group, holoTC correlated significantly to creatinine ($r = + 0.28$, $p < 0.001$).

Table 4. 3. Concentrations of metabolites and vitamins in 63 age-and gestational-age-matched pairs of pregnant women

	Controls (n = 63)	**Preeclampsia (n = 63)**
tHcy, µmol/l	6.0 (4.5-9.7)	9.3 (6.8-14.6)*
Folate, ng/ml	15.9 (5.9-26.6)	7.3 (4.2-12.3)*
Vit B12 status		
Vit B12, pg/ml	218 (144-294)	182 (114-294)
MMA, nmol/L	296 (143-660)	323 (134-618)
HoloTC, pmol/L	23 (9-64)	25 (14-79)
Vit B6 status		
PLP, ng/ml	2.4 (1.2-7.6)	2.0 (0.9-4.2)*
Cys, nmol/L	232 (170-392)	284 (177-556)*

Data are presented as medians (10^{th}-90^{th} percentiles). Subject age, 25 (19-36) years and gestational age , 36 (30-40). * significant difference vs. controls

Levels of Hcy and Cys were found to be elevated in the serum of individuals with subnormal vit B12 status (Stabler et al., 1993). Additionally, normal- to high-normal levels of folate are common in vit B12-deficient subjects. Recently, a high incidence of vit B12 deficiency was reported in Syria (Herrmann et al., 2003; Obeid et al., 2002). Therefore, aiming to eliminate the influence of vit B12 deficiency, we compared B-vitamins levels and the metabolites only in individuals with normal cobalamin status (MMA $\leq$ 271 nmol/L) and renal function (creatinine $\leq$ 0.9 mg/dl). Significantly higher levels of tHcy, Cys and lower levels of folate, vit B6, and vit B12 were found in preeclamptic women as compared to controls (table 4. 4). These differences remained significant after adjusting for the gestation age at inclusion**.**

Table 4. 4. Meth metabolites and B-vitamins of the study groups with MMA < 271 nmol/L

	Normotensive (normal MMA) N = 41	Hypertensive (normal MMA) N = 48
Hcy, µmol/l	5.3 (3.8-6.8)	8.2 (6.6-14.9)*
Folate, ng/ml	17.2 (8.5-27.5)	8.2 (3.5-18.1)*
Vit B12 status		
Vit B12, pg/ml	232 (168-348)	197 (94-406)*
MMA, nmol/L	188 (118-255)	193 (124-261)
HoloTC, pmol/L	30 (19-70)	25 (10-86)
Vit B6 status		
PLP, ng/ml	2.5 (1.6-9.3)	2.0 (0.9-6.7)*
Cys, nmol/L	229 (139-393)	293 (194-524)*

Data are presented as medians (10^{th}-90^{th} percentile). * significant difference vs. controls. Only subjects with normal renal function were included.

4. 4. Correlation analyses

As expected, birth weight correlated strongly with the gestation age in preeclamptic women ($r = + 0.73$, $P < 0.001$). Overall, neither the metabolites (Hcy, Cys, MMA) nor B-vitamins (folate, vit B12, vit B6) showed significant association with maternal age. In normotensive women, gestation age was positively correlated to Hcy, Cys, and MMA, and negatively to serum B-vitamins (figure 4. 4), which may partly explain elevated the metabolites and decreased B-vitamins seen in controls when the pregnancy progresses (table 4. 10). On the contrary, no such correlations were found in preeclamptic women with exception of vit B6 (figure 4. 4).

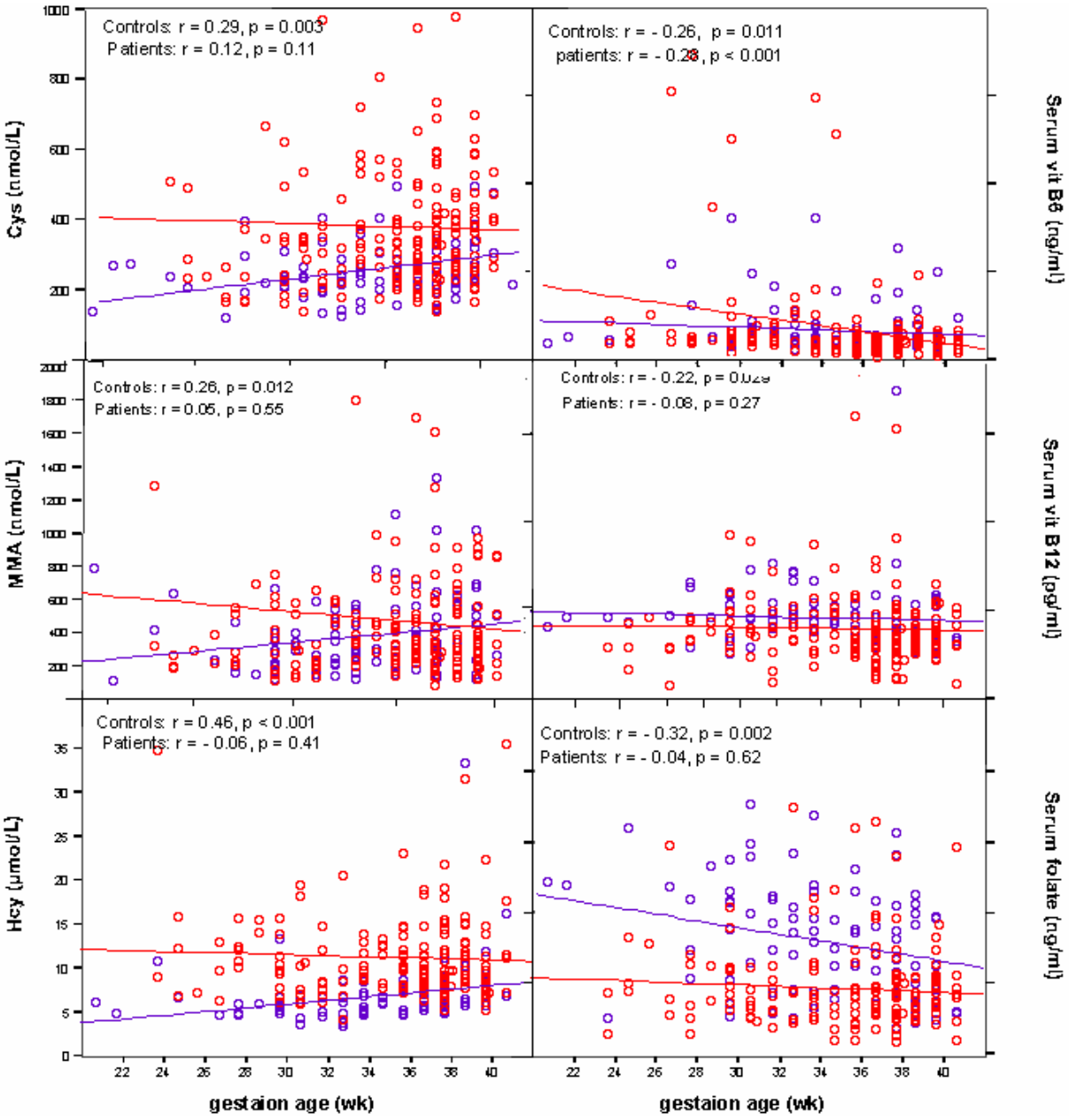


Figure 4. 4. The association between gestation age and the metabolites (left panels) and B-vitamins (right panels) in control and patients groups. o — patients, o — controls

In all women who ever used supplements during pregnancy, longer duration of vitamin supplementation was associated with lower tHcy levels and higher serum folate (figure 4. 5).

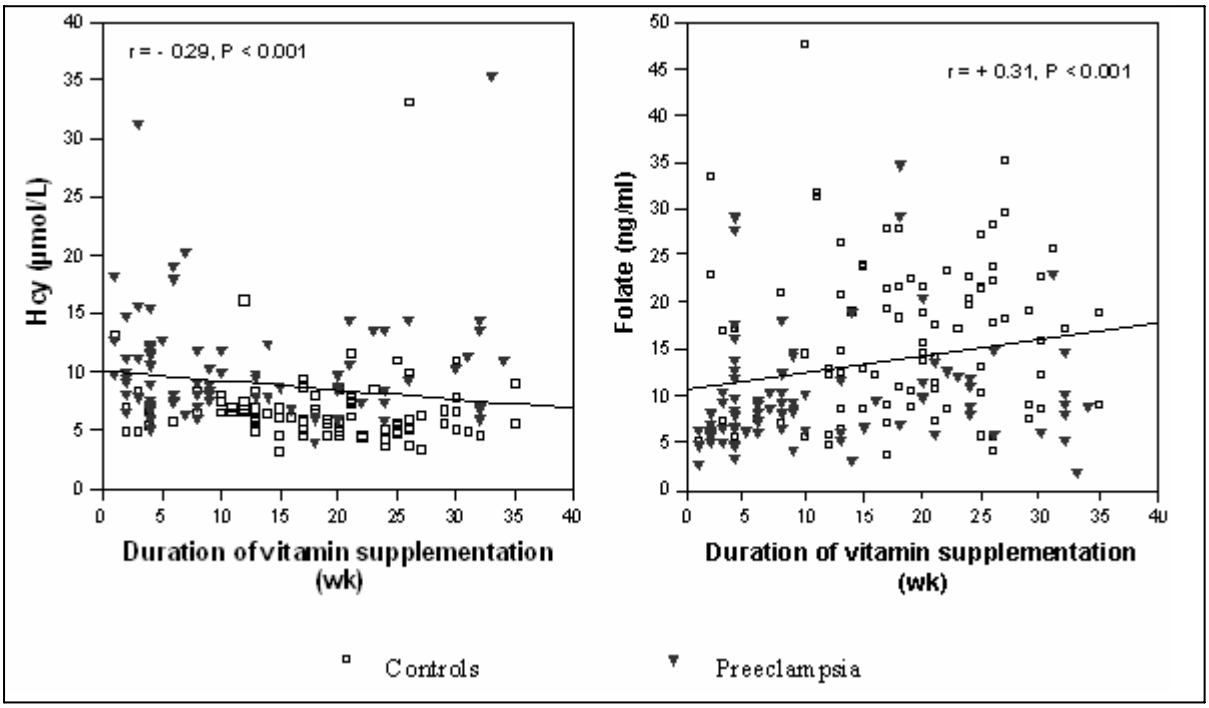


Figure 4. 5. Overall analysis of correlation between duration of vitamin supplementation, tHcy and folate

4. 4. 1. Correlations between Hcy, MMA, vit B12, holoTC, and folate

Serum tHcy concentrations correlated positively with serum folate in healthy and preeclamptic women. The correlation between Hcy and folate was stronger in controls compared to preeclamptic women (r = - 0.49 vs. – 0.29). A significant negative correlation between serum tHcy and holoTC (r = - 0.47, $p < 0.001$) was found among healthy pregnant women, and a marginal negative correlation (r = - 0.27, p = 0.062) was found in preeclamptic women. Likewise, the correlation between serum tHcy and vit B12 was significant only in normotensive women (r = - 0.27, p = 0.033). Nevertheless, Hcy correlated significantly to MMA in both groups. However, the correlation between concentrations of Hcy and MMA was much stronger in controls compared to preeclamptic women (figure 4. 6).

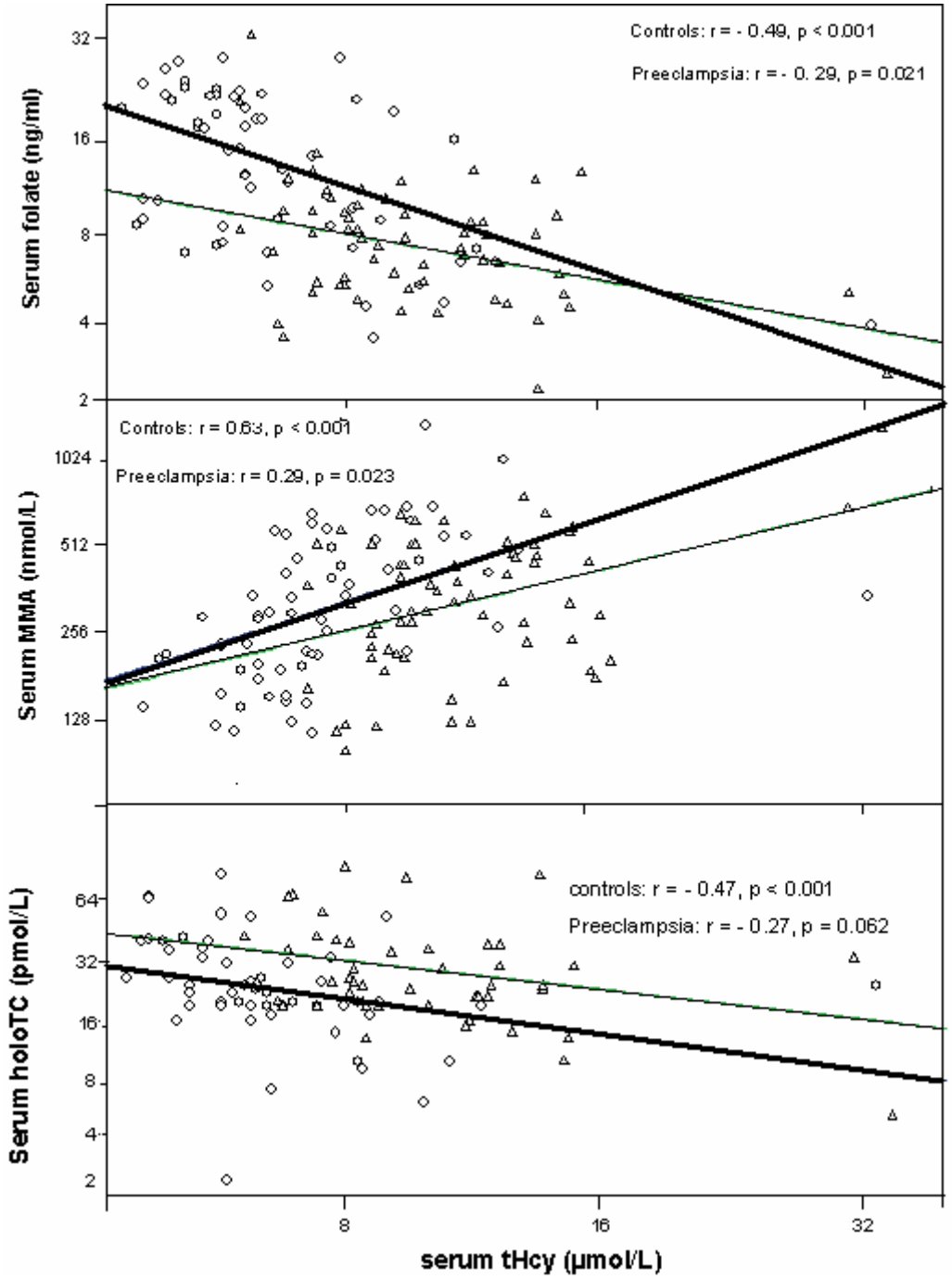


Figure 4. 6. The correlation between serum tHcy and that of holoTC, MMA, and folate in age- and gestation age-matched controls and patients (n = 63 pairs). O ——— controls, △ —— patients

As expected, there were positive correlation between vit B12 and holoTC (controls: r = 0.53, p < 0.001; patients: r = 0.59, p < 0.001), and negative correlation between vit B12 and MMA (controls: r = - 0.33, p = 0.007; patients: r = - 0.34, p = 0.001). Additionally, serum MMA

correlated negatively and significantly to holoTC (figure 4. 7) (controls: r = - 0.40, p = 0.002; preeclampsia: r = - 0.30, p = 0.038).

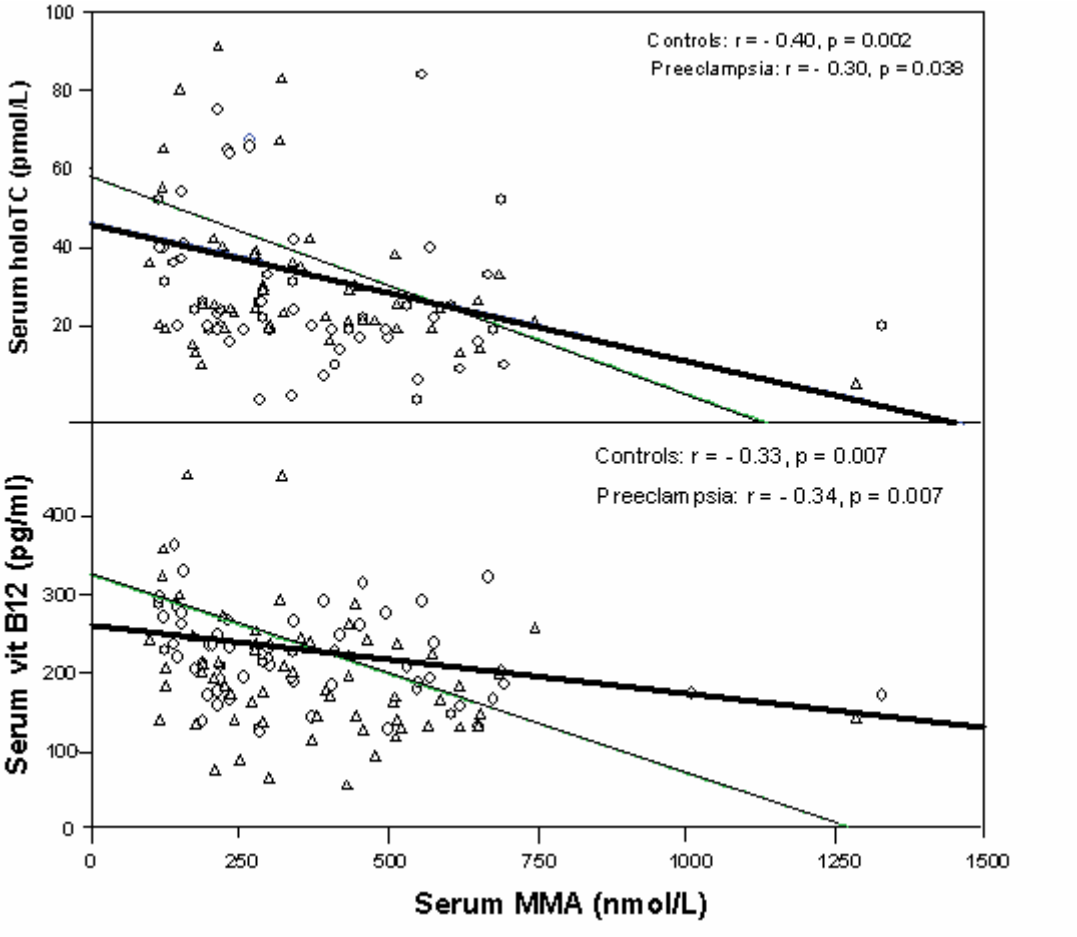


Figure 4. 7. The correlation between serum MMA and that of holoTC, and vit B12 in age-and gestation age-matched controls and patients (n = 63 pairs). O ——— controls, △ —— patients

The correlation between the metabolites and other vitamins are shown in table 4. 5.

Table 4. 5. Spearman rank-rho correlation coefficient of the metabolites and B-vitamins in the whole study groups (A: total population; B: control group; C: preeclamptic group). All correlations were adjusted for maternal and gestational age

A

		Overall (n = 275)									
		Hcy	**Cys**	**MMA**	**vit B12**	**folate**	**holo TC**	**vit B6**	**M.A.**	**G.A.**	**BMI**
Hcy	**r**		**0,54**	**0,38**	**-0,22**	**-0,52**	**-0,3**	**-0,23**	0,03	**0,17**	**0,14**
	P		**<0,001**	**<0,001**	**<0,001**	**<0,001**	**<0,001**	**<0,001**	0,65	**0,005**	**0,032**
Cys	**r**			**0,21**	**-0,12**	**-0,21**	-0,11	**-0,28**	0,08	**0,22**	0,07
	P			**<0,001**	**0,048**	**0,001**	0,12	**<0,001**	0,17	**<0,001**	0,26
MMA	**r**				**-0,31**	**-0,18**	**-0,38**	-0,12	0,01	**0,13**	0,03
	P				**<0,001**	**0,003**	**<0,001**	0,053	0,94	**0,032**	0,61
vit B12	**r**					**0,23**	**0,48**	**0,27**	0,03	**-0,16**	-0,12
	P					**<0,001**	**<0,001**	**<0,001**	0,59	**0,011**	0,062
folate	**r**						**0,31**	**0,15**	0,09	**-0,18**	-0,07
	P						**<0,001**	**0,012**	0,16	**0,004**	0,31
holoTC	**r**							**0,26**	0,06	-0,1	-0,04
	P							**<0,001**	0,35	0,16	0,57
vit B6	**r**								-0,02	**-0,3**	**-0,14**
	P								0,71	**<0,001**	**0,027**
M.A.	**r**									0,03	**0,39**
	P									0,58	**<0,001**
G.A.	**r**										**0,2**
	P										**0,002**

B

		Controls (n = 98)									
		Hcy	**Cys**	**MMA**	**vit B12**	**folate**	**holo TC**	**vit B6**	**M.A.**	**G.A.**	**BMI**
Hcy	**r**		**0.38**	**0,65**	**-0,28**	**-0,41**	**-0,50**	-0,14	0.10	**0,46**	-0,01
	P		**<0,001**	**<0.001**	**0,005**	**<0,001**	**<0,001**	0,16	0,34	**<0,001**	0,93
Cys	**r**			0,18	-0,11	-0,12	-0,05	**-0,31**	**0,22**	**0,29**	0,19
	P			0,068	0,27	0,24	0,69	**0,002**	**0,028**	**0,003**	0,061
MMA	**r**				**-0,38**	**-0,22**	**-0,45**	-0,12	-0,07	**0,26**	-0,13
	P				**<0,001**	**0,029**	**<0,001**	0,25	0,48	**0,012**	0,2
vit B12	**r**					0,06	**0,44**	**0,2**	-0,04	**-0,22**	-0,07
	P					0,56	**<0,001**	**0,047**	0,69	**0,029**	0,52
folate	**r**						**0,32**	0,08	0,12	**-0,32**	-0,03
	P						**0,003**	0,41	0,26	**0,002**	0,76
holoTC	**r**							0,19	0,17	-0,14	0,03
	P							0,086	0,12	0,2	0,77
vit B6	**r**								-0,17	**-0,26**	-0,11
	P								0,087	**0,011**	0,3
M.A.	**r**									0,08	**0,33**
	P									0,45	**0,001**

G.A.	**r**										0,2
	P										0,058

C

		Patients (n = 177)									
		Hcy	**Cys**	**MMA**	**vit B12**	**folate**	**holo TC**	**vit B6**	**M.A.**	**G.A.**	**BMI**
Hcy	**r**		**0,44**	**0,31**	-0,05	**0,32**	**-0,24**	**-0,16**	0,02	-0,06	0,07
	P		**<0,001**	**<0,001**	0,5	**<0,001**	**0,006**	**0,032**	0,78	0,41	0,4
Cys	**r**			**0,18**	0,02	-0,01	-0,08	**-0,19**	0,03	0,12	-0,07
	P			**0,015**	0,81	0,96	0,37	**0,011**	0,69	0,11	0,39
MMA	**r**				**-0,26**	-0,12	**-0,33**	-0,11	0,06	0,05	0,12
	P				**<0,001**	0,12	**<0,001**	0,16	0,47	0,55	0,16
vit B12	**r**					**0,16**	**0,5**	**0,23**	0,05	-0,08	-0,08
	P					**0,032**	**<0,001**	**0,002**	0,55	0,27	0,32
folate	**r**						**0,29**	0,05	0,06	-0,04	0,05
	P						**0,001**	0,53	0,46	0,62	0,55
holoTC	**r**							**0,3**	-0,01	-0,05	-0,06
	P							**<0,001**	0,92	0,57	0,54
Vit B6	**r**								0,04	**-0,28**	-0,14
	P								0,59	**<0,001**	0,11
M.A.	**r**									0,01	**0,44**
	P									0,95	**<0,001**
G.A.	**r**										0,16
	P										0,054

Interaction between folate and vit B12 as determinants of tHcy levels

The influence of folate status on tHcy levels depends on functional vit B12 status. Medians tHcy levels were presented in two subgroups within three folate tertiles. Both subgroups of controls had no significant differences in folate concentration in each tertile of folate. Normotensive pregnant women with normal levels of MMA (MMA ≤ 271 nmol/L) achieved lower tHcy levels at already lower levels of folate compared with normotensive pregnant women with elevated MMA (MMA > 271 nmol/L). Additionally, at the same level of folate pregnant women with abnormal level of MMA showed significantly higher tHcy levels compared to their counterparts with normal MMA levels. Serum vit B6 did not differ between these two subgroups of MMA within each tertile of folate. Of note, serum tHcy correlated significantly to MMA levels ($r = 0.46$, $p = 0.002$) in individuals with normal MMA levels, and to folate ($r = -0.56$, $r < 0.001$) and MMA ($r = 0.39$, $p = 0.003$) in individuals with abnormal MMA levels (data not shown). These observations may indicate the increased requirement for folate in individuals with subnormal vit B12 status (figure 4. 8).

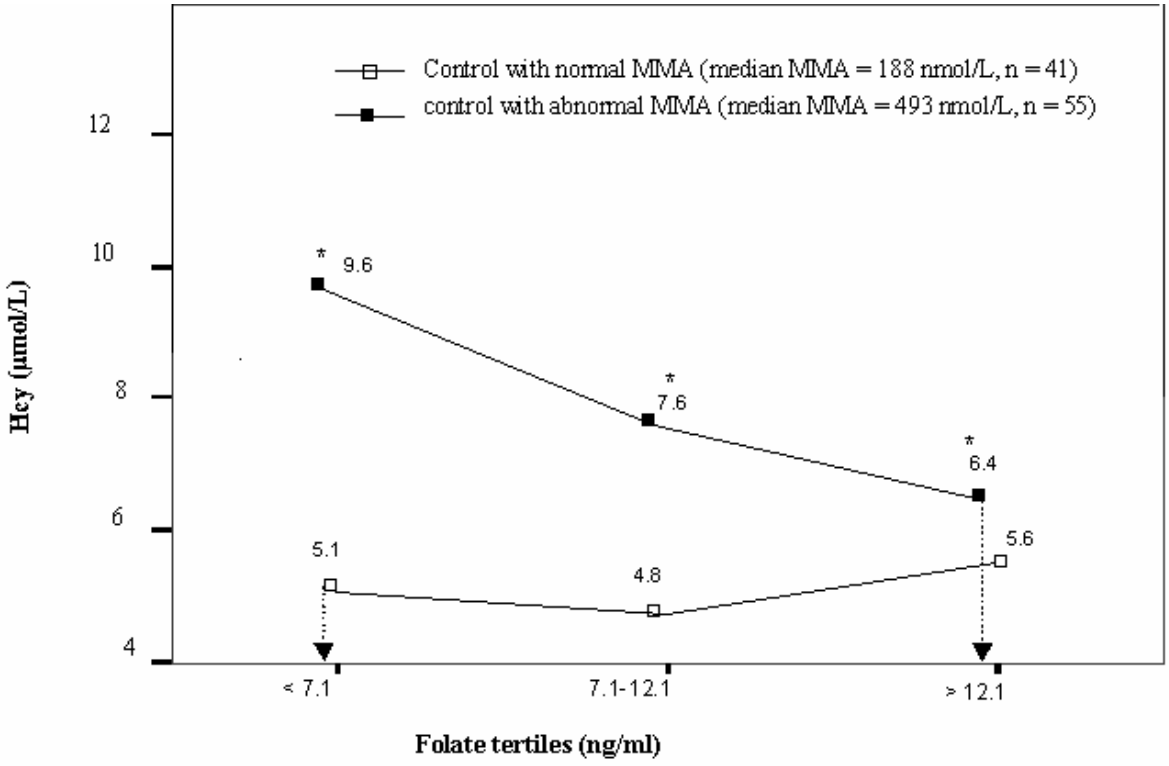


Figure 4. 8. Medians tHcy levels in different tertiles of folate. Lines represent healthy pregnant women; with MMA ≤ 271nmol/L or with MMA > 271nmol/L. Only subjects with

normal renal function (creatinine ≤ 0.9 mg/dl) and folate > 5 mg/ml were included. * significant difference vs. controls within each tertile of folate

Compared with normotensive women, preeclamptic women required higher folate levels to achieve the same levels of Hcy seen in controls (figure 4. 9).

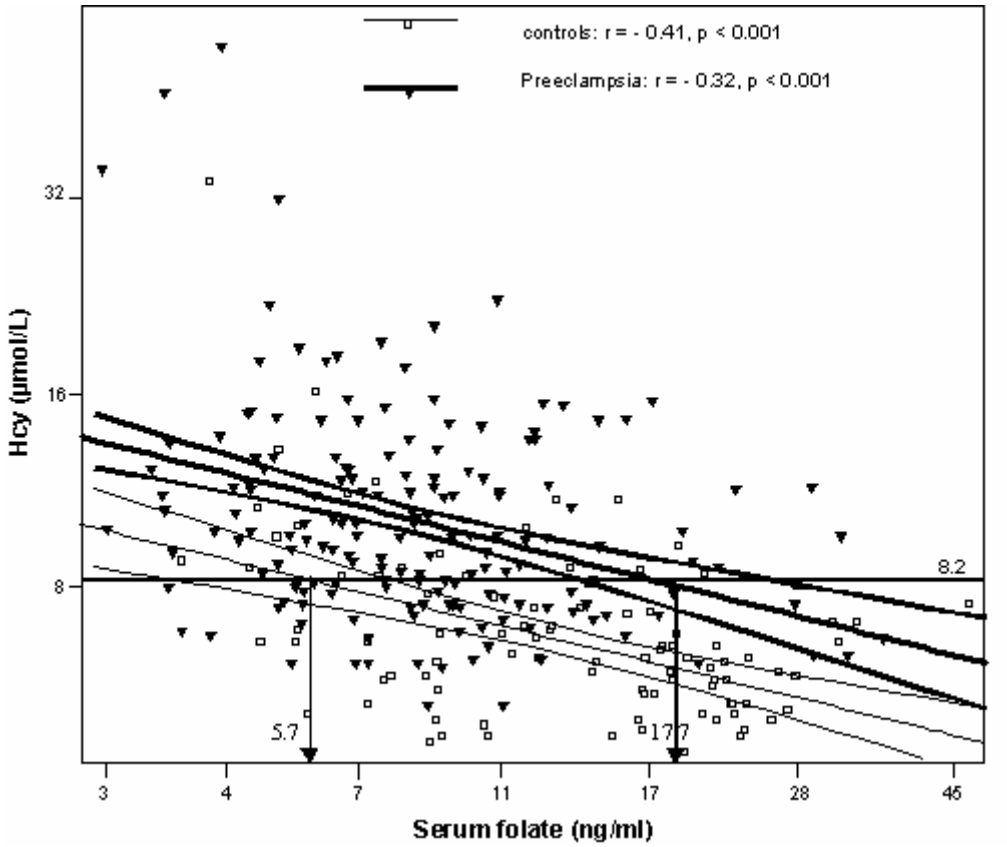


Figure 4. 9. The correlation between tHcy and folate in controls and preeclamptic women. Preeclamptic women had higher folate requirement to maintain similar tHcy levels as that in normotensive women

4. 4. 2. Correlations between creatinine, uric acid, the metabolites and B-vitamins

As expected, creatinine, uric acid, and urea correlated to each other in both groups. Serum creatinine was positively correlated to tHcy, Cys, and MMA in both groups. The correlation between creatinine and holoTC was significant only in preeclamptic women (table 4. 6).

Table 4. 6. Spearman rank-rho correlation coefficient of renal function, the metabolites, and B-vitamins

		Overall (n = 275)			**Controls (n = 98)**			**Patients (n = 177)**		
		Crea	**Uric A.**	**Urea**	**Crea**	**Uric A.**	**Urea**	**Crea**	**Uric A.**	**Urea**
Hcy	**r**	**0,51**	**0,57**	**0,44**	**0,42**	**0,29**	0,042	**0.33**	**0,31**	**0,21**
	P	**<0,001**	**<0,001**	**<0,001**	**<0,001**	**0,003**	0,68	**<0,001**	**<0,001**	**0,006**
Cys	**r**	**0,42**	**0,41**	**0,39**	**0,23**	**0,27**	0,09	**0,36**	**0,25**	**0,28**
	P	**<0,001**	**<0,001**	**<0,001**	**0,021**	**0,006**	0,37	**<0,001**	**0,001**	**<0,001**
MMA	**r**	**0,19**	**0,13**	0,1	**0,28**	0,054	0,041	**0,16**	0,12	0,069
	P	**<0,001**	**0,035**	0,08	**0,004**	0,59	0,69	**0,034**	0,1	0,36
vit B12	**r**	0,037	-0,07	**0,005**	0,099	0,04	**0,27**	**0,21**	**0,18**	**0,21**
	P	0,54	0,27	**0,93**	0,33	0,69	**0,008**	**0,005**	**0,016**	**0,005**
folate	**r**	**-0,21**	-0,26	**-0,19**	**-0,21**	-0,09	-0,058	0,072	0,11	0,13
	P	**<0,001**	<0,001	**0,002**	**0,037**	0,36	0,57	0,34	0,16	0,087
Holo TC	**r**	**0,17**	0,09	**0,19**	0,066	0,05	**0,27**	**0,28**	**0,23**	**0,28**
	P	**0,014**	0,21	**0,006**	0,55	0,65	**0,012**	**0,001**	**0,01**	**0,001**
vit B6	**r**	-0,07	-0,07	0,02	-0,034	0,007	-0,018	0,076	0,13	**0,32**
	P	0,25	0,29	0,69	0,74	0,94	0,86	0,31	0,088	**<0,001**
M.A.	**r**	-0,03	**0,04**	0,002	0,14	**0,23**	0,11	-0,08	-0,023	-0,054
	P	0,68	**0,53**	0,97	0,17	**0,001**	0,27	0,28	0,77	0,48
G.A.	**r**	0,06	**0,09**	-0,03	**0,46**	**0,42**	0,078	-0,2	**-0,19**	**-0,29**
	P	0,36	**0,13**	0,65	**<0,001**	**<0,001**	0,44	0,07	**0,013**	**<0,001**
BMI	**r**	0,06	**0,17**	0,06	0,19	**0,27**	0,032	-0,16	**-0,023**	-0,14
	P	0,39	**0,01**	0,34	0,066	**0,009**	0,76	0,065	**0,79**	0,11
Crea	**r**		**0,71**	**0,63**		**0,52**	**0.33**		**0,6**	**0,54**
	P		**<0,001**	**<0,001**		**<0,001**	**0,001**		**<0,001**	**<0,001**
Uric A.	**r**			**0,67**			**0,23**			**0,51**
	P			**<0,001**			**0,02**			**<0,001**

4. 5. MTHFR genotypes

The prevalence of MTHFR C677T

The frequency of the homozygous 677C→T (T/T) genotype was 8.8 % in all subjects with a mutant allele frequency of 31.1 %. Distribution of the three genotypes did not differ between the groups (p = 0.224, χ^2- test). Table 4. 7 summarizes the distribution of the MTHFR in the three groups.

Table 4. 7. Frequency of MTHFR 677 C→T genotypes in healthy and preeclamptic women

Genotype	All subjects (n = 272)	Controls (n = 97)	Patients (n = 175)
CC	127 (46.7 %)	38 (39.2 %)	89 (50.8 %)
CT	121 (44.5 %)	47 (48.4 %)	74 (42.3 %)
TT	24 (8.8 %)	12 (12.4 %)	12 (6.9 %)

CC: wildtype, CT: heterozygotes, TT:homozygotes
Note. DNA not available for 1 control and 2 preeclamptic women

Folate, vit B12, and tHcy concentrations were studied in relation to the MTHFR genotypes (table 4. 8). The presence of T-allele did not have influence on these variables. In both groups, subjects who were homozygous or heterozygous for the mutant allele did not exhibit significant differences in tHcy, folate, and vit B12 as compared to subjects with CC genotype. Compared to controls, serum tHcy level was significantly higher and folate and vit B12 were significantly lower in preeclamptic women among subjects with CC and CT genotypes, whereas similar concentrations among subjects with TT genotype were seen (table 4. 8). Maternal age and gestational age which are potential confounders of tHcy levels, did not differ between subjects with CC and those with CT or TT genotypes in controls and patients groups

Table 4. 8. Maternal tHcy, folate, and vit B12 concentrations according to the MTHFR genotypes

	Con/PE, n	CC (38/89)	CT (47/74)	TT (12/12)	P[1]- value
Hcy, µmol/L	Controls	6.2	6.1	8.2	0.234
	Patients	9.7	9.6	10.6	0.307
P-value		<0.001	<0.001	0.178	
Folate, ng/ml	Controls	15.6	15.6	7.3	0.068
	Patients	8..1	8.2	6.3	0.463
P-value		<0.001	<0.001	0.410	
Vit B12, pg/ml	Controls	216	217	187	0.312.
	Patients	182	161	217	0.129
P-value		0.023	<0.001	0.843	

Data are presented as medians. CC: wildtype, CT: heterozygotes, TT:homozygotes. P[1]: Kruskal Wallis test by genotype, P: Mann-Whitney test by study groups.

Even that the medians of tHcy did not differ among the three genotypes (CC, CT, TT), the incidence of HHcy (Hcy > 8.2 µmol/L) was highest in subjects with the TT genotype compared to the other two genotypes in each study group (figure 4. 10). Morethat, HHcy was more frequent in preeclamptic women compared to the controls within each MTHFR genotype.

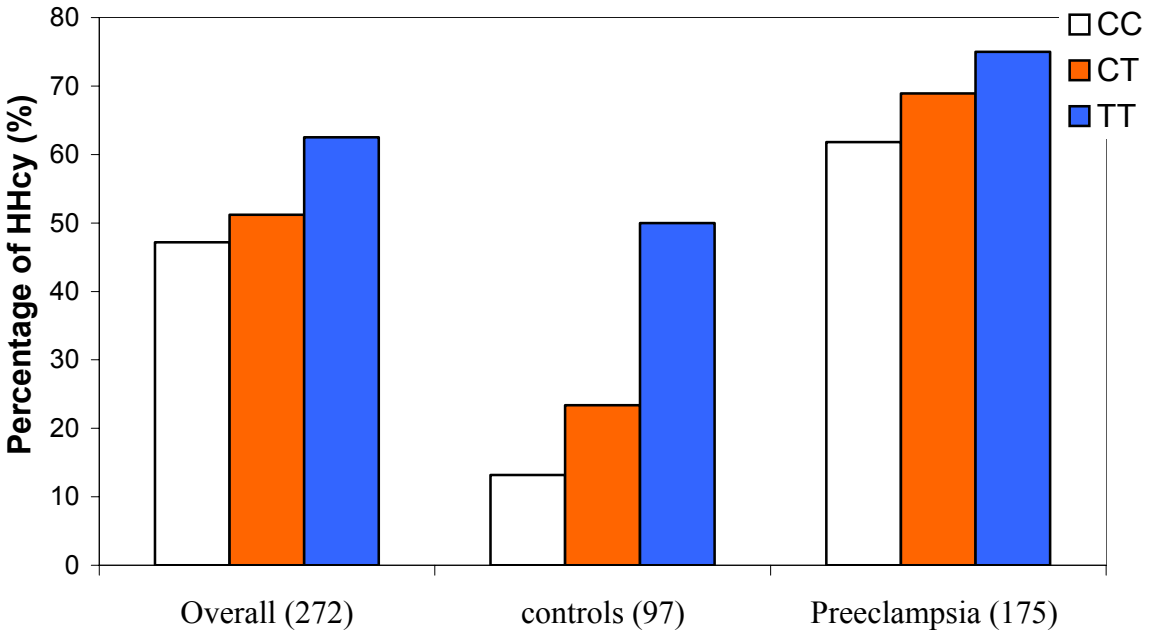


Figure 4. 10. The prevalence of HHcy (Hcy > 8.2 µmol/L) according to the MTHFR genotypes

The interaction between folate status and MTHFR genotypes as determinants of Hcy

Table 4. 9 shows tHcy and folate concentration among MTHFR genotypes in two ranges of folate status. Maternal and gestation age did not differ significantly within MTHFR genotypes (CC, CT, TT) in both folate levels (folate ≤ 8.9 ng/ml, folate > 8.9 ng/ml). The influence of MTHFR TT genotype on tHcy level was seen only when folate was ≤ 8.9 ng/ml. As shown, the TT group had significantly higher tHcy levels than CC (median tHcy:11.3 vs. 9.6 µmol/L, p = 0.027) when folate was ≤ 8.9 ng/ml, whereas this difference disappeared when folate was above 8.9 ng/ml (figure 4. 11). Increased tHcy levels seen in the TT group with folate ≤ 8.9 ng/ml accompanied with significant lower levels of folate (4.5 ng/ml) as compared with either CC (6.5 ng/ml) or CT (6.3 ng/ml) groups (p = 0.004 and 0.006, respectively) (table 4. 9).

Table 4. 9. Serum tHcy and folate concentrations of women according to MTHFR genotypes within two folate range

	Genotypes	Total population (folate ≤ 8.9 ng/ml) N = 136	Total population (folate > 8.9 ng/ml) N = 136
Hcy, µmol/L	CC	9.6[a]	7.4[a]
	CT	9.6[a]	7.0[a]
	TT	11.3*	6.8[a]
P[1]- value		0.083	0.720
Folate, ng/ml	CC	6.5	14.0
	CT	6.3	15.4
	TT	4.5*[†]	13.4

P[1]- value		0.012	0.838
Maternal age, years	CC	25	26
	CT	25	27
	TT	23	32
P[1]- value		0.228	0.731
Gestational age, weeks	CC	37	37
	CT	37	35
	TT	38	36
P[1]- value		0.869	0.055

Data are presented as medians. P[1]: Kruskal Wallis test by genotype. Values with identical superscript letters are not significantly different. * significant as compared with CC, † significant as compared with CT. The cutt-off value of folate represents the median of folate in the total population

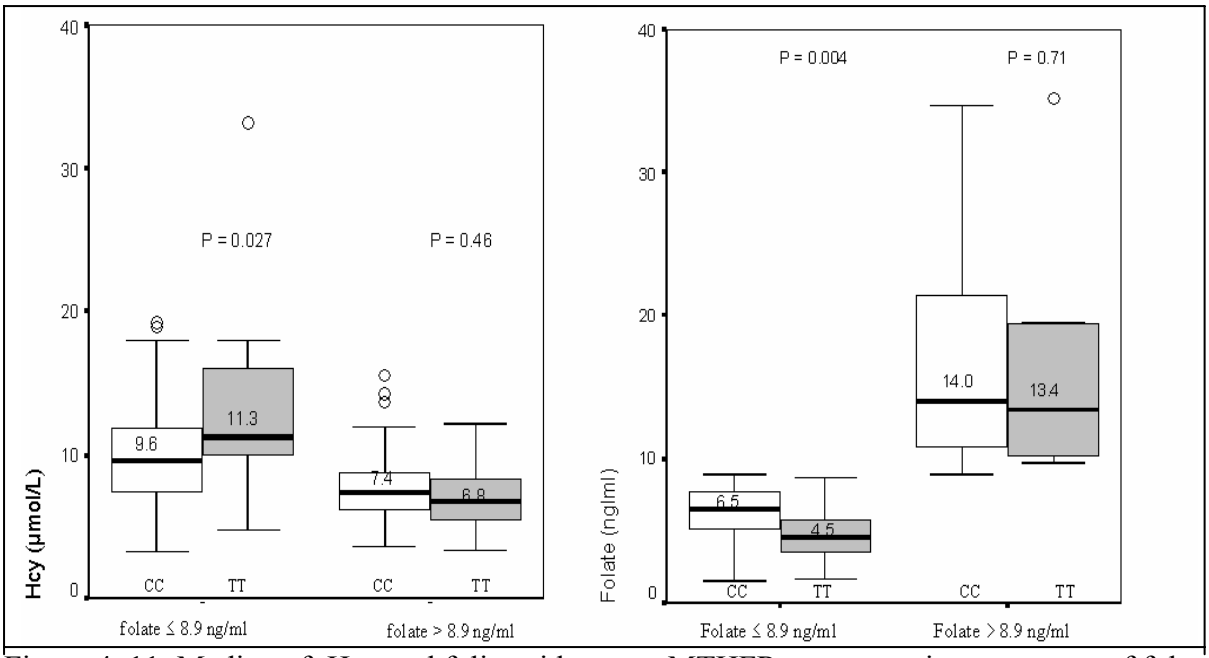


Figure 4. 11. Median of tHcy and folic acid among MTHFR genotypes in two ranges of folate status (folate ≤ 8.9 ng/ml and > 8.9 ng/ml). P represents the significance of difference between CC and TT genotypes

4. 6. Determinants of Hcy, Cys, and MMA levels

Logistic regression analysis with backward elimination was used to determine the factors that independently influenced Hcy, Cys, and MMA levels in healthy and preeclamptic women (table 4. 10). In both groups, Hcy was inversely and independently influenced by cobalamin and folate status, and positively by Cys. In healthy pregnant women, tHcy level was more influenced by cobalamin status than by folate status. Renal function indicated by creatinine

had an independent influence on the Hcy, Cys, and MMA levels only in preeclamptic women, suggesting that preeclampsia related renal dysfunction accounts for some of these metabolites elevations.

Table 4. 10. The final model of the backward regression analysis with Hcy, Cys and MMA as dependent variables

Dependent variables	Independent variables in the final model	Beta	P-value.	R-square
Healthy women				
Hcy	Cys	+ 0.26	0.001	
	MMA	+ 0.23	<0.001	$R^2 = 0.48$
	Folate	- 0.14	0.008	
MMA	Hcy	+ 0.94	<0.001	$R^2 = 0.37$
	Vit B12	- 0.54	0.002	
Cys	Hcy	+ 0.41	<0.001	
	Vit B12	+ 0.27	0.006	$R^2 = 0.25$
	Vit B6	- 0.23	0.016	
Preeclamptic women				
Hcy	Creatinine	+ 0.33	0.005	
	Cys	+ 0.29	<0.001	$R^2 = 0.40$
	Folate	- 0.26	<0.001	
	MMA	+ 0.13	0.004	
MMA	Creatinine	+ 0.49	0.023	$R^2 = 0.31$
	Vit B12	- 0.40	<0.001	
	Hcy	+ 0.39	0.003	
	MTHFR TT	- 0.17	0.039	
	Vitamin use	- 0.14	0.002	
Cys	Hcy	+ 0.34	<0.001	$R^2 = 0.33$
	Creatinine	+ 0.26	0.001	
	Vit B6	- 0.21	0.009	
	BMI	- 0.20	0.017	

Beta: is the regression coefficient and interpreted as the amount of change in the dependent variable with one unite of change in the independent variable. R-square: the coefficient of determination and shows the strength of the relationship between the model and the dependent variables. Variables with skewed distribution were logarithmic transformed for normality. In addition to the variables that appeared in the final model, other variables were entered in the test (duration of vitamin supplementation, maternal age, gestational age, Urea, holoTC, BMI, and MTHFR C→T genotypes).

4. 7. The metabolites and B-vitamins concentrations according to the gestational age

Subjects were stratified according to their gestation age to investigate a possible association between the vitamins and the metabolites in patients and controls of comparable age of gestation (table 4. 11). Significant higher tHcy and Cys levels were seen in preeclamptic women as compared to controls in each category of the gestation age (table 4. 11). Additionally, differences in serum concentrations of folate, vit B12, and vit B6 were observed. Within each study group, pregnant women in the age ≤ 34 wk of gestation had relatively higher B-vitamins levels compared to those who were late in their pregnancy (i.e., those with gestational age > 38 wk). More that, in the control group a significant increase in tHcy, Cys, and MMA concentrations occurred with increasing gestation, whereas preeclamptic women had elevated levels from these metabolites earlier in their gestation. (figure 4. 13). The cut-off value for HHcy was identified in each tertile of gestation age as the 95th percentile of Hcy concentration in normotensive pregnant women who had normal renal function. Accordingly, the cut-off values were 10.3, 11.0, and 15.7 µmol/L in the first, second, and the third tertile of gestation age, respectively. Using these values, HHcy was found in 41.8 %, 40.3 %, and in 8 % of preeclamptic women who were in the first, second, and the third tertile of gestation age, respectively. As shown in figure 4.12, the 95th percentile of Hcy concentrations was higher in preeclamptic women as compared to controls in each tertile of gestation age.

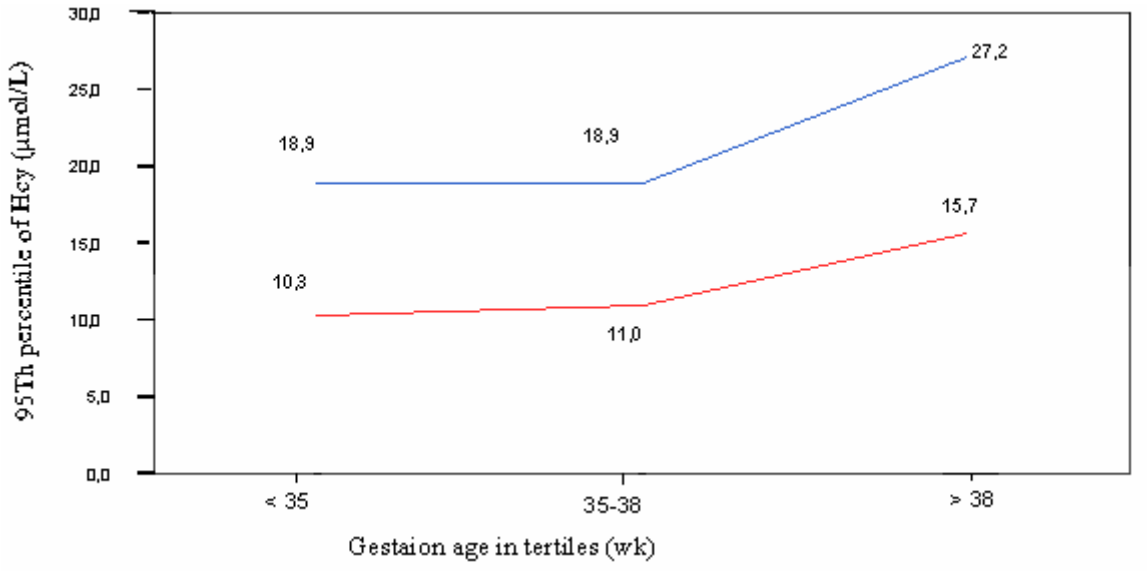


Figure 4. 12. the 95Th percentile of Hcy concentrations in each tertile of gestation age

Table 4. 11. Serum concentration of the metabolites and vitamins according to the gestation

		20-34 weeks	35-38 weeks	>38 weeks	P[1]- value
Controls/patients, n		46/55	30/72	22/50	
Hcy (µmol/L)	Controls	5.6 (10.9%)	6.4 (16.7%)	8.0 (50.0%)	< 0.001
	Patients	9.8 (6.7%)	9.6 (62.5%)	9.3 (66.0%)	0.890
P-value		< 0.001	< 0.001	0.017	
Cys (nmol/L)	Controls	226 (10.9%)	241(23.3%)	270 (40.9%)	0.018
	Patients	315 (56.4%)	325 (56.9%)	374 (62.0%)	0.367
P-value		< 0.001	0.003	0.013	
MMA (nmol/L)	Controls	263 (47.8%)	337 (65.5%)	469 (68.2%)	0.031
	Patients	320 (59.3%)	346 (77.5%)	339 (65.3%)	0.437
P-value		0.141	0.690	0.654	
Vit B12 (pg/ml)	Controls	226 (30.4%)	208 (60.0%)	187 (68.2%)	0.042
	Patients	186 (60.0%)	169 (65.7%)	164 (78.0%)	0.473
P-value		0.003	0.018	0.092	
Folate (ng/ml)	Controls	18.6 (4.3%)	14.4 (3.3%)	10.1 (9.1%)	0.012
	Patients	8.3 (18.2%)	6.6 (22.9%)	8.0 (16.0%)	0.241
P-value		< 0.001	< 0.001	0.002	
HoloTC (pmol/L)	Controls	20 (70.3%)	22 (80.8%)	24 (81.0%)	0.480
	Patients	21 (77.3%)	22 (76.9%)	21 (81.8%)	0.942
P-value		0.301	0.774	0.993	
PLP (nmol/L)	Controls	2.6 (80.4%)	2.1 (86.7%)	2.2 (90.9%)	0.015
	Patients	2.1 (75.9%)	1.8 (95.7%)	1.9 (98.0%)	< 0.001
P-value		0.228	0.037	0.097	

Data are presented as medians. P[1]: significant by categories of gestational age, P: significant by study groups. % refers to the percent of prevalence of abnormal metabolites or vitamin deficiency

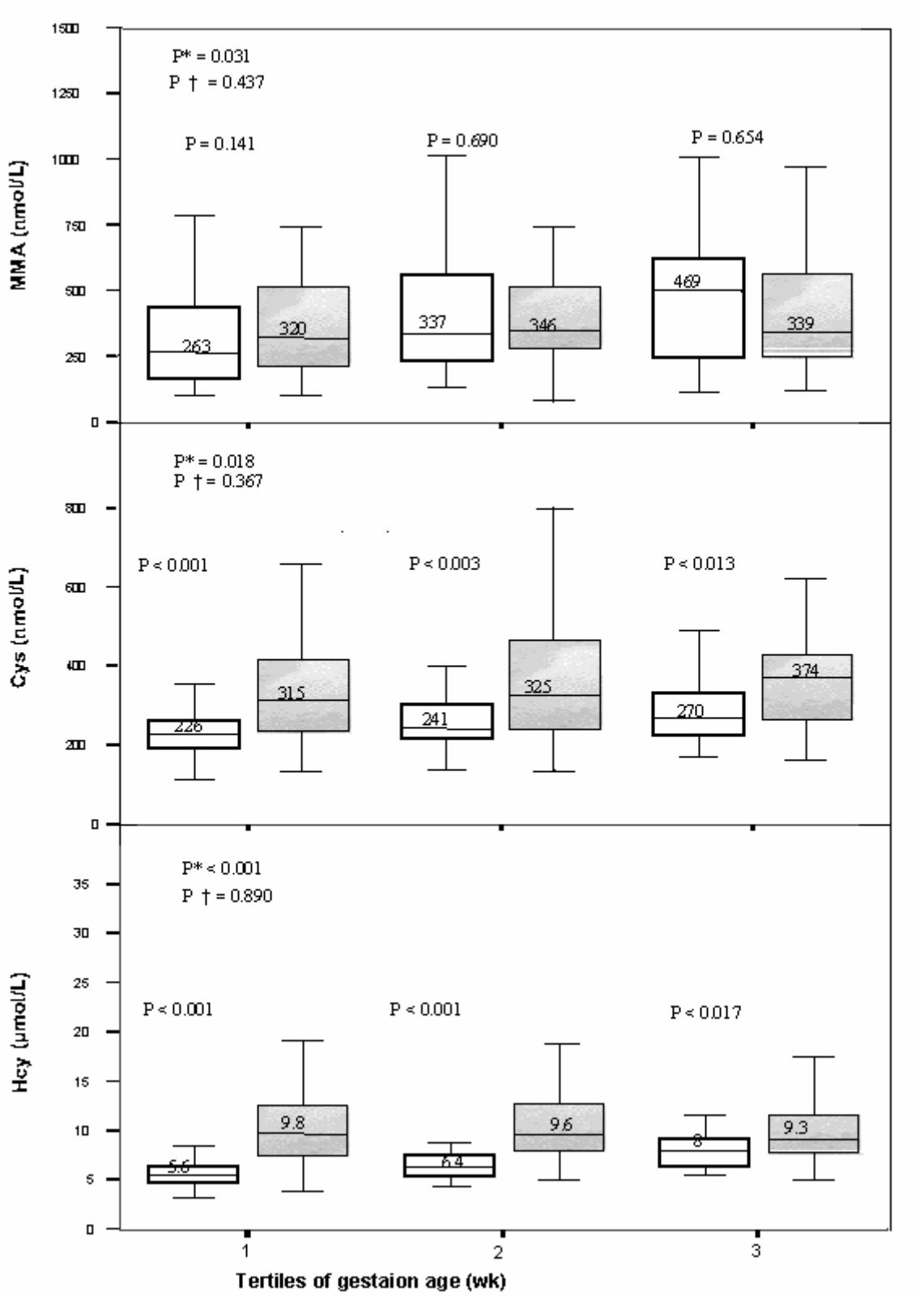


Figure 4. 13. Medians of Hcy, Cys, and MMA in relation to tertiles of gestation age. P*: in controls, p†: in preeclamptic women

4. 8. Odds ratio for HHcy

Logistic regression analysis on the pooled data were applied to identify the effect of B-vitamins and MTHFR genotypes on the risk of HHcy (Hcy > 8.2 μmol/L) in the pregnant women of the present study. For this purpose each subject classified once according to the quartiles of folate determined by the distribution of folate in the total population, and once according to the quartiles of MMA determined by the distribution of MMA in the total population. As shown in table 4. 12, the MTHFR genotypes and vit B6 had no significant influence on the risk of HHcy. In contrast, folate and vit B12 deficiency had significant influences, and the risk of HHcy associated with elevated MMA levels was higher than the risk associated with decreased folate levels. Pregnant women who were within the highest quartile of MMA had higher risk of HHcy than pregnant women who were within the lowest quartile of folate (9.78-fold and 7.03-fold increased risk of HHcy, respectively). This risk was higher when decreased folate levels was associated with elevated serum MMA levels or TT genotype (table 4. 12).

Table 4. 12. the odds ratio of HHcy risk (Hcy > 8.2 μmol/L) in pooled data

	Adjusted OR (95 % CI)[b]	P value
MTHFR genotypes		
MTHFR CC	1.0 (referent)	
MTHFR CT	1.52 (0.69-3.34)	0.29
MTHFR TT	2.28 (0.66-7.89)	0.19
Vit B6, ng/ml		
$Q_4 \geq 3.0$	1.0 (referent)	
Q_3 [2.2-2.9]	1.64 (0.50-5.32)	0.41
Q_2 [1.7-2.1]	2.05 (0.66-6.38)	0.22
$Q_1 \leq 1.6$	2.16 (0.71-6.51)	0.17
Folate, ng/ml		
$Q_4 \geq 14.91$	1.0 (referent)	
Q_3 [8.93-14.86]	2.81 (0.78-10.09)	0.114
Q_2 [6.20-8.90]	5.99 (1.68-21.36)	0.006
$Q_1 \leq 6.13$	7.03 (1.94-25.49)	0.003
MMA, nmol/L		
$Q_1 \leq 217$	1.0 (referent)	
Q_2 [218-337]	1.44 (0.50-4.09)	0.50
Q_3 [339-537]	4.55 (1.56-13.29)	0.006
$Q_4 \geq 540$	9.78 (3.10-30.86)	< 0.001
Combination (folate/MMA)		
Folate ≥ 14.91/MMA< 540	1.0 (referent)	
Folate < 14.91/MMA ≥ 540	39.40 (4.92-315.71)	< 0.001
MTHFR/folate		
MTHFR CC/folate ≥ 14.91	1.0 (referent)	
MTHFR TT/folate < 14.91	70.36 (3.54-1398.16)	0.005
MTHFR/MMA		
MTHFR CC/MMA < 540	1.0 (referent)	
MTHFR TT/MMA ≥ 540	5.89 (0.58-59.42)	0.13

The model was adjusted for potential confoundings: Maternal age, gestation age, study groups, creatinine, MMA, folate, vit B6, and MTHFR genotypes.

4. 9. Odds ratio for preeclampsia

To estimate the odds ratio for PE according to the different variables of the current study (Hcy, folate, vit B12, and MTHFR genotypes), each subjects was classified once according to the quartiles of Hcy determined by the distribution of Hcy in controls, once according to the quartiles of folic acid determined by the distribution of folate in controls, and once according to the quartiles of MMA determined by the distribution of MMA in controls (table 4. 13).

There was a significant association between maternal tHcy and folate status and the risk of preeclampsia. After adjustment for the potential confounding, women in the highest quartile of Hcy or in the lowest quartile of folate experienced increased risk of preeclampsia as compared with women in the lowest quartile of Hcy and in the highest quartile of folate, respectively (OR for Hcy = 21.6 (3.7-125.3); OR for folate = 9.9 (2.53-39.44)). After adjustment for the potential confounding, there was no clear association of preeclampsia risk and vit B12 status indicated by MMA. Logistic regression analysis was applied again to analyse the combined effect of folate status and MTHFR genotype on the occurrence of preeclampsia. As shown in table 4. 13, maternal folate concentration had a greater influence than MTHFR genotypes as a determinant of preeclampsia risk. Compared to women with folate $\geq$ 8.9 ng/ml and CC genotype (the referent group), women with low folate (folate < 8.9 ng/ml) and CC genotype experienced 4.8-fold increased risk of preeclampsia.

Table 4. 13. the odds ratio of preeclampsia risk

	controls N = 97	patients N = 175	Adjusted OR (95 % CI)[b]	P value
HCY, µmol/L				
Q1 <5.2	24	3	1.0 (referent)	
Q2 [5.2-6.1]	24	8	2.6 (0.39-16.5)	0.321
Q3 [6.2-7.7]	25	33	7.3 (1.32-40.0)	0.023
Q4 >7.8	24	128	21.6 (3.7-125.3)	0.001
Folate, ng/ml				
Q_4 >21.3	25	8	1.0 (referent)	
Q_3 [14.7-21.3]	24	13	1.1 (0.22-5.28)	0.924
Q_2 [8.7-14.6]	24	49	5.6 (1.41-22.55)	0.014
Q_1 <8.7	24	102	9.9 (2.53-39.44)	0.001
MMA, nmol/L				
Q_1 <199	24	28	1.0 (referent)	
Q_2 [199-297]	25	41	1.5 (0.48-4.76)	0.48
Q_3 [298-531]	25	58	1.4 (0.47-3.99)	0.56
Q_4 >531	25	45	0.4 (0.138-1.43)	0.17
MTHFR genotypes				
MTHFR CC	38	86	1.0 (referent)	
MTHFR CT	47	74	0.65 (0.28-1.48)	0.301
MTHFR TT	12	12	0.14 (0.031-0.68)	0.014
MTHFR/folate				
CC/folate ≥8.9 ng/ml	31	36	1.0 (referent)	
CC/folate <8.9 ng/ml	7	50	4.8 (1.56-14.5)	0.006
TT/folate ≥8.9 ng/ml.	6	5	0.8 (0.16-3.82)	0.76
TT/folate< 8.9 ng/ml	6	7	1.5 (0.31-6.99)	0.63

The model was adjusted for maternal age, gestational age, BMI, total parity, reported vitamin use, MTHFR, and creatinine. Furthermore, MMA and folate were entered in the model for folate and MMA, respectively.

5. DISCUSSION

The present study was carried out to investigate the role of HHcy and the underlying causes in preeclampsia in a population of Syrian pregnant women. B-vitamins status and other associated metabolites in 98 normal pregnant and in 177 preeclamptic Syrian women were analysed. Higher concentrations of Hcy, Cys, and MMA were closely linked to a lower status of the B-vitamins. Serum concentrations of folate and vit B6 were significantly lower and tHcy and Cys were significantly higher in preeclamptic as compared to normotensive women. Noteworthy, pregnant women were less likely to have folate deficiency (14 %), whereas a high prevalence of subnormal cobalamin status was found, indicated by elevated serum MMA (64.6 %) and low holoTC (77.7 %). HHcy (Hcy > 8.2 µmol/L) was seen in 65.2 % and 22 % of patients and controls, respectively. The findings underline that low B-vitamin status and HHcy are potential contributing factors for preeclampsia in Syrian pregnant women.

5. 1. Homocysteine

5. 1. 1. Hcy in normal pregnancy

Normal range of tHcy in women was identified in several studies, with an accepted mean value of 9 µmol/L (Holmes et al., 2005; Murphy et al., 2004; 2002; Bates et al., 2002; Nygard et al., 1995). In the present study, healthy pregnant women were stratified into three subgroups according to the gestation age. First group included normotensive women with gestation age ≤ 34 wk, second group included normotensive women with gestation age between 35 and 38 wk, and the third group included normotensive women with gestation age > 38 wk (table 4. 11). As others have shown (Holmes et al., 2005; Murphy et al., 2004; 2002; Walker et al., 1999; Anderson et al., 1992), serum tHcy levels in normal pregnant women were lower (median value = 6.0 µmol/L) than that reported in non-pregnant women. Several explanation have been proposed for the lower tHcy concentrations in pregnancy (see above), but till now the exact mechanism is still not totally clarified.

Through different tertiles of gestation age, serum tHcy demonstrated a significant increase: median values were 5.6, 6.4, and 8.0 µmol/L, respectively, ($P < 0.001$). Increased tHcy levels with increasing gestation were also found in other studies of a longitudinal design (Holmes et al., 2005; Ellison et al., 2004; Murphy et al., 2004). In these studies serum tHcy levels increased in the third trimester to reach its preconception levels at the onset of labour (Murphy et al., 2004), and two days after the delivery (Holmes et al., 2005). Some authors suggested that this elevation in tHcy plays a physiologic role in the preparation for labour,

since a significant influence of Hcy on the contractions of the myometrium has been recently reported elsewhere (Ayar et al., 2003). So far, the reason behind this elevation in tHcy during normal pregnancy is still unidentified. However, in the present study serum Hcy correlated negatively with serum folate and vit B12 and positively with MMA (table 4. 5, A). Additionally, asymptomatic women in late pregnancy had significantly higher level of MMA and lower levels of folate compared to those at earlier stage of gestation (table 4. 11). These findings suggest that vitamins depletion occurred in a part of the normotensive women throughout the pregnancy may contribute to the increase in tHcy levels seen in pregnant women (Milman et al., 2006; 2006 a). Of note, tHcy concentrations in normotensive women increased with increasing gestation by about 43 % (from 5.6 to 8.0 μmol/L). Increased tHcy concentrations was associated with decreased serum folate concentrations by about 46 % (from 18.6 to 10.1 ng/ml), whereas vit B12 concentration displayed a small decrease, about 17 % (from 226 to 187 pg/ml) (table 4. 11). This indicates that increased tHcy levels in normal pregnant women is more influenced by the decline in serum folate concentrations rather than by vit B12 deficiency.

5. 1. 2. Hcy in pregnancy complicated with preeclampsia

Serum tHcy levels in preeclamptic women were significantly higher than those in normotensive counterparts (table 4. 3), with higher prevalence of HHcy (Hcy > 8.2 μmol/L) in patients (65.2 %) compared to controls (22 %) (figure 4. 1). Several studies have found elevated tHcy levels in preeclamptic women (Rajkovic et al., 1999; 1997; Leeda et al., 1998; Powers et al., 1998), while others did not (Herrmann et al., 2004; Hietala et al., 2001; Sorensen et al., 1999). Hcy metabolism is mainly influenced by B-vitamins availability (Murphy et al., 2004; McMullin et al., 2001; Andersson et al., 1992; Leeda et al., 1998) and renal function (Guttormsen et al., 1997). Therefore, higher tHcy levels in patients compared to controls could be related either to inhibited Hcy metabolism or to failure of mechanisms that lower Hcy during normal pregnancy. Since preeclamptic women had significantly lower level of folate and vit B6 compared to controls (table 4. 3) accompanied with renal changes, the possibility that Hcy was not actively catabolized in preeclamptic women seems more plausible (the relation between Hcy and either of B-vitamins or renal function will be discussed later). Furthermore, it is well accepted that the reference range for Hcy during pregnancy is lower than that in non-pregnant women.

A recent study showed that women with higher tHcy levels in the preconception period were more likely to develop preeclampsia during their pregnancy (Ronnenberg et al., 2002).

Additionally, previous prospective studies demonstrated that elevated tHcy plasma levels may predict, in the early second trimester period, the subsequent development of preeclampsia (Cotter et al., 2001; Sorensen et al., 1999). Therefore, it may be argued that tHcy elevation precedes and predisposes to preeclampsia rather than being an indicator of preeclampsia. However, concentrations of tHcy in preeclamptic women of the present study and many previous reports were not in the range that may cause vascular damage. Additionally, reduced plasma volume and fluid loss from the intravascular compartment associated to preeclampsia may cause serum tHcy elevation. Furthermore, oxidative stress, which is reported to increase in preeclampsia (Hubel CA., 1999; Power et al., 1998) impairs selectively the MS function resulting in impairment of Hcy remethylation (McCaddon et al., 2002). The impairment of this metabolic function might explain the aetiology of HHcy seen in preeclampsia in several studies.

In conclusion, normal pregnancy associated with increased tHcy level with increasing gestation, and Hcy metabolism was more influenced by folate status rather than by vit B12 status. The significant lower folate and vit B6 concentrations in preeclamptic women suggest that Hcy in patients group was not actively catabolized.

5. 2. B-vitamins

5. 2. 1. Folate

Serum folate concentration in normotensive women decreased significantly with increasing gestation with significant lower values in women in late pregnancy compared to those at earlier stages of gestation (table 4. 11). This decrease in folate concentrations may be explained, as others have suggested, by the accelerated breakdown of this vitamin because of its participation in cellular biosynthesis (Higgins et al., 2000; McPartlin et al., 1993), or by the pregnancy-related hemodilution (koebnick et al., 2001; Hall et al., 1976). Other studies showed that concentration of folate decreased from the fifth month of pregnancy onwards, and continue to decrease even at the post-partum stage (Lopez-Quesada et al., 2003; Ackurt et al., 1995; Bates et al., 1986). The highest catabolism rate of folate was seen in the third trimester where the maximal increase in fetal mass is occurred (Higgins et al., 2000). In contrast to normotensive pregnant women, serum folate concentrations in preeclamptic women did not decrease significantly during pregnancy (table 4. 11). However, the hemoconcentration associated to preeclampsia may hide such a decrease in serum folate causing, consequently, unrecognised folate deficiency (Koebnick et al., 2001).

Folic acid-supplemented women had significantly higher folate concentrations than un-supplemented women in both groups (controls: 15.8 vs. 8.8, p = 0.048; patients: 8.5 vs. 5.7, P < 0.001). Additionally, other studies reported that maternal folate concentrations is mainly dependent on folate intake, and higher folate intake associated with higher serum folate concentrations (Ellison et al., 2004; walker et al., 1999; Bronstrup et al., 1998 a; Scholl et al., 1996). Therefore, the lower folate concentration seen in preeclamptic women compared to controls is most probably because of increased folate requirements associated with lower folate intake. The present results showed that asymptomatic women took vitamin supplementation more frequently than preeclamptic women did (91 % of controls compared with 63 % of patients took supplementation, table 4. 1). More that, the duration of vitamin supplementation in normotensive women was significantly longer than that of patients (median duration of vitamin supplementation: controls = 19 wk; Patients = 8 wk, P < 0.001; figure 4. 5). Additionally, the consumption of folate, which acts as antioxidant by scavengering free radicals, as a response to increased oxidative stress in preeclmpsia increases the folate requirement and thus causes lower folate levels (Moat et al., 2006; Joshi et al., 2001).

Serum folate concentrations were significantly and inversely associated with Hcy in both groups (controls: r = - 0.41, p < 0.001; patients: r = - 0.32, p < 0.001). Lack or low level of folate inhibits the remethylation of Hcy into Meth resulting in elevated Hcy levels (Finkelstein JD., 1998). Thus, the higher tHcy levels in preeclamptic women may be partly explained by the significant lower levels of folate in patients compared to controls. Folic acid supplementation during pregnancy enhances RBC and serum folate status and the reduction in tHcy concentration (Holmes et al., 2005; Murphy et al., 2004; 2002). The present results do however confirm the reduction in Hcy levels due to folic acid supplementation (figure 4. 3). These findings are of great importance because reducing Hcy levels by folic acid supplementation is a safe and cheap policy and may reduce maternal complications associated to elevated tHcy levels (Hernandez-Diaz et al., 2002). Recently, folic acid supplementation was found to reduce the incidence of gestational hypertension (Hernandez-Diaz et al., 2002) and preeclampsia (sanchez et al., 2001).

Despite that supplemented patients had serum folate concentrations similar to un-supplemented controls (8.5 vs. 8.8 ng/ml), Hcy elevation was more pronounced in supplemented patients (10.0 vs. 8.3 µmol/L). This refers to that Hcy elevation seen in preeclamptic women is not entirely explained by lower folate status but other factors like renal function, combined low micronutrient status may be involved. Thus folic acid

supplementation alone was probably not sufficient to prevent Hcy elevation in women who developed preeclampsia. It is well established that folate and vit B12 may impact Hcy remethylation in an interactive manner (Obeid et al., 2002). Available data indicate that normal-to high-normal concentrations of serum folate are frequent in vit B12-deficient subjects (Herrmann et al., 2003 b) and unless both micronutrients are available, the accumulation of Hcy can not be prevented (Tefferi and Pruthi, 1994).

5. 2. 2. Vit B12

Serum vit B 12 concentrations gradually decreased throughout pregnancy. Decreased serum vit B12 concentrations was comparable in control and patient groups. In control, vit B12 concentration decreased from a value which was shortly above the cut-off value of vit B12 deficiency, reaching deficient concentrations in the third trimester, whereas vit B12 concentration in preeclamptic women was pathologically lowered at earlier stage. Several studies reported decreased vit B12 concentrations with increasing gestation, and that about 20-30 % of uncomplicated pregnancy associated with lower than normal serum vit B12 concentration (Chery et al., 2002; Koebnick et al., 2002; Cikot et al., 2001, Ball and Giles, 1964). The decreased vit B12 concentration throughout pregnancy was attributed to the active transport across the placenta (Monsen et al., 2001; Baker et al., 1958), changes in capacity and saturation of vit B12-binding proteins (Koebnick et al., 2002), and hemodilution (koebnick et al., 2001).

Several authors suggested that decreased serum vit B12 concentrations during normal pregnancy does not necessarily indicate a vit B12 deficiency (Koebnick et al., 2002). The present results, however, argue against this suggestion. Along with decreasing serum vit B12, serum MMA concentrations displayed a significant increase (table 4. 11), confirming a gradual decline in the intracellular vit B12 concentrations. Elevated serum MMA concentrations were found in 57.6 % of the normotensive pregnant women, whereas 45.0 % had serum vit B12 < 211 ng/ml. Additionally, 22.0 % of normal pregnant women had elevated MMA levels despite normal serum vit B12 concentrations. Thus, in subjects with normal renal function, concentration of MMA is more sensitive in diagnosing an intracellular vit B12 deficiency than the measurement of serum vit B12 concentration. Nevertheless, backward regression analysis showed that MMA level is significantly dependent on serum vit B12, but it is not specific parameter of intracellular vit B12 deficiency, since other factors had a significant influence on MMA levels (table 4. 10).

Concentrations of MMA in our subjects was much higher than values reported from American (Adams et al., 1995) or European pregnant women (McMullin et al., 2001; Monsen et al., 2001). Similar metabolic abnormalities have been reported in pregnant women from other populations of poor socio-economic status (Guerra-Shinohara et al., 2004; Bondevik et al., 2001). Pregnant women are at increased risk of developing subclinical vit B12 deficiency, particularly when pregnancy is associated with inadequate dietary intake of vit B12 (Chery et al., 2002). Serum MMA levels significantly increased in normotensive women with increasing pregnancy, and abnormal levels of serum MMA were seen in preeclamptic women at earlier stage of pregnancy (table 4. 11). These indicate a marginal preconception vit B12 status associated with inadequate supply (Monsen et al., 2001). Serum MMA concentrations in preeclamptic women was influenced by several factors (table 4. 10), and these factors should be considered during estimation the vit B12 status depending on MMA levels. Firstly, backward regression analysis showed that creatinine independently and significantly influenced MMA concentrations. According to Rasmussen, MMA is eliminated by the glomerular filtration and passive reabsorption by the tubules, and conditions of renal insufficiency cause higher concentrations of MMA independent of vit B12 status (Rasmussen et al., 1990; 1989). Therefore, one may argue that glomerular endothelial cells damage characteristic of the kidney in preeclamptic women may cause higher concentrations of MMA independent of vit B12 status. But concentrations of MMA did not differ significantly between the preeclamptic and the control women at any time of gestation (table 4. 11). Therefore, it is more probable that MMA elevation is due to inadequate vit B12 status. Additionally, MMA correlated positively with Cys in preeclamptic women. Secondly, the condition of hemoconcentration or hypovolemia, like that seen in preeclampsia and thyroid disease are another possible reasons for light to mild elevation in MMA concentrations (Norman EJ., 1998). Unfortunately, in this regard the current study is limited. The GFR and maternal haematocrit values were not measured. Additionally, the interview-based questionnaires can not confirm the presence of any of diseases known to influence MMA levels. However, the low cobalamin status in the pregnant women of the current study is not unexpected because these women were taken from a population where vit B12 deficiency is endemic (Herrmann et al., 2003).

5. 2. 3. Vit B6

Normal pregnancy is associated with decreased maternal concentration of vit B6, particularly in the third trimester (Cleary et al., 1975; Shane and Contractor, 1975; Hamfelt and Tuvemo,

1972). According to Cikot et al. (2001) pregnancy induces a continuous decrease in pyridoxal phosphate concentration (the physiologically active vit B6), reaching about 23 % at the end of pregnancy. In the present study plasma vit B6 deficiency was found in a high frequency in both groups (85 % of controls and 89.8 % of preeclamptic women had plasma vit B6 < 4.3 ng/ml). Decreased formation of pyridoxal-5′-phosphate in the liver or/and increased serum phosphatase activity, especially placental isoenzyme, contribute to the decreased plasma vit B6 during pregnancy (Barnard et al., 1987; Anderson et al., 1980). Additionally, the correlation analysis showed that vit B6 correlated significantly and inversely to gestation age (table 4. 5). A recent study showed that vit B6 was higher (6-fold) in the infants than in the maternal blood (Obeid et al., 2005). In order to maintain maternal plasma concentration within the normal range throughout pregnancy, American institute of medicine recommended a daily supplementation of 1.9 mg vit B6 during pregnancy, which is higher than the recommended dosage for nonpregnant women by 0.6 mg (Institute of Medicine, USA, 1999). In this context, Chang SJ. (1999) found that in healthy pregnant women a daily supplement of 2 mg pyridoxine hydrochloride provides the adequacy of maternal and neonatal vitamin B6 status and the satisfactory growth of neonates at birth.

Plasma vit B6 concentrations were significantly lower in preeclamptic women compared to controls (2.0 vs. 2.4 ng/ml, P = 0.001). This result is in accordance with the results obtained by others. Brophy and Siiteri, (1975) found that pyridoxal phosphate concentrations in peripheral and cord blood obtained at the time of delivery were significantly lower in preeclamptic women compared to controls. Of note, the reported concentrations of PLP in their study were higher than the concentrations found in the present study, suggesting that Syrian pregnant women may have lower vit B6 status before pregnancy. Limit evidence is available regarding the role of vit B6 in preeclampsia (Vasdev et al., 1999; Brophy and Siiteri, 1975). The administration of vit B6 during pregnancy has been reported to be beneficial in decreasing the incidence of preeclampsia. Wachstein and Graffeo, (1956) found that a daily supplementation of a normal diet with 10 mg of pyridoxine hydrochloride during pregnancy caused significant decrease in the incidence of preeclmpsia (from 4-fold to 1.4-fold). Hillman et al. (1963) found however that the single supplementation with vit B6 had no influence on the incidence of preeclampsia.

Serum vit B6 correlated inversely and significantly with Hcy only in patients group, indicating that decreased vit B6 concentrations is another possible reason, or participate together with other reasons, for tHcy elevation in preeclamptic women (Miller et al., 1992). Low vit B6 concentration impairs the production of the methyl group necessary for Hcy

remethylation by inhibiting the serin-hydroxymethyl-transferase enzyme in the folate cycle (Martinez et al., 2000), leading to a disturbed remethylation of Hcy and increased its serum concentrations.

In case of vit B6 deficiency the degradation of Cys is inhibited more effectively than its synthesis resulting in Cys trap (Martinez et al., 2000; Ubbink et al., 1996). Plasma vit B6 correlated significantly with Cys in both groups (table 4. 5). Despite that the median values of vit B6 in both groups were so far below the value which is commonly used as a primary indicator of PLP inadequacy, 20 nmol/L, Cys levels were significantly lower in normotensive women than in preeclamptic women (table 4. 3). This indicates sensitivity for vit B6 deficiency in preeclamptic women which can be explained by the activation of transsulfuration pathway due to increased oxidative stress (Vitvitsky et al., 2003). The activation of transsulfuration pathway is an autocorrective response that leads to maintain or even to increase the intracellular glutathion pool in cells challenged by oxidative stress. The regression analysis showed that Cys concentrations were significantly and independently modulated by Hcy, creatinine, vit B6 and BMI in preeclamptic women, and by Hcy , vit B12, and vit B6 in normotensive women (table 4. 10). This data indicates that Cys level is not specific indicator for vit B6 deficiency, and in case of normal renal function, Cys is an indicator for B-vitamin deficiency in general. The absence of the relation between renal function and Cys in normotensive women confirms the importance of adequate renal function in controlling Cys levels in pregnancy.

5. 3. Renal function and complicated pregnancy

Serum creatinine concentrations were significantly higher in preeclamptic women compared to controls (0.71 vs. 0.57 mg/dl, $p < 0.001$), indicating a reduced glomerular filtration rate in patients group. It was reported that in pregnancy complicated with preeclampsia glomerular filtration rate (GFR) and renal plasma flow (RPF) decrease by 30 % to 40 % compared with normal pregnancy (Moran et al., 2003). This change in GFR was attributed to the abnormal glomerular morphology “endotheliosis” characteristic of preeclampsia (Robert JM., 1999).

Kidney is provided with the whole necessary Hcy metabolising enzymes. According to Guttormsen et al. the renal uptake and metabolism of Hcy could account for approximately 70 % of the daily Hcy elimination. Therefore, Hcy concentration is influenced by the renal clearance (Arnadottir et al., 1996).

In the current study serum creatinine correlated highly significantly with Hcy (table 4. 6). This correlation confirms the role of the kidney as an important organ for Hcy metabolism.

Additionally, creatinine and Hcy are metabolically linked. The synthesis of creatinine from creatine is associated with simultaneous Hcy production (Stead et al., 2001; Mudd and Poole, 1975). However, according to the current results the correlation of creatinine to Hcy seems to be due to the role of creatinine as a marker of GFR, and not due to its link to Hcy production. This is because creatinine lost its predictive value in normotensive women who had intact renal function and GFR, whereas it was one of the strongest predictors of fasting tHcy levels in preeclamptic women (table 4. 10).

Increased serum concentrations of uric acid is usually used as a clinical marker in diagnosing preeclampsia (Yoneyama et al., 2002; Many et al., 1996; Hickman et al., 1982), and this elevation is correlated with the severity of disease (Pipkin and Roberts, 2000). Likewise, in this study serum uric acid levels were significantly higher in eclamptic women as compared to preeclamptic women (7.3 vs. 6.5 mg/dl, respectively, $P < 0.001$, data not shown). In normal situation uric acid is considered as a potent anti-oxidant. In the case of the depletion of other antioxidants, like in preeclampsia, it impairs the endothelial function by paradoxically acting as a pro-oxidant (Santos et al., 1999). Therefore, it is considered not only a marker of renal function but it is a risk factor for the progression of the disease, and recently was correlated with several prenatal complications (Yassaee F., 2003).

Uric acid is freely filtered by the glomeruli with reabsorption in the proximal tubule. Increased levels of uric acid found in preeclampsia are due to an increase in proximal tubular reabsorption and a decrease of tubular secretion associated with decreased GFR (Conrad and Lindheimer, 1999). Foreman et al. suggested that the removal of Hcy in the normal kidney takes place in the proximal tubular cells (foreman et al., 1982). In this study uric acid correlated significantly to tHcy levels suggesting that the altered tubular function participates in the elevation of tHcy found in preeclamptic women. Nevertheless, the recent study of Yoneyama et al. suggested increased uric acid production due to increased activity of plasma 5`-nucleotidase enzyme in preeclampsia (Yoneyama et al., 2002). Therefore, one may argue that preeclamptic women in the current study have intact renal function and the significant elevation in tHcy concentrations in preeclampsia group is not explained by the impaired renal function. However, uric acid correlated highly significantly with creatinine in all groups and both were significantly higher in patients compared to healthy pregnant women (table 4. 2).

In conclusion, mild renal dysfunction was an important determinant of tHcy, indicated by the positive correlation between creatinine, uric acid and tHcy. Therefore we can not exclude the possibility that preeclampsia related renal dysfunction accounts for Hcy elevations noted among cases versus controls.

5. 4. Effects of the interaction between MTHFR polymorphism, folate, and vit B12 on Hcy levels

In this study the frequency of the T677 allele was 31.1 % which is comparable to the frequency reported in western population (Schneider et al., 1998). Lower tHcy levels seen in controls compared to preeclamptic women can not be attributed to a lower MTHFR T allele frequency. This because the frequency of the mutant allele did not differ significantly between controls and preeclamptic women (table 4. 7). Additionally, lower tHcy levels were even found in normal pregnant women comparing each MTHFR genotype independently (table 4. 8).

MTHFR enzyme catalyzes the conversion of CH_2-THF to CH_3-THF. The homozygous MTHFR TT genotype reduces MTHFR activity resulting in lower CH_3-THF, the only methyl donor in the remethylation of Hcy into Meth, and higher tHcy levels. In the present study (table 4. 9), pregnant women with MTHFR TT genotype had significantly higher serum tHcy and lower folate levels than those with MTHFR CC genotype only when their serum folate levels were ≤ 8.9 ng/ml (this value represents the median folate in total population), and these differences disappeared when their folate concentrations were above the median, indicating that the influence of MTHFR TT genotype on tHcy and folate levels was modified by serum folate status. These results are in agreement with other studies (Bailey and Georg, 1999; Brattstrom et al., 1998), and gives a pattern of gene-nutrient interaction that influences tHcy levels in this population of Syrian pregnant women (Kim et al., 2004). One explanation for these observations is that higher folate status increases the stability of the mutated MTHFR enzyme, thus making its activity comparable to that of CC or CT, i.e., folate directly affects the mutated MTHFR enzyme (Jacques et al., 1996). Another explanation is that folate protects mutant enzyme against flavine adenine dinucleotide (FAD) loss, and consequently against thermal inactivation, i.e., indirect effect of folate (Hustad et al., 2000; Guenther et al., 1999).

In addition to folate, a secondary gene-nutrient interaction between C677T-MTHFR and vit B12 has been postulated (Lucock et al., 2001). In the current study, a higher risk of HHcy was found in vit B12-deficient subjects with TT genotype compared with CC subjects who had higher vit B12 levels. The risk of HHcy in pregnant women with TT genotype increased to 5.89 when TT pregnant women were within the highest quartile of MMA (table 4. 12). Nevertheless, the effect of the interaction between MTHFR and vit B12 did not reach the magnitude of the effect of the interaction between folate and MTHFR (table 4. 12). One explanation for this observation could be, as suggested by Herrmann et al. (2003 a), that vit

B12 is required in TT individuals for the reactivation of CH_3-THF pool rather than for directly affecting the mutated MTHFR enzyme.

In addition to folate and vit B12, vit B2 is involved in the folate cycle as a cofactor required for the maximal catalytic activity of the MTHFR enzyme. High serum levels of vit B2 was found to attenuate HHcy due to MTHFR TT genotype. Additionally, animal studies showed a reduction in the activity of MTHFR and decreased the availability of 5-CH_3-THF in the liver of vit B2-deficient rats (Bates and Fuller, 1986 a). Therefore, the measurement of vit B2, in combination with folate and vit B12, should be considered in the analysis of the influence of MTHFR genotype on tHcy concentration. Unfortunately, the present study is limited in this point where no measurements of serum vit B2 are available.

In conclusion , the present study showed that tHcy concentrations did not differ significantly with the MTHFR genotype, and the influence of TT on tHcy levels was modulated by folate and vit B12 status as TT subjects with low folate and vit B12 status had increased risk of HHcy.

5. 5. MTHFR polymorphism, folate, vit B12, and the risk of preeclampsia

It is hypothesised that MTHFR 677 C→T is a potential risk factor for preeclampsia (Online Mendelian Inheritance, OMIM). Table 4. 13 showed that MTHFR genotype was not associated with the risk of preeclalampsia, which argues against the usefulness of maternal MTHFR polymorphism in predicting the risk of preeclampsia among pregnant women of the present study. Several investigators have found an association between MTHFR 677 C→T and the risk of preeclampsia (Grandone et al., 1997; Sohda et al., 1997), whereas others did not (Yilmaz et al., 2004; Prasmusinto et al., 2002; Zusterzeel et al., 2000; Powers et al., 1999). In contrast to MTHFR 677 C→T polymorphism, increased risk of preeclampsia was associated with increased levels of tHcy. Women with tHcy levels above 7.8 µmol/L were 21.6 times more likely to have preeclampsia compared with women whose tHcy levels were lower than 5.2 µmol/L (table 4. 13). Additionally, maternal folate concentrations had a significant role in preeclampsia risk. The calculated odds ratio (OR) for preeclampsia risk for different quartiles of folate concentrations showed a higher risk of preeclampsia at lower folate concentrations, with odds ratios ranging from 1.1 in the third quartile to 9.9 in the lowest quartile. Furthermore, we found that the risk of developing preeclampsia in women with CC genotype increased from 1 to 4.8 in the presence of low folate, while the risk increased in women with TT genotype only from 0.8 to 1.5 with low folate (table 4. 13). This

adds further evidence that the risk of preeclampsia was not associated with the MTHFR genotype. Higher serum MMA levels, however, were not associated with an elevated risk of preeclampsia, which is in consistent with previous studies (Sanchez et al., 2001; Rajkovic et al., 1997). Several studies found no association between maternal serum folate and the risk of preeclampsia (Powers et al., 1998; Rajkovic et al., 1997). The OR associated to decreased maternal folate concentration is lower than that associated with higher tHcy levels. This observation may be explained by the role of folate as antioxidant and its inverse relationship to Hcy (Selhub et al., 1993), Whereas HHcy is known to promote endothelial dysfunction, thereby increasing the risk of preeclampsia (Robert and cooper, 2001; Roberts et al., 1999). Due to the retrospective design of this study it was not possible to determine whether these differences in maternal tHcy and folate levels are causal for preeclampsia or caused by preeclampsia. However, a prospective study by Sorensen et al. demonstrated that Hcy-elevation precedes preeclampsia by approximately 8-16 weeks (Sorensen et al., 1999).
In conclusion, low maternal folate concentration and high Hcy levels were associated with an increased risk of preeclampsia. Results from the present study and few other (Sanchez et al., 2001; Rajkovic et al., 2000; Ray and Laskin, 1999) suggest that folic acid and other B vitamins may be important in the pathogenesis of preeclampsia.

5. 6. Limitations and strengths of the study

The results obtained from this study must be interpreted with some caution due to several limitations. First, the patients and controls were not matched for the gestation age which was later in the control group than in patients group. Changes in maternal tHcy levels according to the gestation age were reported in several studies (Holmes et al., 2005; Murphy et al., 2004; Walker et al., 1999). However gestation age correlated significantly with tHcy in controls but not in patients group. Therefore, the higher gestation age in patients compared to controls (37 vs. 35 wk; $P < 0.05$) can not explain the elevated tHcy levels found in patients. Because the blood pressure of the pregnant women was not the outcome of interest in the present study, the individual values of blood pressure in the normal pregnant women were missed (table 4. 1). However, the controls selection was based on the available data registered by the resident doctors, which insured the normal blood pressure of the selected women.

One strength of the current study is that subjects were homogenous group of Syrian women. of similar educational background and socio-economic status. Therefore, ethnicity as a possible confounder for preeclampsia is excluded in this study (Eskenazi et al., 1991).

Moreover, by use of the questionnaire, important information about diet and lifestyle factors such as smoking, coffee or/and alcohol consumption, and exercises could be obtained. These factors are known to influence the biochemical factors (Nurk et al., 2004; de Bree et al., 2001). It was reported that B-vitamins status in women varied significantly depending on the season in which blood was sampled (Jiang et al., 2005; Ronnenberg et al., 2000). These variations were attributed to the seasonal variations in the availability of B-vitamins-rich foods. In order to avoid this variation in B-vitamins status, the blood samples of normotensive women were collected in parallel to the blood samples of the preeclamptic women. Other important strength is that blood samples were collected from women who had fasted for at least 12 hours. This point of importance because dietary factors may affect circulating tHcy levels (Ueland et al., 1993).

The current study and few others (Wannous and Arous, 2001; Bakour et al., 1998) included socioeconomically disadvantaged Syrian women admitted to cost-free hospitals operated by the Syrian government. These women were also of low education level and were not likely to visit antenatal care services at early pregnancy. Therefore, these data might probably not reflect the nation wide situation. However, folate, vit B12, and vit B6 intakes should be increased in women of childbearing age from this population.

Taken together, the present study refers to a high incidence of HHcy in Syrian preeclamptic women. HHcy was closely related to a poor nutritional status (folate, vit B12, vit B6). The limited effect of folate supplementation on serum concentrations of Hcy was partly related to a short duration of usage. Folate effect on Hcy level was also counterbalanced by a low status of vit B12 and B6. Further studies should clarify the impact of combined vitamin supplementation on some pregnancy complications and outcome, including preeclampsia, pre-term deliveries and low birth weight. Finally, the effect of poor maternal nutritional status on some health aspects of the newborns needs further investigations.

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7. ACKNOWLEDGEMENT

I would like to acknowledge many people who have directly or indirectly contributed to development of the work in this thesis

I would like to thank Prof. Dr. med. habil. Dr. rer. nat. W. Herrmann for generous hospitality in Germany, his support and patience. His critical questions and suggestions have always inspired me to think about this topic at a deeper level.

I am deeply indebted to Dr. M. Herrmann who offered me so much advice and patiently supervising me.

I would like also to express my appreciation to Prof. Dr. F. Sitzmann for his invaluable support and kindly help, and for Prof. Dr. M. Jouma who offered me his time and participated in the study design.

I would like to give my heartfelt thanks to Prof. Dr. J. Geisel for his help and support.

A sincere thank goes to Dr. U. Hübner. His unselfish help has helped me greatly in overcoming many obstacles I encountered (thank you Dr. Hübner).

I am very grateful for Mrs. M. Bodis, Prof. Dr. R. Obeid, Mrs. Dr. H. Schorr, and Mr. JP. Knapp for their technical help.

I would like also to thank Mrs. M. Druck, Mrs. M. Gareiss, Mrs. M. Sand-Hill, and Mr. R. Schnell for their generous help and patient.

I would like also to thank the whole staff of the maternal and obstetrical hospital (Damascus) for their help, in particular Prof. Dr. M. N. Yasmina, Prof. Dr. M. Al-Tabah, Prof. Dr. A. Abd Al-Salam, and Dr. M. Hanhoun, and so I would like to give my thanks to all the subjects who participated in this study.

I am deeply thank my family for their love, unconditional support and encouragement. They had confidence in me when I doubted myself.

Homburg, June 19, 2006

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PUPLICATIONS LIST

Herrmann W, Isber S, Obeid R, Herrmann M, Jouma M (2005) Concentrations of homocysteine, related metabolites and asymmetric dimethylarginine in preeclamptic women with poor nutritional status. Clin Chem Lab Med 43 (10): 1139-1146

Geisel J, Schorr H, Bodis M, Isber S, Hubner U, Knapp JP, Obeid R, Herrmann W (2005) The vegetarian lifestyle and DNA methylation. Clin Chem Lab Med 43 (10): 1164-1169.