DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS
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HOMOCYSTEINE AND HYPERTENSION: Associations between homocysteine and essential hypertension in treated and untreated hypertensive patients with and without coronary artery disease

PIIBE MUDA

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals (I–IV):

I Muda P, Kampus P, Zilmer K, Soopõld Ü, Tikk S, Õim M, Kalder M, Zilmer M, Teesalu R. Homotsüsteiin ägeda koronaarse sündroomiga patsientidel. Eesti Arst 2002; 12: 776–779.

II Muda P, Kampus P, Zilmer M, Zilmer K, Kairane C, Ristimäe T, Fischer K, Teesalu R. Homocysteine and red blood cell glutathione as indices for middle-aged untreated essential hypertension patients. J Hypertens 2003; 21: 2329–2333.

III Muda P, Kampus P, Zilmer M, Ristimäe T, Fischer K, Zilmer K, Kairane C, Teesalu R. Effect of antihypertensive treatment with candesartan or amlodipine on glutathione and its redox status, homocysteine and vitamin concentrations in patients with essential hypertension. J Hypertens 2005; 23: 105–112.

IV Muda P, Kampus P, Teesalu R, Zilmer K, Ristimäe T, Fischer K, Zilmer M. Effect of candesartan and amlodipine on oxidized low-density lipoprotein level in patients with mild to moderate essential hypertension. J Hypertens (submitted for publication).

V Muda P, Zilmer M, Teesalu R. Hüperhomotsüsteineemia — uus haiguste riskifaktor. Eesti Arst 2001; 6: 303–309.

ABBREVIATIONS

ACS	Acute coronary syndrome
ACE	Angiotensin converting enzyme
AdoHcy	S-adenosyl-homocysteine
AdoMet	S-adenosyl-methionine
AT II	Angiotensin II
BMI	Body mass index
BP	Blood pressure
CBS	Cystathionine-beta-synthase
CD	Conjugated dienes
CAD	Coronary artery disease
CVD	Cardiovascular disease
CV	Cardiovascular
Cys	Cysteine
DBP	Diastolic blood pressure
EDTA	Ethylenediaminetetraacetic acid
eNOS	Endothelial nitric oxide synthase
GSH	Glutathione
GSSG	Oxidized glutathione
GPx	Glutathione peroxidase
Hcy	Homocysteine
HDL	High density lipoprotein
HtHcy	Hyperhomocysteinemia
LDL	Low density lipoprotein
LDL-BDC	Baseline diene conjugation in circulating low density lipoproteins
Met	Methionine
MI	Myocardial infarction
MTHFR	Methylenetetrahydrofolate reductase
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
NFκβ	Nuclear factor kappa-β
NO	Nitric oxide
OxLDL	Oxidized low-density lipoprotein
OxS	Oxidative stress
RBC	Red blood cell
RBC-GSH	Red blood cell reduced glutathione
RBC-GSSG	Red blood cell oxidized glutathione
RBC-TGSH	Red blood cell total amount of glutathione
ROS	Reactive oxygen species
SBP	Systolic blood pressure
TG	Triglycerides
VSMC	Vascular smooth muscle cell

1. INTRODUCTION

Hypertension is one of the principal and modifiable conventional risk factors for cardiovascular disease (CVD) (Kannel *et al*. 1986). Its etiology has not been fully elucidated mostly because of as yet unknown genetic variation and multiple nonhereditary factors that have important and modifiable influences on blood pressure (BP)(Carretero 2000).

Among the non-conventional risk factors homocysteine (Hcy) has shown clinical impact. There is evidence that hyperhomocysteinemia (HtHcy) is also an important independent risk factor for atherosclerosis and thrombotic disease (Boushey *et al.* 1995, Graham *et al.* 1997).

Hcy is an intermediate generated during the metabolism of methionine (Met), an essential sulfur containing amino acid. Vitamins B_6 and B_{12} and folic acid are important cofactors in Hcy metabolism (van der Griend *et al.* 2000). Deficiency of these vitamins is a frequent cause of HtHcy (Stanger *et al.* 2003).

The results from prospective studies concerning the association between plasma total Hcy concentration and acute coronary event are conflicting. Voutilainen *et al.* did not found association between Hcy level and an increased risk of the first coronary event (2000). On the contrary, there exist studies there the risk of the first stroke and myocardial infarction (MI) is increased directly with total Hcy level (Bots *et al.* 1999, Aronow and Ahn 2000). Nor is it clear at which Hcy level such association may be revealed.

Assessing the relationship between hypertension and Hcy, concomitant cardiovascular (CV) factors (e.g. obesity and blood lipids), level of cofactors important in Hcy metabolism and presence of excessive oxidative stress (OxS) should be taken into account. In determining the level of OxS, several markers should be measured (Dotan *et al.* 2004).

It has been demonstrated that in the presence of traditional CV risk factors Hcy may play a permissive role in the endothelial damage even within the traditionally used reference range (Schlaich *et al.* 2000, Konukoglu *et al.* 2003). These effects of Hcy seem to be related to increased OxS (Kanani *et al.* 1999, Virdis *et al*. 2001, Mujumdar *et al.* 2001). In an experimental study Hcy has been demonstrated to enhance low-density lipoprotein (LDL) oxidation (Pfanzagl *et al.* 2003).

Cellular reduced glutathione (GSH) has an important role in protection of endothelial cells from excess of free oxygen radicals, leading to prevention against endothelial dysfunction in the arteries exposed to profound OxS (Murphy *et al.* 1991, Meister 1994). The glutathione redox buffer modulates cell response to redox changes, while glutathione redox status appears crucial in maintaining cellular viability (Jefferies *et al.* 2003). An experimental study has shown that Hcy metabolism provides about half of glutathione (Mosharov *et al.* 2000). Several links have been reported recently between hypertension and glutathione (Nemeth *et al.* 2001, Turi *et al.* 2003).

According to available data, the possible relationships between the level of plasma Hcy and the level of red blood cell (RBC) GSH (RBC-GSH) have not been investigated in humans including hypertensive subjects. As the associations between Hcy and GSH have been assessed mainly in *in vitro* studies, it remains unclear whether they are also valid in the human body.

An elevation of Hcy or a decrease in cellular GSH are unfavourable side effects of drugs. Antihypertensive drugs are not similar regarding their effect on Hcy or GSH levels (Golik *et al.* 1995, Jacques *et al.* 2001). Data about the effect of antihypertensive drugs on Hcy level have been contradictory: there exist reports on absence of change or decrease in Hcy level as well as on increase in Hcy level (Westphal *et al*. 2003, Korkmaz *et al.* 2003).

Angiotensin II (AT II) type 1 (AT_1) receptor antagonists and calcium channel blockers are among the first choice drugs for treatment of essential hypertension (Cifkova *et al.* 2003). The AT_1 receptor antagonist candesartan and the calcium channel blocker amlodipine are effective in reducing BP in patients with essential hypertension (Kloner *et al.* 2001). Both drugs also possess some antioxidative properties (Koh *et al.* 2003, Mason 2002).

However, there remain several unanswered questions regarding the effect of antihypertensive drugs on plasma Hcy, on the main intracellular OxS marker GSH, on lipid peroxidation markers and on Hcy metabolism-related vitamins.

2. REVIEW OF THE LITERATURE

2.1. Homocysteine in the pathogenesis of the cardiovascular diseases

2.1.1. Homocysteine and its metabolism

Hcy, an essential sulfur-containing amino acid, is generated during metabolism of methionine (Met). It is present in four forms: about 1% circulates as a free thiol, 70–80% are disulfide-bound to plasma protein, chiefly albumin, and the remaining 20–30% combine with itself, to form the dimer homocystine, or with other thiols, including cysteine (Cys), with which it forms Hcy-Cys mixed disulfide. The term "total plasma Hcy" refers to the combined pool of all four forms of Hcy (Ueland 1995).

Metabolism of Hcy is shown in Figure 1. A principal stage in the metabolism of Met is formation of activated Met or S-adenosylmethionine (AdoMet). The latter is the main methyl group donor for a variety of compounds in over 100 methylation reactions catalysed by methyltransferases (Bolander-Gouaille 2002). This reaction takes place wherever methyl group is needed. Demethylation of AdoMet results in the formation of S-adenosylhomocysteine (AdoHcy), a potent inhibitor of methyltransferase. The AdoHcy is metabolised to adenosine and Hcy. Further metabolism of Hcy occurs through two pathways: remethylation back to Met and transsulfuration.

The Hcy can be remethylated to Met after receiving a methyl group from methyltetrahydrofolate or betaine. This remethylation requires the transfer of a methyl group from *N*-5-methyltetrahydrofolate (a coenzyme form of folic acid), catalysed by Met synthase, an enzyme that requires vitamin B_{12} as a cofactor (Figure 1). The remethylation cycle occurs in all tissues (Finkelstein and Martin, 2000). In the liver and kidney of rats, portions of Hcy could be remethylated by an alternative route in which betaine (derived from choline) serves as the methyl donor and is catalysed by betaine-Hcy methyltransferase.

In the transsulfuration pathway (Figure 1), Hcy is the substrate of the vitamin B_6-dependent cystathionine β-synthase (CBS) reaction (Selhub 1999). This enzyme condensates Hcy and serine to form cystathionine. This is a critical step in the pathway because it is irreversible under physiological conditions; from this point on, Hcy is committed to follow this pathway. In the last step of the transsulfuration pathway, cystathionine is cleaved by γ-cystathionase, another vitamin B_6-dependent enzyme to form Cys. Cys is used mainly to produce GSH (also taurine and eventually inorganic sulfates can be produced). Transsulfuration has a limited distribution, occurring primarily in the liver, kidney, small intestine and pancreas. These tissues contain CBS and γ-cystathionase and are involved in synthesis of GSH, a process that consumes a principal amount of Cys (Finkelstein and Martin 2000).

Availability of Met appears to be the principal determinant regulating the activity of transsulfuration and the remethylation pathways.

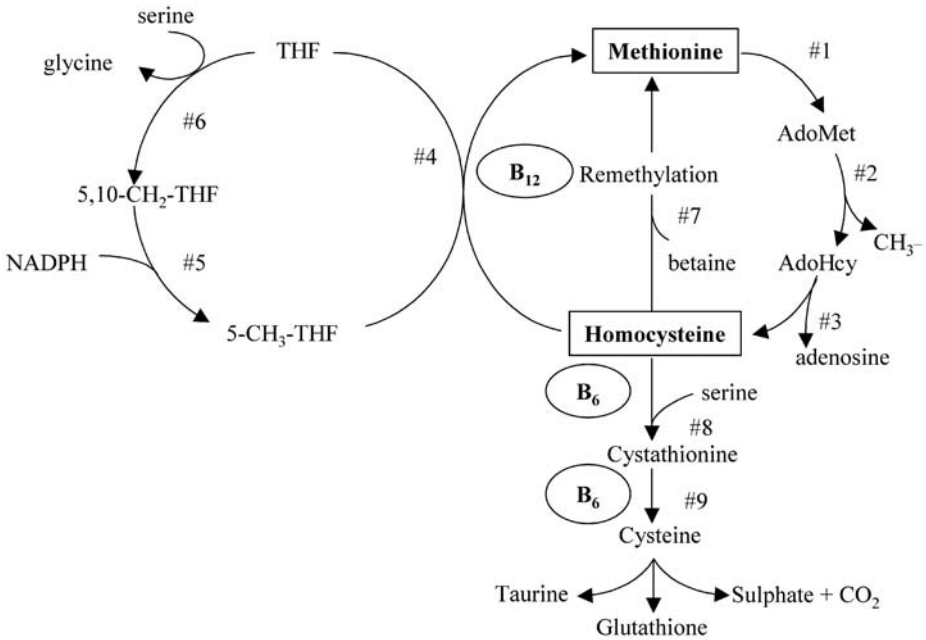

Figure 1. Metabolism of homocysteine.
Explanations are given in the text. AdoMet – S-adenosyl-methionine, AdoHcy – S-adenosyl-homocysteine, THF – tetrahydrofolate, 5, 10-CH_2-THF – 5, 10-methylene-tetrahydrofolate, 5-CH_3-THF – 5-methyl-tetrahydrofolate, NADPH – nicotinamide adenine dinucleotide phosphate, #1 – methionine-adonosine transferase, #2 – methyltransferase, #3 – adenosine- homocysteine hydrolase, #4 – methionine synthase, #5 – methylene-tetrahydro-folate reductase, #6 – serine-glycine-hydroxymethyl-transferase, #7 – betaine-homocysteine methyltransferase, #8 – cystathionine-β-synthase, #9 – γ-cystathionase.

The main regulatory control appears to be exerted at the level of Hcy: when Met is in deficit, Hcy is remethylated by Met synthase or betaine-Hcy methyl-transferase; when Met is in excess, metabolism of Hcy occurs via another pathway.

Hcy metabolism is closely associated with GSH metabolism. It has been postulated that under profound OxS, Hcy transsulfuration is favoured over remethylation, thereby increasing the supply of Cys for GSH synthesis (Finkel-stein and Martin 2000). Approximately half of the intracellular GSH pool in human liver cells is derived from Hcy via the transsulfuration pathway (Mosharov *et al.* 2000). Hence, transsulfuration provides a direct link between

Hcy and GSH, the major redox buffer in mammalian cells (Meister 1994, Mosharov *et al.* 2000). As the associations between Hcy and GSH have been assessed mainly in *in vitro* studies, it remains unclear whether they are also valid in the human body.

The Hcy may also induce intracellular suppression of the GSH antioxidant defence system, and possibly reduce Hcy-derived GSH synthesis (Mosharov *et al.* 2000). In *in vitro* studies, increasing Hcy concentrations decreased intracellular GSH concentrations in endothelial cells (Hultberg *et al.* 1997). The Hcy inhibits the expression of the antioxidant enzyme cellular glutathione peroxidase (GPx) *in vitro* and *in vivo* (Dayal *et al.* 2002), which can lead to an increase in reactive oxygen species (ROS) that inactivate nitric oxide (NO) and promote endothelial dysfunction. It is shown that Hcy induces human vascular smooth muscle cell (VSMC) proliferation and induces collagen expression in a dose- and time-dependent manner. This proliferation was reversed by the addition of the antioxidant N-acetylcysteine. Induction of the above mentioned collagen production was reversed by the addition of N-acetylcysteine and GSH (Tyagi 1998).

A study in rats has identified the kidney as a major site for removal and metabolism of Hcy. It seems that there occurs metabolic channelling, leading the Hcy removed from the blood by the kidney, to be metabolised primarily through the transsulfuration pathway (Medina *et al.* 2001). Renal impairment commonly causes HtHcy (Bostom and Lathrop, 1997). This may contribute to the high incidence of vascular complications in patients with chronic renal failure (Hankey and Eikelboom, 1999). Renal excretion does not seem to be an important route of Hcy elimination. Only about 1% of Hcy filtered by glomeruli is normally found in the urine. The rest is reabsorbed and metabolised. Thus, the kidneys are Hcy-metabolising rather than Hcy-excreting (Bolander-Gouaille 2002).

2.1.2. Measurement of homocysteine

2.1.2.1. Methods for measurement of homocysteine

Almost all determinations of Hcy in blood are performed on plasma instead of serum. Because of the continuous production of Hcy in the RBC, which is released to the extracellular compartment, faster centrifugation is required and sampling on a dry tube is avoided. Keeping samples cooled on ice until centrifugation also prevents increase in Hcy. After removal of the blood cells, Hcy in plasma or serum is stable (Refsum *et al.* 2004). The most widely used anticoagulant is ethylenediaminetetraacetic acid (EDTA). Several methods are available for the measurement of Hcy: gas chromatography–mass spectrometry with isotopic dilution, liquid chromatography with tandem mass spectrometry, high performance liquid chromatography with fluorescence or electrochemical detection, fluorescence polarization immunoassay, enzyme-linked immuno-

assay, capillary electrophoresis with laser-induced fluorescence and immunochemiluminescence method. Measurement of Hcy in plasma by an immunoassay appears to have become the preferred analytical approach.

Protein-bound Hcy is reduced to free Hcy and is enzymatically converted to AdoHcy by the action of AdoHcy hydrolase. All Hcy immunoassays share, to date, a single commercially available antibody (raised against the AdoHcy compound), and the AdoHcy hydrolase, licensed from Axis (Axis Biochemicals, Grünerløkka, Norway). This is an advantage for homogeneity of the results obtained with these different methods (Ducros *et al.* 2002).

2.1.2.2. Factors affecting homocysteine measurement

Food intake

A light meal (e.g. breakfast) does not influence plasma Hcy concentrations in healthy people (Ubbink *et al*. 1992, Guttormsen *et al.* 1994). Intake of a heavy, highly animal protein-rich meal may increase plasma Hcy level by 10–15% after 6–8 h (Guttormsen *et al.* 1994). Fasting status and the time from the last meal may influence the levels of Hcy, which should be considered in studies of Hcy as a risk factor for CV and other diseases (Nurk *et al.* 2001).

Posture

Blood samples collected in the supine position have up to 10% lower mean Hcy concentration than those collected in the sitting position (Rasmussen and Moller 2000). A possible explanation is that plasma albumin which binds Hcy, is reduced in the supine position (Refsum *et al.* 2004).

Diurnal and seasonal changes

Serum Hcy levels revealed a statistically significant circadian rhythm with peak values occurring during the evening and the lowest levels occurring during the morning (Bremner *et al.* 2000). A possible explanation of this diurnal variation is that a highly animal protein-rich meal may increase plasma Hcy by 10–15% after 6–8 h (Refsum *et al.* 2004). Two studies report the absence of significant seasonal variation in plasma Hcy level (Clarke *et al.* 1998, McKinley *et al.* 2001). Although there occurred low seasonal variation in folate status, there was no corresponding seasonal variation in plasma Hcy (McKinley *et al.* 2001).

Intraindividual variability

Intraindividual variability of Hcy is very low (Garg *et al.* 1997, Clarke *et al.* 1998). Garg *et al*. found that an individual's plasma Hcy concentration is relatively constant over at least 1 month, and a single measurement characterizes average concentration reasonably well (1997). On the other hand, epidemiological studies based on single Hcy measurements may underestimate the magnitude of any risk associations with disease by 10–15% (Clarke *et al.* 1998). Without appropriate correction, risk is underestimated by about one-fifth after 2 years and by one-half after 10 years (Clarke *et al.* 2001).

2.1.3. Hyperhomocysteinemia as a clinical problem

2.1.3.1. Definition of the hyperhomocysteinemia

Previously Kang *et al*. classified several types of HtHcy in relation to total plasma Hcy concentrations (1992). They defined HtHcy as severe for concentrations higher than 100 μmol/l, intermediate for concentrations between 30 and 100 μmol/l, and moderate for concentrations of 15–30 μmol/l, and the reference total plasma Hcy range as 5–15 μmol/l. In general, the reference intervals are calculated as the 2.5^{th}–97.5^{th} percentile interval (or 95% reference interval) for presumed healthy individuals. Reference intervals may be established for different populations to account for important differences, including those related to the non-modifiable factors such as age, gender or ethnicity, as well as the modifiable factors, such as permanent nutritional status, lifestyle and concomitant diseases. Hcy concentration increases throughout life and approximately doubles from childhood to old age. Men have approximately 2 μmol/l higher mean Hcy concentration than women. Plasma Hcy concentrations differ among ethnic groups but the effect of the upper reference limit is relatively small between the groups living in the same area and having a similar diet. In most adults who do not eat food fortified with folic acid, the upper reference limit is 15 μmol/l. In adults, aged 15–65 years, with a good vitamin status or a healthy lifestyle, the suggested upper reference limit for Hcy is 12 μmol/L (Refsum *et al.* 2004). Moderate HtHcy (plasma Hcy >12 μmol/l) is found in 5 to 10 percent of the general population and in up to 40 percent of patients with CVD (Stanger *et al.* 2003). It is difficult to specify reference ranges in the usual sense because plasma Hcy levels below 10 μmol/l are already associated with a graded increase in risk or manifestations of CVD (Nygard *et al*. 1997a, Boushey *et al.* 1995). Each μmol/l increment in plasma Hcy concentration is associated with a 6–7 percent risk increase (Bots *et al.* 1999). Starting at the plasma Hcy concentration of approximately 10 μmol/l, an associated risk increase follows a dose-response relationship without specific threshold level (Boushey *et al.* 1995, Nygard *et al.* 1997a, Bostom *et al.* 1999). However, differentiated prophylactic and therapeutic risk ranges for CVD can be defined for clinical practice. Plasma Hcy levels >12 μmol/l and <30 μmol/l are traditionally referred to as “moderate HtHcy”. As the synergistic interactions of Hcy in case of coexistence of additional CV risk factors produce an overproportional risk increase in total risk, in these subjects the suggested upper limit for Hcy is 10 μmol/l. The target populations at risk for CVD include those with smoking habit, arterial hypertension, hyperlipidemia, renal insufficiency, diabetes, metabolic syndrome and family history of CVD (Stanger *et al*. 2003).

2.1.3.2. Causes of hyperhomocysteinemia

Disturbances in intracellular Hcy metabolism lead in most cases to elevated Hcy concentrations. These disturbances may have inherited or acquired reasons (van der Griend *et al.* 2000).

Inherited causes of HtHcy

The most common inherited cause is a point mutation in methylenetetrahydrofolate reductase (MTHFR) gene, which is, remarkably, not always associated with significantly increased CV risk. The underlying genetic defect was identified as a C-to-T missence mutation at nucleotide 677, which substitutes valine for alanine (Kang *et al.* 1988). Homozygous mutants, with a prevalence of approximately 12% in Caucasians, have 50% residual enzyme activity and increased thermolability of this activity compared with the wild-type enzyme. Subjects with the homozygous mutant genotype have higher Hcy concentrations, especially with suboptimal folate intake. Approximately one percent of the general population are heterozygotes for mutations in the CBS gene. In enzymatic studies heterozygosity for CBS deficiency has been presumed to be the cause of HtHcy in premature vascular disease patients. A possible compensation for the genetic predisposition to HtHcy by adequate folate intake offers a rationale for therapeutic intervention (van der Griend *et al.* 2000). Most other genetic polymorphisms in the enzymes related to Hcy are very rare and have a low impact on Hcy concentrations (Lievers *et al.* 2003).

Acquired causes of HtHcy

Age and gender

Increasing age and the male gender are associated with higher Hcy level. Part of the relationship with age in women might be explained by menopause, since Hcy concentration was found to be higher in post-menopausal women compared with premenopausal women (De Bree *et al.* 2002).

Smoking.

Smoking is associated with increased Hcy level (Nygard *et al.* 1995).

Physical activity

Physical activity is probably not or weakly inversely associated with Hcy concentration. An intervention study showed that intensive exercise does not affect Hcy concentration. This can be explained by the fact that exercising is generally associated with a healthier lifestyle, and a healthier lifestyle with lower Hcy concentration (De Bree *et al.* 2002).

Nutritional factors

Deficiencies of the B-vitamins involved in Hcy metabolism, i.e., methylcobalamin (vitamin B_{12}), pyridoxal 5'-phosphate (vitamin B_6) and especially folate (as a *N*-5-methyltetrahydrofolate), lead to elevated Hcy concentrations. Particularly in the elderly, inadequate status of these nutritionally modifiable coenzymes in Hcy metabolism appears a major determinant of HtHcy (van der Griend *et al.* 2000). Coffee consumption is positively associated with Hcy concentration both in men and women in most observational studies. Caffeine might be a factor that elevates Hcy concentration as it may inhibit the conversion of Hcy to Cys by acting as a vitamin B_6 antagonist. In a large population-based survey conducted in Norway, Hcy concentrations increased with age (1 μmol/l per decade), with heavy coffee consumption (2 μmol/l when ≥9 cups per day), and with heavy smoking (up to 2 μmol/l in women smoking ≥20 cigarettes per day) (Nygard *et al.* 1995, Nygard *et al.* 1997b). Alcohol consumption is probably associated with Hcy concentration in a J-shaped fashion: moderate alcohol consumers have lower Hcy concentration compared with non-drinkers, whereas alcoholics have elevated Hcy concentrations (De Bree *et al.* 2002).

Kidney dysfunction

The most frequent clinical cause of HtHcy after nutritional deficiencies of folate and vitamin B_{12} is renal failure. Plasma levels of Hcy increase in even mild renal insufficiency (creatinine clearance <60 ml/min). The basis of HtHcy in renal failure is not completely clear, although several processes may explain the high correlation between kidney function and Hcy concentration; the kidney may influence or regulate Hcy metabolism in other tissues and it may convert a major amount of the Hcy present in blood. Decreased Hcy excretion in urine seems unlikely, since urinary Hcy clearance makes up only 0.3% of creatinine clearance (van der Griend *et al.* 2002). Renal reabsorption of Hcy in the tubular cells only occurs for the nonprotein-bound disulfide forms (about 30% of plasma Hcy concentration). The redox status of the tubular cells allows a reduction of the disulfides, which makes Hcy available for conversion via transsulfuration or remethylation pathway (De Bree *et al.* 2002).

Intestinal Diseases

Several gastrointestinal disorders may lead to a deficiency of folate or vitamin B_{12}, or both, which in turn will result in higher Hcy concentrations. The intestinal diseases associated with higher Hcy levels are ulcerative colitis, Crohn's disease, celiac disease, and inflammatory bowel disease. Treatment of patients with these diseases often involves gastrointestinal surgery, which may further elevate Hcy levels. In addition, bacterial overgrowth, pelvic and abdominal radiotherapy, and increased gastric pH may lead to diminished B-vitamin uptake (De Bree *et al.* 2002).

Endocrine Disorders
Insulin-dependent diabetes is accompanied by high Hcy concentrations only in advanced stages of the disease. In these stages, also creatinine levels are elevated and patients develop macroalbuminuria. Levels of Hcy were higher in hypothyroidism and lower in hyperthyroidism. This finding may be related to the influence of the thyroid function on metabolic turnover; however, other factors as B-vitamins status and kidney function may have been involved.

Rheumatoid Arthritis
HtHcy is commonly observed in rheumatoid arthritis but is not always necessarily dependent on methotrexate use. The origin of HtHcy in these patients is not clear, as a combination of drug use, vitamin deficiencies, MTHFR 677C>T genotype and gastrointestinal dysfunction, all may play some role here.

Proliferating Diseases
Diseases like cancer and psoriasis are associated with higher Hcy concentrations. These conditions are accompanied by rapidly dividing cells, which have a high demand for methyl groups to methylate vital cell components, including proteins. When Met donates a methyl group, Hcy is produced. Another process that may lead to higher Hcy concentrations in these diseases is that one-carbon units from tetrahydrofolate are preferentially used for synthesis of DNA and RNA, at the expense of Hcy remethylation (De Bree *et al.* 2002).

Drugs
Numerous drugs have an impact on Hcy metabolism, especially when acting as direct or indirect antagonists of vitamin cofactors and enzyme activities, but also as a consequence of disulfide exchange reactions, impairment of absorption and enzyme induction (Stanger *et al.* 2003). Association between use of antihypertensive drugs and Hcy will be discussed in a separate chapter.

2.1.3.3. Deleterious effects of the hyperhomocysteinemia

Effects on the endothelium
Damage to the endothelium is considered to be a principal aspect of the atherosclerotic process, which precedes overt manifestation of the disease (Ross, 1993). Experimental data have shown that HtHcy may induce endothelial dysfunction via high-grade OxS (Virdis *et al.* 2001, Kanani *et al.* 1999). One of the deleterious mechanisms of endothelial damage over HtHcy is increased ROS production. It was shown on a model of cultured porcine aortic endothelial cells that Hcy induced increase in endothelial cell superoxide anion levels, which was completely inhibited by the concomitant incubation with vitamin C. Thus, the inhibitory effect of Hcy on endothelium-dependent relaxation is due to increase in the endothelial cell intracellular levels of the superoxide anion

and provides a possible mechanism for endothelial dysfunction associated with HtHcy (Lang *et al*. 2000).

In humans, HtHcy impairs endothelium-dependent vasodilatation in the brachial artery as well as in the forearm microcirculation of normotensive subjects (Tawakol *et al*. 1997, Woo *et al.* 1997, Bellamy *et al*. 1998, Chambers *et al.* 1999, Kanani *et al.* 1999, Chao *et al.* 2000), an effect prevented by administration of the antioxidant vitamin C (Chambers *et al.* 1999, Kanani *et al.* 1999). This suggests that the endothelial dysfunction induced by HtHcy involves OxS. Abnormal vasomotor response is believed to be an early step in the formation of atherosclerotic lesions (Lang *et al.* 2000).

Hcy-induced vascular OxS may be further aggravated by Hcy-mediated specific decrease in the expression of the cellular isoform of GPx, as shown *in vitro* and *in vivo* (Rodrigo *et al.* 2003). Endothelial GPx reduces both hydrogen and lipid peroxides to their corresponding alcohols. It also prevents oxidative inactivation of NO. Hcy reduces significantly the activity of GPx with simultaneous decrease in GPx mRNA level. The Hcy is the only thiol that inhibits GPx activity *in vitro* (Upchurch *et al.* 1997).

Voutilainen *et al.* tested the hypothesis that high plasma Hcy in men is associated with increased *in vivo* systemic lipid peroxidation. They measured plasma F2-isoprostanes and found that elevated fasting plasma Hcy is associated with enhanced systemic lipid peroxidation (1999).

Elevated plasma Hcy may pose high-grade OxS leading to development of vascular damage. A component of this effect may be disturbance of the extracellular redox state. In patients with severe HtHcy, both plasma Cys and plasma total antioxidant capacity were inversely correlated with Hcy. Thus HtHcy may pose OxS not only through the direct cytotoxicity of Hcy but also through associated decrease in plasma Cys (Moat *et al.* 2001).

In healthy individuals the relationship between Hcy and OxS is not clear. In this case lowering of plasma Hcy through folate supplementation was not associated with any significant change in the measures of antioxidant activity (plasma and RBC GPx and RBC superoxide dismutase activity) or oxidant damage (plasma malondialdehyde), although an improvement in plasma total antioxidant capacity just failed to reach significance (Moat *et al.* 2003).

In normal conditions, NO combines with Hcy to form S-nitroso-Hcy, a potent vasodilator and platelet inhibitor. However, this protective action of NO is undermined by long-term exposure to HtHcy, which induces NO consumption and thus leads to unopposed Hcy-mediated oxidative damage (Loscalzo 1996). Moreover, Hcy selectively impairs the capacity of endothelial cells to detoxify ROS, thus rendering NO more susceptible to oxidative inactivation (Upchurch *et al.* 1997). Recently, it was found that Hcy-dependent lipid peroxidation is independent of H_2O_2 and alterations in GPx activity, but dependent on superoxide. Mechanistically, the pro-oxidant effect of Hcy appears to involve endothelial NO synthase (eNOS), as it is blocked by eNOS inhibitor l-N(G)-nitroarginine methyl ester. Thus, Hcy promotes development

of high-grade OxS in endothelial cells via an eNOS-dependent mechanism (Heydrick *et al*. 2004).

HtHcy may stimulate formation of asymmetric dimethylarginine, an endogenous inhibitor of eNOS. Bogaty *et al*. demonstrated that acute elevation of Hcy concentration by Met load in healthy subjects impaired vascular endothelial function by a mechanism in which an elevated concentration of asymmetric dimethylarginine may be involved (2001).

The endothelium also synthesises clotting factors. The balance between procoagulant and anticoagulant mechanisms is vital to the maintenance of vascular hemostasis. Hcy may upset this balance and predispose to thrombogenesis via a number of mechanisms. Hcy stimulates the procoagulant factor V, increases the activity of tissue factor, reduces anticoagulant mechanisms like antithrombin III and activated protein C, and impairs fibrinolysis (Thambyrajah and Townend, 2000).

Effects on smooth muscle cells and on the elastic properties of the vascular wall

The patophysiological mechanisms of HtHcy-induced hypertension also encompass the stimulation of VSMC proliferation and the alterations of the elastic properties of the vascular wall (Rolland *et al.* 1995). Hcy increases intima-media thickness (Voutilainen *et al*. 1998), produces endothelial cell desquamation and increases monocyte adhesion to the vessel wall. VSMC have a redox-sensitive Hcy receptor that regulates collagen expression. The redox state of these cells is controlled by the receptors of nuclear factor kappa-β (NFκβ) that are induced by Hcy. Hcy creates high-grade OxS by altering the redox thiol status of the cell, thereby activating the NFκβ, possibly by Hcy generated ROS (Rodrigo *et al.* 2003). In coronary arteries Hcy increases tumor necrosis factor-α expression which enhances OxS through upregulating NADPH-oxidase (an enzyme which is a potent player in ROS production) and inducible eNOS. VSMC remodel the existing and new extracellular matrix and it has been suggested that Hcy induces constrictive collagen remodelling (Rodrigo *et al.* 2003). *In vitro* it has been demonstrated that HtHcy induces a reduced vascular elastic compliance through diminution of the vascular elastin/collagen ratio and activation of elastinolytic gelatinase A, a matrix metalloproteinase (Mujumdar *et al.* 2001). Also, Hcy has been shown to block aldehyde groups in elastin, thereby inhibiting the cross-linking necessary to stabilize elastin (Rodrigo *et al.* 2003). Hcy enhances collagen synthesis and accumulation (Majors *et al.* 1997). The underlying mechanisms of these processes are not known. Majors *et al.* demonstrated recently that increase in collagen accumulation in Hcy-treated cultures might involve alternative mechanisms not involving ROS (2002).

Hcy induces release of intracellular calcium in VSMC and may induce proliferation of these cells. Depletion of extracellular calcium did not alter the effect of Hcy on intracellular calcium; however, thapsigargin pretreatment,

which depletes intracellular calcium stores, abolished the effect of Hcy, demonstrating its dependence on intracellular calcium stores. Also the effect of Hcy on collagen production correlated with its effect on intracellular calcium. To determine the effect of Hcy on the ability of VSMC to respond to a potent agonist such as AT II, VSMC were pretreated with Hcy and exposed to a range of AT II concentrations, which normally have no effect on intracellular calcium. After Hcy pretreatment, VSMC were extremely responsive to AT II at concentrations below the physiologic range (Mujumdar *et al.* 2000). AT II, via its actions on the AT_1 receptor, promotes atherosclerotic process at virtually all stages of the disease. AT_1 receptor activation leads to production of ROS, as well as OxS, in the vessel wall. One of the important consequences of increased superoxide production in response to ATII is inactivation of NO (Nickenig and Harrison 2002). Chronic Met treatment increased plasma Hcy concentration, lead to increased AT II-induced contraction, which appeared to be related to the release of vasoconstrictor prostanoid(s) as indomethacin inhibits enhancement in contractile response to AT II (Bonaventura *et al.* 2004).

Effect on myocardium

Hcy displays also relationship with development of left ventricular hypertrophy, often accompanying hypertension. In an animal study in the absence of other hypertrophic stimuli, short-term intermediate HtHcy caused pathological hypertrophy and a remodelling of both ventricles with diastolic dysfunction of the left ventricle. These results demonstrate that Hcy has direct adverse effects on cardiac structure and function (Joseph *et al.* 2003).

2.2. Homocysteine and its associations with acute coronary syndrome

2.2.1. Definition of acute coronary syndrome

Acute coronary syndrome (ACS) has evolved as a useful operational term to refer to any constellation of clinical symptoms that are compatible with acute myocardial ischemia. It encompasses acute MI (elevation and depression of the ST-segment, Q wave and non–Q wave) as well as unstable angina. The interaction between the vulnerable atherosclerotic plaque and thrombus formation forms the basis of ACS.

Vulnerability of the fibrous cap is determined mainly by circumferential wall stress, lesion characteristics and blood flow characteristics. Plaque disruption is not a purely mechanical process. Inflammation is also important. Once a plaque ruptures, local and systemic predispositions to thrombogenesis are the final factors that lead to ACS. The local factors include blood flow characteristics (shear stress and tensile stress) and plaque composition. The disruption of lipid-

rich plaques facilitates the interaction between tissue factor and flowing blood, triggering activation of the coagulation cascade, thrombin generation and thrombus formation (Corti *et al*. 2002).

2.2.2. Hyperhomocysteinemia as a cardiovascular risk factor in patients with coronary artery disease

Over past several years, there has been cumulating evidence indicating that even moderate elevation of total plasma Hcy level is a risk factor for coronary artery disease (CAD) (Stampfer *et al.* 1992, Graham *et al.* 1997, Nygard *et al.* 1997a). A prospective study with 14,916 participating male physicians with no prior MI or stroke showed that moderately high levels of plasma Hcy (mean Hcy 11 μmol/l) are associated with the subsequent risk of MI independent of other CV risk factors (Stampfer *et al.* 1992). A similar result was obtained for women (Knekt *et al.* 2001). It has been demonstrated that HtHcy is a strong predictor of mortality both in patients with angiographically confirmed CAD (Nygard *et al.* 1997a) and ACS (Omland *et al.* 2000). Recently, it has been reported that irrespective of concomitant CV risk factors, in middle-aged women Hcy is an independent risk factor for MI and, in particular, for mortality due to MI. An interesting fact is that comparing patients with the highest Hcy level with others, significant differences both for acute MI and for death due to acute MI were apparent after 15 years of follow-up (Zylberstein *et al.* 2004). Ultimately, there seems to be no definite threshold in the linear relation between Hcy level and CV risk. Hcy was reported to increase CV risk from concentrations as low as 9 μmol/l (Nygard *et al*. 1997a), with more data of an elevated risk above 11-15 μmol/l (Stubbs *et al.* 2000; Graham *et al.* 1997, Stampfer *et al.* 1992).

In some studies Hcy is associated with degree of coronary atherosclerosis (Montalescot *et al.* 1997, Yoo *et al.* 1999, Rifai *et al.* 1999, Yu *et al.* 2000). Another study found such correlation only among patients with low CV risk profiles (Tsai *et al.* 2000). Some studies do not confirm such association (Nikfardjam *et al.* 2001, Bozkurt *et al.* 2003).

The results from prospective studies concerning the association between plasma total Hcy concentration and acute coronary event are conflicting. Nor is it clear at which Hcy level such association may be revealed. Voutilainen *et al.* found in a prospective case-control study that plasma Hcy was not associated with an increased risk of the first coronary events in the middle-aged male population in eastern Finland (2000). On the contrary, according to the Rotterdam Study the risk of the first stroke and MI increased directly with increasing total Hcy. The linear correlation coefficient suggested a risk increase of 6% to 7% for every 1-micromol/l increase in total Hcy (Bots *et al.* 1999). In older persons increased plasma Hcy was found to be an independent risk factor for

new coronary events with and without prior CAD in a prospective study (Aronow and Ahn 2000).

Impairment of coronary blood flow reserve has been shown to be an early manifestation of atherosclerosis and CAD. On the basis of a stepwise regression model, severely oxidized LDL (OxLDL), Hcy and triglycerides (TG) were significant predictors of coronary blood flow reserve in apparently healthy adults suggesting the important role of OxLDL and plasma Hcy in early impairment of coronary reactivity in young adults (Laaksonen *et al.* 2002).

2.3. Homocysteine and its associations with hypertension

2.3.1. Hypertension and the risk of cardiovascular disease

Hypertension is an established risk factor for all clinical manifestations of atherosclerosis. It is a common and powerful independent risk factor for development of CAD, stroke, peripheral artery disease and heart failure. CVD sequels occur at 2- to 4-fold increased rate compared with normotensive persons of the same age. BP is critical in atherogenesis because atherosclerosis seldom occurs in the low-pressure segments of the circulation. Also, animal experiments indicate that lipid-induced atherogenesis can be accelerated or retarded by raising or lowering BP. Elevated BP is related to development of CVD in a continuous graded fashion, with no indication of a critical value. The risk of CVD increases with each increment in BP, even within the high normal range (Kannel and Wilson 2003). In the past, hypertension has been defined as a systolic BP (SBP) ≥140 mmHg or a diastolic BP (DBP) ≥90 mmHg, based on actual observations, indicating that above these levels, CVD death rates were at least doubled in comparison with BP levels below 120/80 mmHg (Izzo and Black 2003). Recently, a metaanalysis of 61 long-term clinical trials reconfirmed the same risk relationship in treated individuals: the rates of the CVD events for the trial participants with BP below 120/80 mmHg were twofold lower than for those with BP above 140/90 mmHg (Lewington *et al.* 2002). Therefore, even in patients with high normal BP (SBP 130–139 mmHg, DBP 85–89 mmHg) initiating antihypertensive therapy in presence of more than three concomitant CV risk factors, diabetes or target organ damage is currently recommended. This suggestion is based on the evidence that lowering of BP reduces CV risk in subjects with CAD, stroke or diabetes (Cifkova *et al.* 2003).

Hypertension seldom occurs in isolation from the other CVD risk factors. It tends to occur in association with other atherogenic risk factors that promote its occurrence and strongly influences its CVD impact. Hypertension appears to be metabolically linked to dyslipidemia, glucose intolerance, abdominal obesity, hyperinsulinemia and hyperuricemia, among others (Kannel and Wilson 2003). During the past years an increasing body of evidence has accumulated

suggesting the contribution of non-traditional and alternative CV risk factors, including Hcy and prolonged high-grade OxS, to development of essential hypertension (Rodrigo *et al*. 2003).

2.3.2. Associations between homocysteine and hypertension

Several studies, some of them population-based, have associated plasma Hcy levels to BP, especially SBP. The Third National Health and Nutrition Examination Survey (1998–1994) revealed that Hcy had an independent positive association with BP after adjusting for the CV risk factors. One standard deviation (approximately 5 μmol/l) increase in Hcy was associated with 0.5 and 0.7 mmHg increase in DBP and SBP, respectively, in men and with 0.7 and 1.2 mmHg increase in women, which was independent of renal function and status of B-vitamins. Similarly, higher levels of Hcy were associated with an increased risk of hypertension. Comparison of the highest and the lowest quintiles of Hcy revealed that women had a threefold increase in the risk of hypertension, while men had a twofold increase (Lim and Cassano 2002).

Moreover, observations that Hcy-lowering therapies with folic acid-based treatments are followed by decrease in BP allow to suppose that the link between Hcy and BP is causal, which is important, since Hcy levels can be easily lowered by folic acid-based regimens.

Mechanisms that could explain the relationship between Hcy and BP include mainly Hcy-induced arteriolar constriction, renal dysfunction and increased sodium reabsorption, as well as increased arterial stiffness. However, there is only circumstantial evidence that these mechanisms act in humans. In addition, confounding by subtle renal dysfunction or by unmeasured dietary and lifestyle factors cannot be excluded as an explanation for the association between Hcy and BP (Stehouwer and van Guldener 2003). However, the association between Hcy and BP remains when patients with renal dysfunction are excluded (Nygard *et al.* 1995, Sutton-Tyrrell *et al.* 1997), or after adjusting for lifestyle factors such as smoking or alcohol consumption (van Guldener *et al.* 2003).

In 1989 Araki *et al.* suggested that the levels of plasma Hcy in conjunction with hypertension could be among the risk factors for atherosclerotic cerebral infarction. In the same study it was reported that hypertensive subjects without cerebral infarction had significantly higher Hcy levels than normotensive controls. Several studies have found that hypertensive subjects have significantly higher plasma Hcy levels than controls (Malinow *et al.* 1995, Sharabi *et al.* 1999, Mendis *et al.* 1999, Sheu *et al.* 2000, Lip *et al* 2001, Wocial *et al.* 2002, Turi *et al.* 2003, Kennedy *et al.* 2003). The same finding was reported for hypertensive children (Glowinska *et al.* 2003) and in the case of hypertension associated with pregnancy (Steegers-Theunissen *et al.* 2004).

Malinow *et al.* reported the relationship between SBP and Hcy in 1995. In the Hordaland Homocysteine Study with more than 16,000 healthy participants, plasma Hcy level showed a positive linear association with both SBP and DBP (Nygard *et al.* 1995). Acute HtHcy induced by Met loading has been shown to increase pulse pressure from 49 to 53 mmHg in healthy men (Davis *et al*. 2001)

A study including 2104 Framingham Heart Study participants (mean age 57 years; proportion of women 58%), who were free of hypertension, MI, heart failure, atrial fibrillation, or renal failure at baseline, did not confirm the causal relationship between Hcy and hypertension. In unadjusted analyses, a Hcy value higher by one standard deviation was associated with increased odds of development of hypertension (odds ratio (OR) with 95% confidence interval (CI) 1.18 (1.05; 1.32), and with increased odds of BP progression (1.17 (1.07; 1.27)). In multivariate models this relationship was not significant. Therefore, the authors concluded that there was no major relation of baseline plasma Hcy levels to the incidence of hypertension or to longitudinal BP progression in a large, community-based cohort of non-hypertensive individuals after adjustment for age, sex, and the other important covariates (Sundstrom *et al.* 2003).

Another group of investigators had an opposite opinion. Plasma Hcy levels were significantly elevated not only in patients with essential hypertension but also in their normotensive siblings when compared with normotensive controls without familial predisposion to hypertension. Thus, plasma Hcy may serve as a marker for development of essential hypertension (Jain *et al.* 2003).

In hypertensive patients absence of nocturnal BP decrease is emerging as an index for future target organ damage (Verdecchia *et al.* 1994). Non-dippers had slightly higher mean plasma Hcy values, but this difference was not statistically significant (Tsioufis *et al.* 2002).

In patients with isolated systolic hypertension, after adjusting for potential confounders, Hcy remained significantly associated with systolic hypertension. The authors hypothesized a causal relationship between HtHcy and isolated systolic hypertension through arterial stiffening (Sutton-Tyrrell *et al.* 1997). Bortolotto *et al.* confirmed this hypothesis (1999).

In patients with end-stage renal disease a reduction of plasma Hcy concentration by acetylcysteine was significantly correlated with a reduction of pulse pressure (Scholze *et al.* 2003). In hypertensive patients a strong relationship was found between Hcy and renal function (Bortolotto *et al.* 1999). An animal study suggested that elevated plasma Hcy may be an important pathogenic factor for glomerular damage in hypertension irrespective of arterial pressure, leading to increase in urinary protein excretion and expansion of glomerular mesangium, glomerular hypercellularity, capillary collapse, and fibrous deposition (Li *et al.* 2002).

Data about the interactions between Hcy and hypertension in CAD patients are so far conflicting. The multicentre European Concerted Action Project (750 patients with atherosclerotic vascular disease, 800 controls) revealed that elevated plasma Hcy level interacts strongly with smoking and hypertension (Graham *et al.* 1997).

Folsom *et al*. reported that Hcy was weakly but positively associated with hypertension (1998). The Rotterdam study showed that the incidence of MI was associated with an elevated Hcy level and this association was more pronounced among subjects with hypertension (Bots *et al.* 1999). Such association was not found for patients with premature CAD (Genest *et al.* 1990, Dalery *et al.* 1995); nor was it established in the Physicians' Health Study (Stampfer *et al.* 1992).

Sharabi *et al*. reported that hypertensive patients with a documented history of cerebral or cardiac events did not differ from age and gender matched hypertensive patients without evidence of any cerebral or cardiac event with regard to Hcy level. They concluded that HtHcy is not a feature of hypertensive patients with atherothrombotic events and did not support opinions about additional or synergistic effects between these two independent risk factors (1999). In another prospective study, it was reported that hypertensive patients who experienced MI or CV death, had a slightly higher mean Hcy value than those free of such events (Lip *et al*. 2001). A similar finding was obtained for patients with stroke. Brattström *et al*. showed that plasma Hcy values were unrelated to the presence of hypertension in a group of stroke survivors (1992). Guo *et al.* reported an opposite finding. They found that all classical CV risk factors, including hypertension, were related to elevated Hcy level in patients with early CAD (2003). Moreover, high plasma Hcy level and concomitant hypertension were associated with recurrent stroke among patients presenting with acute ischemic stroke (Mizrahi *et al.* 2003).

Patients with both hypertension and high levels of Hcy (> 11.3 μmol/l, median value) had more severe coronary atherosclerosis and more diffuse atherosclerosis compared with coronary patients without this association (Montalescot *et al.* 1997).

Further studies are needed to clarify the associations between Hcy and hypertension in patients with and without CAD.

2.3.3. Associations between glutathione and hypertension

It has been shown that hypertension is directly associated with the elevated RBC glutathione redox ratio both in gestational hypertension (Nemeth *et al.* 2001) and in juvenile hypertensive patients (Turi *et al*. 2003). GSH depletion is known to result in perturbation of the NO system and causes severe hypertension in normal animals (Vaziri *et al.* 2000). GSH supplementation, given by intravenous infusion, selectively improved human endothelial dysfunction by enhancing NO effects (Prasad *et al.* 1999, Kugiyama *et al.* 1998). Moreover, GSH infusion caused a reduction of BP in adult hypertensive patients (Ceriello *et al.* 1991).

2.3.4. Influence of the antihypertensive treatment on plasma homocysteine

Data about effects of antihypertensive drugs on Hcy level are sparse: there exist reports on absence of change or decrease in Hcy level as well as on increase in Hcy level (Westphal *et al.* 2003, Korkmaz *et al.* 2003).

Elevation of Hcy level is an unwanted side effect of some antihypertensive drugs. In the Framingham Offspring Study, the persons who were using antihypertensive medication had higher plasma Hcy levels than those who were not taking such medications. Such a relationship was likely not due to impaired renal function because the association was completely unaffected by adjustment for serum creatinine concentrations (Jacques *et al.* 2001). The Rotterdam Study reported 1.2 μmol/l higher plasma Hcy level in participants who took anti-hypertensive medication (Vermeer *et al.* 2002).

Short-term treatment for four weeks with the angiotensin-converting enzyme (ACE) inhibitor captopril increased Hcy level by 0.8 μmol/l, but this change was statistically insignificant (Westphal *et al.* 2003).

Use of the beta-blocker metoprolol reduced Hcy level after one month of treatment and this decline continued until the end of the study after five months. In the spironolactone group, Hcy level decreased slightly over one month and remained unchanged until the end of the study (Korkmaz *et al.* 2003). Patients with premature CAD who were not taking any beta-adrenergic blocking drug had insignificantly higher mean Hcy level compared with patients who were taking this class of drugs (Genest *et al.* 1990).

Several studies report that use of thiazide type diuretics is associated with elevation of Hcy (Morrow and Grimsley 1999, Westphal *et al.* 2003).

AT_1 receptor activation leads to free radical release in the vessel wall. AT II deteriorates endothelium dependent vasodilation by increasing the production of ROS (Griendling *et al.* 1994, Rajagopalan *et al.* 1996). In patients with essential hypertension OxS caused a reduction in NO availability, and consequently reduced vasodilation to acetylcholine (Taddei *et al.* 1998). This AT II induced effect on endothelial function is mediated by the AT_1 receptor. A study conducted by Ghiadoni *et al.* confirmed the presence of endothelial dysfunction in essential hypertension and demonstrated that treatment with the AT_1 blocker candesartan increased NO release and reduced the vasoconstricting effects of endogenous endothelin-1 (2000).

2.3.5. Influence of the antihypertensive treatment on glutathione

GSH is a tripeptide synthesised in the liver from the precursor amino acids glutamate, glycine and Cys. GSH is easily oxidized and can be regenerated very rapidly. Due to these characteristics, the functions of GSH are the following: 1)

it is the main intracellular antioxidant, 2) it modulates cell proliferation and immune responses, 3) it helps to regulate signal transduction within cells through redox sensitive molecules such as NFκβ.

The redox state of cells reflects the balance between the levels of oxidation and reduction. The GSH buffer system (ratio of oxidized glutathione — GSSG to GSH (GSSG/GSH)) modulates cell response to redox changes. The glutathione redox status is important in the regulation of most cellular metabolic processes including activation. As cells constantly generate ROS during aerobic metabolism, the glutathione redox status is crucial in maintaining cellular viability (Jefferies *et al.* 2003).

Drugs altering GSH levels or the activity of any GSH-dependent enzymes, may initiate deterioration of antioxidant defence. In animal (Helliwell *et al.* 1985) and *in vitro* (Jurima-Romet *et al.* 1991) studies ACE inhibitors captopril and enalapril have been shown to affect liver GSH levels. Captopril induced a dose dependent depletion of liver GSH, while enalapril elicited a concentration-related reduction of intracellular GSH in primary cultures of rat hepatocytes.

In patients with essential hypertension, during long-term treatment (6 months) with enalapril, the GSH level decreased significantly, while no change was noted in the captopril treated group. In the captopril group GPx activity rose significantly. No change was found in GPx activity in the enalapril group. Plasma lipid peroxidation decreased significantly in both groups (Golik *et al.* 1995).

2.3.6. Overview of the study drugs

Candesartan cilexetil

Candesartan cilexetil is a potent AT_1 receptor blocker. Candesartan cilexetil is an ester prodrug that is converted to an active form of carboxylic acid, candesartan, after administration (Gavras 2000). It lowers BP in a dose-related manner, and maintains its antihypertensive efficacy over long-term treatment (Reif *et al.* 1998). During candesartan treatment, systemic and renal hemodynamics improved in patients with hypertension. Despite the reduction in BP, glomerular filtration rate and renal plasma flow increased in association with a pronounced decrease in renal vascular resistance. (Fridman *et al.* 1997). Candesartan improved insulin sensitivity in patients with hypertension but did not affect glucose homeostasis or the serum lipid profile in persons with coexisting type II diabetes mellitus (McClellan and Goa 1998).

The renin-angiotensin system may contribute to atherogenesis through promotion of endothelial dysfunction. The plausible mechanisms are that AT II promotes superoxide anion generation, endothelial dysfunction, inflammation, and impaired fibrinolysis. An *in vitro* study on human vascular endothelial cells showed that AT II inhibited dose- and time-dependently human vascular endothelial cell motility, altered adversely the intracellular glutathione redox

status, increased generation of ROS and reduced NO metabolite concentrations in culture media. Candesartan attenuated the inhibitory action exerted by AT II on endothelial cell motility, reversed the increase in intracellular OxS, and restored NO availability (Desideri *et al.* 2003).

Inhibition of the AT_1 receptor in hypertensive patients reversed endothelial dysfunction, measured as an improvement in flow-mediated dilation, and reduced the levels of OxS and inflammatory cytokines, suggesting that AT_1 receptor blocker therapy has antiatherogenic effects. There were no significant correlations between these changes and the reduction of SBP or DBP (Koh *et al*. 2003). Similar findings were obtained from a study conducted by Ghiadoni *et al.* (2000) and by Dohi *et al.* (2003). These results suggest that candesartan reduces OxS and inflammation in hypertensive patients independently of its effects on BP.

In liver homogenates from spontaneously hypertensive rats, candesartan reduced malondialdehyde, a marker of lipid peroxidation, and increased the GSH/GSSG ratio without affecting GPx activity (Cediel *et al*. 2003).

Also there are reports about the effect of candesartan on Hcy level in hypertensive patients with type II diabetes, where no statistically significant changes were found in Hcy after one (–0.3 μmol/l) or twelve (–0.9 μmol/l) months of treatment (Derosa *et al.* 2003).

Candesartan attenuated the cell-injurious effects of OxLDL (Li *et al.* 2000) and lowered OxLDL level in VSMC (Watanabe *et al.* 2001).

Amlodipine besylate

Amlodipine belongs to the dihydropyridine class of calcium channel blockers. Like other members of its class, amlodipine inhibits calcium influx into VSMC via L-type calcium channels. In patients with mild to moderate hypertension the drug has a sustained and gradual onset of antihypertensive effect (Haria and Wagstaff 1995).

Amlodipine has been shown to limit progression of arteriosclerosis and to reduce the incidence of CV events. The mechanisms underlying the beneficial effects of amlodipine, however, remain unclear. Recently, it was found in an animal study that amlodipine reduced eNOS inhibitor induced vascular inflammation, OxS, and prevented arteriosclerosis (Kataoka *et al.* 2004).

In patients with essential hypertension amlodipine reduced OxS, evaluated by measurement of plasma malondialdehyde and lipoperoxides and increased plasma antioxidant capacity. In spite of decrease in OxS, conduit artery endothelium-dependent vasodilation did not improve (Ghiadoni *et al.* 2003).

Experimental evidence suggests that calcium channel antagonists exert an antioxidative effect and therefore could protect endothelial cells against free-radical injury. In AT II-infused rats, amlodipine displayed antihypertensive and antioxidant activity, which effectively inhibited many of the OxS-dependent mechanisms (ROS production, endothelial dysfunction) involved in AT II-mediated CV injury (Zhou *et al.* 2004). In hypertensive patients, amlodipine

improved endothelial function (On *et al.* 2003). In an animal study amlodipine significantly inhibited proliferation of VSMC (Lai *et al.* 2002).

It is reported that the effects of amlodipine may be mediated in part by the prostanoid endothelium-derived factor and NO, via preservation of endogenous antioxidant activity, via smooth muscle cell membrane stabilization and via endothelial cell protection (Mason 2002). It has been shown in experimental studies that amlodipine is able to suppress oxidisability of LDL *in vitro* (Chen *et al.* 1997) and to inhibit binding of OxLDL to model membranes (Phillips *et al.* 2003).

3. AIMS OF THE STUDY

Possible associations between hypertension and Hcy are still not clear. Also exists no univocal opinion which Hcy level should be considered harmful and whether there exist patient subgroups depending on presence or absence of concomitant CAD. How can antihypertensive treatment with AT_1 receptor blockers or calcium channel blockers may influence plasma Hcy level and factors related to its metabolism, is not completely known.

Therefore, present study had the following aims:

1. To evaluate patients with ACS and with or without essential hypertension according to plasma Hcy level for determining 1) the prevalence of HtHcy among these subjects, 2) possible associations between Hcy and the other CV risk factors as well as 3) impact of concomitant hypertension and Hcy on the recurrent acute coronary event.

2. To estimate the relationships between plasma Hcy and cellular GSH (including the glutathione redox status) in hypertensive subjects without any other traditional CV risk factors in order to find out whether associations between Hcy and GSH found in the experimental studies are valid in a clinical setting.

3. To evaluate whether the AT_1 receptor antagonist candesartan and the calcium channel blocker amlodipine affect plasma Hcy, cellular GSH and the glutathione redox status as well as vitamin levels during antihypertensive treatment in a homogeneous group of hypertensive patients.

4. To compare the effects of amlodipine and candesartan on OxLDL, on conjugated dienes (CD) and on baseline diene conjugation in circulating low-density lipoproteins (LDL-BDC) with the aim to find out how lipid peroxidation is associated with antihypertensive treatment.

4. MATERIALS AND METHODS

4.1. Study subjects

4.1.1. Patients with acute coronary syndrome

Between January 16th and June 16th 2000, we recruited 107 consecutive patients with ACS hospitalised in the Department of Emergency Cardiology, Tartu University Clinics. Eleven patients were excluded from the study due to concomitant malignancies or renal insufficiency (creatinine >160 μmol/l). The present study comprised 96 patients (54 men/ 42 women, mean age ± standard deviation (SD) 64.5±12.2 years). The data about CV risk factors (smoking habits, hypertension, diabetes) and the history of previous MI were recorded for each patient. ACS was considered recurrent if a new acute MI or unstable angina developed in the patient with previously diagnosed MI. The diagnosis of hypertension was based on the presence of antihypertensive treatment or SBP ≥140 mmHg and/or DBP ≥90 mmHg. The ACS group consisted of 54 patients with the first MI, 17 patients with recurrent MI and 25 with unstable angina. MI was diagnosed in accordance with the ACC/AHA criteria (ACC/AHA Guidelines, 1999). Unstable angina was diagnosed if the patient had chest pain with ischemic genesis but the criteria for diagnosing acute MI were not met. Patients with ACS were divided into two groups according to the median value of Hcy, which was 11.1 μmol/l.

4.1.2. Patients with uncomplicated essential hypertension

Two groups of patients with mild to moderate essential hypertension were recruited using a consecutive sampling design. The patients were studied at the Department of Cardiology, University of Tartu. The first group of patients (N=16) was recruited between September 2000 and June 2001. In these patients baseline investigations were performed. The second study group consisted of 49 patients who received, after baseline investigations, antihypertensive treatment for 16 weeks. They were recruited between September 2001 and December 2002. The selection criteria for both study groups were identical. The entire study population consisted of 65 out-patients (59 men/ 6 women, mean age 52.1±7.1 years) with untreated mild to moderate essential hypertension. All subjects who responded to the advertisement and in whom the inclusion criteria were met were recruited on a consecutive basis at the department of cardiology, University of Tartu, Estonia. The diagnosis of hypertension was established on the basis of SBP >140 mmHg and/or DBP >90 mmHg measured during three different visits. Patients were considered eligible if they had mild to moderate essential hypertension, were aged 40–65 years and had been free of previous

antihypertensive treatment for at least two months. Patients with diabetes (based upon glucose tolerance test), a history of CVD or cerebrovascular disease, heart failure (left ventricular ejection fraction <50%), hypercholesterolemia (total cholesterol (T-Chol) >6.5 mmol/L), other systemic diseases, recent/current infection, anemia and secondary hypertension were excluded (N=34). Routine clinical, hematological and radiological examinations excluded the secondary forms of hypertension. The information of the family history of hypertension and other CVD was collected for all study subjects.

All study subjects were non-smokers with a body mass index (BMI) of <30 kg/m^2. None of the patients had clinical evidence suggestive of CAD, based upon history, electrocardiography, exercise test and echocardiography. There was no left ventricular dysfunction on echocardiography or microalbuminuria on urine analysis. The subjects who were taking any medical vitamin preparations or drugs were not included. No dietary restrictions were imposed.

In Paper II, 16 patients from the first group and 32 patients from the second group were included. In Papers III and IV all 49 patients from the second study group were included.

4.1.3. Controls

The total number of the control subjects was 76. The control group for comparison with the patients with ACS (Paper I) comprised 60 sex-matched apparently healthy subjects (35 men, 25 women, mean age 48.2±5.6 years) with low CV risk (no history and objective findings of any heart disease, BP <140/90 mmHg, T-Chol levels <6.5 mmol/L and normal electrocardiogram).

The control group for comparison with the patients with hypertension studied in Paper II consisted of 28 healthy age-matched male consecutive volunteers (mean age 51.6±5.5 years). The subjects studied in Paper III were 32 (27 men, 5 women, mean age 51.0±5.5 years) healthy sex- and age-matched consecutive volunteers who responded to the advertisement. All controls demonstrated normal findings at physical and biochemical examinations, and had normal BP values (SBP <136 mm Hg and DBP < 88 mmHg).

All controls for comparison with the uncomplicated hypertensive patients were non-smokers with a BMI of <30 kg/m^2. None of them had clinical evidence suggestive of CAD, based upon history, electrocardiography, exercise test and echocardiography. The subjects who were taking any medical preparations of vitamins or drugs were not included. No dietary restrictions were imposed. The information of the family history of hypertension and other CVD was collected.

4.2. Methods

4.2.1. Design of the clinical studies

Cross-sectional studies (Papers I, II). The studies 1) for evaluation of the association between Hcy, BP and other CV risk factors and 2) for assessing the relationships between hypertension, Hcy and GSH were designed as cross-sectional studies.

Study with candesartan and amlodipine (Papers III, IV). The study was designed as a randomised double-blind double-dummy study for comparing the effects of the AT_1 receptor blocker candesartan (Atacand®) and the calcium channel blocker amlodipine (Norvasc®) on Hcy, vitamins, intracellular OxS and serum lipid peroxidation markers. Both study drugs were provided in a double-dummy formula by AstraZeneca AB, Sweden.

During the 4-week run-in period patients did not receive any treatment and were seen for repeated measurements of BP, for the performing of the exercise stress test, glucose tolerance test, echocardiography and ultrasound investigation of the renal arteries. After run-in period, forty-nine patients were enrolled. The patients were randomly assigned to receive either one tablet of candesartan 8 mg and one capsule of placebo (N=25), or one capsule of amlodipine 5 mg and one tablet of placebo (N=24) for 16 weeks. The patients were seen at weeks 2, 6, 10 and 16. The dose of the study drug was doubled at week 2 or 6 if SBP was >140 mmHg or DBP >90 mmHg. The maximum daily dose of candesartan used in this study was 16 mg and that of amlodipine was 10 mg. All visits took place at 08:00–09:00 a.m. before the patient had taken the daily dose of the study medication. At each visit the BP values were recorded. A complete physical and laboratory examination was performed at the initial visit, at randomisation and at the end of the study. The metabolic profile, Hcy, vitamins, GSH and lipid peroxidation markers were measured at baseline, at week 2 and at the end of the study.

The study protocols were approved by the Ethics Committee of the Faculty of Medicine, University of Tartu.

4.2.2. Blood pressure measurements

BP was determined with a mercury sphygmomanometer, with the subject seated for 10 minutes before the measurement. DBP was recorded at the disappearance of Korotkoff sounds (phase 5). The mean of three readings taken with 2 min intervals was used.

4.2.3. Laboratory analyses

The subjects were studied and the plasma samples were collected between 8:00 and 9:00 a.m., after overnight fast. The blood samples were processed within 30 min. The measurements for biochemical analyses and vitamins were performed at the Unified Centre of Laboratories of Tartu University Clinics. The measurements of Hcy, lipid peroxidation markers, GSH and its redox status were performed at the Department of Biochemistry of the University of Tartu.

4.2.3.1. Measurement of homocysteine

Hcy was measured by using enzyme immunoassay method (Axis-Shield Diagnostics Ltd, Dundee, UK). For Hcy measurement, blood was drawn into tubes containing EDTA, placed on ice and then centrifuged. The plasma samples were stored at –70°C until analysis.

4.2.3.2. Measurement of glutathione

The RBC-GSH was measured by using enzymatic method as described previously (Annuk *et al*. 2001). Protein was removed from 0.3 ml of heparinised whole blood by adding an equal volume of a 10% solution of metaphosphoric acid in water, leaving the mixture at room temperature for 10 min, and then centrifuging it (4°C, 1200 x *g*, 10 min). The supernatant was carefully collected and stored at –20°C. The sample was divided into two parts for the measurement of the total amount of RBC GSH (RBC-TGSH) and RBC-GSSG. To assay RBC-TGSH or RBC-GSSG, the supernatant was mixed with 0.895 ml of 0.2 M sodium phosphate buffer (pH 7.5) containing 0.01 M EDTA and with 0.5 ml of the same buffer containing 0.5 U GSH-reductase and 0.3 mM NADPH. The reaction was initiated by the addition of 0.1 ml of 1 mM 5,5'dithiobis-(2-nitrobenzoic acid). The change in optical density was measured at 412 nm after 10 min and quantitated in comparison with the standard curve. The concentration of RBC-GSH was calculated as the difference between RBC-TGSH and RBC-GSSG.

4.2.3.3. Measurement of lipid peroxidation markers

For the measurement of OxLDL, blood was drawn into tubes containing EDTA. The plasma samples for the measurement of lipid peroxidation markers were stored at –70°C until analysis. OxLDL levels were measured using an enzyme-linked immunosorbent assay kit (Mercodia, AB, Uppsala, Sweden).

For LDL-BDC measurement, first serum LDL was precipitated as described by Ahotupa *et al.* 1998. Before precipitation of LDL, the serum samples (to which 1 mg/ml of EDTA were added) and the precipitation reagents were allowed to equilibrate at room temperature. 1 ml of the sample was added to 7 ml of the heparin-citrate buffer. The precipitation buffer consisted of 0.064 M trisodium citrate adjusted to pH 5.05 with 5 M HCl, and contained 50,000 IU/l heparin. After mixing with a Vortex mixer the suspension was allowed to stand for 10 min at room temperature. The insoluble lipoproteins were sedimented by centrifugation at 1000 g for 10 min. The pellet was resuspended in 1 ml of 0.1 M Na-phosphate buffer, pH 8.0, containing 0.9% NaCl. Thereafter the lipids were extracted from the LDL samples (0.1 ml) by chloroform – methanol (2:1), dried under nitrogen, then redissolved in cyclohexane, and analysed spectrophotometrically at 234 nm. The absorbance units (difference $A_{234} - A_{300}$) were converted to the molar units using the molar extinction coefficient 2.95x $10^4\ M^{-1}\ cm^{-1}$. The ratio of LDL-BDC to LDL was calculated.

The level of CD was measured according to the method described by Starkopf *et al.* 1995. The samples 0.15ml + 0.15 ml 0.9% NaCl (reagent blank contains only isotonic saline) were incubated at 37º C for 30 min, 0.25% BHT (0.015 ml) was added, the samples were extracted with heptane/isopropanol (1:1, whole volume 1.8 ml) and acidified by 0.5 ml 5N HCl. After extraction with cold heptane (1.6 ml), the samples were centrifuged (for 5 min at 3000 rpm) and the absorbance of the heptane fraction was measured at 234 nm.

4.2.3.4. Measurement of vitamins

The concentrations of plasma folic acid and vitamin B_{12} were measured by chemiluminescence method with the Immulite 2000 Analyser (Diagnostic Products Corporation, California, USA). The concentration of RBC folic acid was measured both in the whole blood and in the serum by chemiluminescence method with the Immulite 2000 Analyser (Diagnostic Products Corporation, USA), and RBC folic acid in ng/ml was calculated according to the formula: RBC folic acid = (R-{S x (100-H) / 100} x (100/H), where R denotes whole blood folic acid, H is hematocrit and S is the level of the patient's serum folic acid.

4.2.3.5. Other biochemical analyses

Serum creatinine, total protein, uric acid and glucose concentrations were measured with the Hitachi 912 automated analyser (Roche Diagnostics). The following methods were used: Roche creatinine Jaffe compensated method for creatinine, Human Biuret method for total protein, Roche uric acid plus assay for uric acid and Human GOD-PAP method for glucose.

Serum lipids: LDL and high-density lipoprotein cholesterol (HDL) (Roche Diagnostics LDL and HDL assay), T-Chol (Human cholesterol liquicolor

assay), and TG (Roche Diagnostics GPO-PAP triglyceride assay) were measured using a Hitachi 912 automated analyser (Roche Diagnostics). Fibrinogen concentration was measured by Clauss clotting method using a Stago Compact analyser (Diagnostica Stago, France). RBC count, haematocrit and hemoglobin were analysed using a Sysmex XE 2100 analyser (Sysmex Corporation, Japan).

4.2.4. Statistical methods

In Paper I, the data are summarised by mean ± SD. For categorical variables, the frequencies were compared by χ^2 test. For the normally distributed variables t-test was used to evaluate the differences in means between two groups. Mann-Whitney U-test and Duncan tests were used as nonparametric tests for the comparison of the group means. To estimate association between HtHcy as binary dependent and other cardiovascular risk factors, logistic regression models were used.

In Paper II–IV, the normally distributed data are summarised by mean ± SD; the non-normally distributed data are summarised by geometric mean with the 95% confidence intervals (95% CI). In Paper II, the distributions of Hcy, RBC count, RBC folic acid and creatinine were skewed and were log-transformed for analysis as required to achieve approximate normality. Unpaired two-tailed t-test was performed to compare the means of the cases and the controls for the study variables. Pearson coefficients of correlation between RBC-GSH and the other variables were calculated. Stepwise multiple regression analysis was performed to assess the relationships between RBC-GSH as the dependent variable and the other study parameters as the independent variables.

In Paper III, differences between the control group and the treatment group at baseline were estimated by *t*-test for the normally distributed variables and by non-parametric Mann–Whitney *U*-test for the non-normally distributed variables. In Papers III and IV, to account for repeated measures, multilevel linear regression analysis was used to test for treatment differences in average BP and biochemical variables at three different time points.

Changes in BP values and biochemical variables were calculated as the difference between the baseline values and the values at the end of the study. The Pearson coefficients of correlation were obtained to estimate the associations between the changes in BP and biochemical variables. To test whether the associations remain the same after adjusting for the treatment group indicator, multiple linear regression analysis was used.

To compare the individuals in whom increase in Hcy concentration was at least ≥2 μmol/l and those without such changes, *t*-test was used for the normally distributed data and Mann–Whitney *U*-test for the non-normally distributed data. In both groups, divided according to the changes in Hcy level, changes in the biochemical variables over time were estimated by paired t-test for the

normally distributed variables and Wilcoxon matched pairs test for the non-normally distributed data. Logistic regression was used to assess the determinants of increase in Hcy concentration of at least 2 μmol/l.

All statistical analyses were conducted using the software R, version 1.6.0. for Windows. The level of significance was defined as $p<0.05$ (two-tailed).

5. RESULTS AND DISCUSSION

5.1. Homocysteine and its associations with cardiovascular risk factors in patients with acute coronary syndrome (Paper I)

5.1.1. Homocysteine and other biochemical markers in patients with acute coronary syndrome

The demographic data and the biochemical variables for comparison of patients with ACS and healthy controls are shown in Table 1.

Table 1. Demographic data and biochemical variables in the patients with ACS and in the healthy controls.

Variable	ACS N=96	Control N=60	p
Age, yrs.	64.5±12.2	48.2±5.6	0.0001
Men, %	56.3	58.3	0.79
Smokers, %	21.8	15.0	0.30
Hypertension, %	50.0	0.0	
Diabetes, %	11.5	0.0	
Previous MI, %	31.3	0.0	
Hcy, μmol/L	12.2±5.2	8.3±2.7	0.0001
Hcy >15 μmol/l, %	20.4	5.0	0.01
Hcy > 11,1 μmol/l, %	50.0	13.3	0.0001
Creatinine, μmol/l	108.0±20.3	92.4±13.5	0.0001
Fibrinogen, g/l	3.9±1.4	3.1±0.5	0.0001
Uric acid, μmol/l	294.6±80.5	231.9±72.8	0.0001
Blood glucose, mmol/l	7.3±2.5	5.1±0.5	0.0001
T-Chol, mmol/l	5.3±1.2	5.3±0.7	0.88
LDL, mmol/l	3.4±1.1	3.4±0.7	0.71
HDL, mmol/l	1.2±0.4	1.4±0.3	0.0001
TG, mmol/l	1.7±1.1	1.1±0.6	0.0001

The patients with ACS were significantly older and had significantly higher plasma Hcy level than the controls. Also all other studied biochemical markers except T-Chol and LDL were significantly higher in the cases than in the controls.

5.1.2. Associations between homocysteine values, the other biochemical parameters and concomitant cardiovascular risk factors in patients with acute coronary syndrome

Differences in the biochemical parameters in the ACS group according to median Hcy are shown in Table 2.

Table 2. Serum creatinine, uric acid and TG according to median Hcy level in the patients with acute coronary syndrome.

Variable	Hcy <11.1 μmol/l	Hcy ≥11.1 μmol/l	p
Hcy, μmol/l	8.6±1.6	15.7±5.1	
Creatinine, μmol/l	102.9±15.8	113.3±22.4	0.01
Uric acid, μmol/l	269.3±76.5	315.7±78.8	0.001
TG, mmol/l	1.4±0.7	1.9±1.2	0.001

ACS patients with Hcy ≥11.1μmol/l had significantly higher values of creatinine, uric acid and TG than those with Hcy < 11.1 μmol/l.

In patients with ACS, HtHcy was associated with diabetes (odds ratio – OR 5.0, 95%CI 1.01-24.70, $p<0.04$). Gender, smoking, concomitant hypertension and former MI were not associated with elevated Hcy levels.

5.1.3. Associations between homocysteine and hypertension in patients with recurrent acute coronary syndrome

Patients with recurrent ACS (new ACS in a patient with a history of previous MI) did not differ regarding their Hcy values from patients with the first ACS. Also the patients with recurrent ACS did not differ from patients with the first ACS by age, blood lipids, fibrinogen, blood glucose, creatinine or uric acid (data not shown). In monofactorial logistic regression, gender, smoking, concomitant diabetes or hypertension did not have relationship with recurrent ACS. Nor was any relationship found between Hcy and new ACS in patients with concomitant hypertension. Also no relationship was found between new ACS and age or the other biochemical variables.

Normotensive ACS patients with recurrent ACS (n=11) had significantly higher values of Hcy than patients with the first ACS (n=37) (Hcy 14.7±7.1 vs 11.0±4.3 μmol/l, respectively, $p<0.04$). The other concomitant risk factors had no impact on the occurrence of new ACS.

5.1.4. Discussion

The present study showed that the patients with ACS had average plasma Hcy levels 3.9 μmol/l higher than controls. However, in the whole ACS group, a new ACS was not associated with elevated levels of plasma Hcy. Mean Hcy level in ACS patients was 12.2 μmol/l which is similar to the mean Hcy values (11.0–13.6μmol/l) found in other studies (Stubbs *et al.* 2000; Omland *et al* 2000; Al-Obaidi *et al.* 2000) where patients with ACS were involved.

According to this study, HtHcy, defined as 11.1 μmol/l, was not a risk factor for a new ACS in hypertensive patients and there was found no support for synergistic effects between these two risk factors. This is consistent with the results of the study conducted by Al-Obaidi *et al.* where hypertensive ACS patients did not differ significantly from normotensive subjects with regard to mean plasma Hcy (2000). In older persons (> 80 yrs) increased plasma Hcy was found to be an independent risk factor for ACS (Aronow and Ahn 2000). The Rotterdam study showed that the incidence of MI was associated with elevated Hcy level and this association was more pronounced among those with hypertension (Bots *et al.* 1999). In an autopsy study of patients with coronary thrombus Hcy correlated with the presence of hypertension in univariate analysis but this association did not remain significant in multivariate analysis (Burke *et al.* 2002).

Most studies confirming the synergistic association between Hcy level and hypertension have been carried out in patients with stable coronary heart disease (Graham *et al.* 1997, Sharabi *et al.* 1999).

The studied patients with recurrent ACS did not differ from the patients with the first ACS. This is consistent with the results of a study conducted by Bogaty *et al.* where patients with recurrent ACS did not differ from subjects with long-standing stable angina with regard to mean Hcy value (2001).

An interesting finding was that the normotensive patients with recurrent ACS had higher values of Hcy than the hypertensive patients with the first ACS. The reasons for this remain unclear. The relationship between HtHcy and the other CV risk factors in enhancing risk of ACS is not clear. A possible explanation for this obscurity might be methodological differences. Another explanation is the different prevalence of the genetic mutations that lead to the enzymatic defects causing HtHcy.

The patients with CAD had significantly higher creatinine level than the controls. Mild to moderate renal insufficiency is an independent risk factor for CV events in postmenopausal women with known CAD (Shlipak *et al.* 2001). The same finding was present in the Cardiovascular Health Study where after adjusting for the other CV risk factors moderately elevated creatinine remained a significant predictor of all-cause and CV mortality, total CVD, claudication, and congestive heart failure in elderly patients, indicating that increased risk is apparent early in renal disease (Fried *et al.* 2003).

The metabolic background (elevated glucose, TG, creatinine and decreased level of HDL), found in our CAD patients was similar to the characteristics of CAD patients in other studies (Erren *et al.* 1999, Stubbs *et al.* 2000, Ozkan *et al.* 2002). The studied patients with CAD and with elevated Hcy level had significantly higher levels of creatinine, uric acid and TG. This is consistent with an earlier study where in patients with angiographically confirmed CAD Hcy was strongly related to serum creatinine level (Nygard *et al.* 1997a). Brattström and Wilcken have suggested that impaired renal function due to hypertension and atherosclerosis is an important cause of elevated plasma Hcy found in vascular disease patients (2000). The reasons are the following. Atherogenesis and elevation of BP commonly develop silently over many years before the emergence of clinically evident vascular events. These processes also lead to nephrosclerosis and a degree of deterioration of renal function, which is highly relevant to the plasma clearance of Hcy. For these reasons, presence of vascular disease itself may contribute to an elevation in circulating Hcy by leading to a decline in renal function. This means that because of reduced renal function, patients with either occult or clinically evident CVD and normal serum creatinine concentrations may have elevated circulating Hcy concentrations (Brattström and Wilcken 2000).

Uric acid is also considered a risk factor for CAD (Bickel *et al.* 2002). With regard to uric acid, in subjects without a history of atherosclerotic disease, uric acid was a predictor of plasma Hcy concentration (Malinow *et al.* 1995, Motti *et al.* 1998). Some studies confirm the relationship between uric acid and CAD (Fang and Alderman 2000, Freedman *et al.* 1995), while some do not (Culleton *et al.* 1999). Maxwell *et al*. found that association between uric acid and CAD may be a consequence of an impairment of vascular NO activity. The authors set the hypothesis that in the case where the endothelium is healthy and OxS is low, NO activity would be expected to be sufficient to put a brake on xanthine oxidase activity restricting the production of uric acid. In the presence of risk factors, however, OxS increases, vascular NO activity wanes, and the brake on xanthine oxidase activity is removed. The subsequent enhanced uric acid production then helps to restore OxS toward normal (Maxwell *et al.* 2001). This hypothesis is consistent with a study conducted by Nieto *et al.* who demonstrated that individuals with atherosclerosis had higher serum antioxidant capacity than matched controls. This difference was almost entirely explained by increased serum uric acid (2000).

There exist only a few data about the associations between Hcy and TG. Stanger *et al.* found that CAD patients with HtHcy do not differ from patients with normal plasma Hcy levels (2000). The difference between their study and ours is that cut-off levels for HtHcy are remarkably different (14.0 vs 11.1 μmol/l).

The percentage of patients with diabetes is similar to that of another study where patients with ACS were studied (Al-Obaidi *et al.* 2000). Recently it was

reported that in patients with type II diabetes, plasma Hcy level was a strong and independent risk factor for CAD events (Soinio *et al.* 2004). Syndrome X, a cluster of several metabolic disorders including hyperinsulinemia, hypertriglyceridemia, and hypertension, is associated with severe vascular morbidity. In a rat model Hcy was positively and significantly correlated with any original component of syndrome X indicating that HtHcy is an integral component of this rat model of syndrome X. It is thus highly likely that HtHcy is an integral component of the human syndrome X as well (Oron-Herman *et al.* 2003). Although such subgroup analysis was not performed in our ACS patients, this may explain the higher TG value and the larger proportion of patients with diabetes in the HtHcy group.

Elevation of fibrinogen level in ACS patients is explainable as part of the acute phase response (Ridker 1999), but higher fibrinogen level is also considered a CV risk factor (Ernst and Resch 1993)

The patients with recurrent ACS did not differ from the patients with the first ACS by age, blood lipids, fibrinogen, blood glucose, creatinine or uric acid. This finding is different from that of a study conducted by Aronow and Ahn where patients with recurrent ACS had significantly higher T-Chol, LDL and TG, lower HDL levels, and the percentage of patients with concomitant obesity, hypertension and diabetes was higher (2000).

5.2. Homocysteine and its associations with glutathione and blood pressure (Paper II)

5.2.1. Homocysteine and its associations with cellular oxidative stress, level of vitamins and hypertension

5.2.1.1. Homocysteine, vitamins, glutathione and its redox status in patients with essential hypertension

Data for comparison of the hypertensive patients and the healthy controls are shown in Table 3.

The untreated hypertensive patients had significantly higher Hcy levels compared with the healthy controls. After adjusting Hcy for age and BMI, the difference between the cases and the controls became more pronounced ($p<0.008$). The untreated hypertensive patients and the normotensive controls did not differ with regard to age, T-Chol, HDL, LDL, serum creatinine, protein, RBC count, hematocrit or hemoglobin. The patients with essential hypertension had significantly higher BMI as well as elevated TG and blood glucose.

Table 3. Clinical characteristics of the study groups

Variable	Patients N=48	Controls N=28	p
Age, y	53.0±7.2	51.6±5.5	0.33
BMI, kg/m^2	27.7±2.0	24.7±3.0	0.0001
SBP, mmHg	147.9±12.9	117.9±10.8	0.0001
DBP, mmHg	97.1±7.3	76.9±6.7	0.0001
T-Chol, mmol/l	5.6±0.8	5.3±0.8	0.14
LDL, mmol/l	3.7±0.7	3.4±0.8	0.06
HDL, mmol/l	1.3 (1.2;1.4)	1.4 (1.2;1.8)	0.13
TG, mmol/l	1.4 (1.3; 1.8)	0.9 (0.8;1.1)	0.0001
Glucose, mmol/l	5.6±0.4	5.0±0.4	0.0001
Protein, g/l	73.0±5.4	71.3±3.8	0.11
Creatinine, μmol/l	102.2 (98.8;107.8)	96.9 (93.3;101.6)	0.08
RBC count, x10^{12}/l	4.9 (4.8;5.0)	4.8 (4.6;4.9)	0.06
Hemoglobin, g/l	147.6±8.2	143.6±13.9	0.18
Hematocrit, %	43.7±2.4	42.3±3.6	0.06

As shown in Table 4, the RBC-GSH levels were significantly lower and the RBC-GSSG levels higher in the subjects with essential hypertension when compared with the age-matched controls. After adjusting RBC-GSH level for age and BMI, the difference between the cases and the controls remained significant ($p<0.04$). In the hypertensive patients, the RBC glutathione redox status, expressed as RBC-GSSG/RBC-GSH, was twice as high as that of the control subjects indicating cellular OxS. The groups did not differ with regard to cellular folic acid level.

Table 4. Plasma homocysteine, red blood cell glutathione and folic acid in hypertensive vs. normotensive subjects

Variable	Patients N=48	Controls N=28	p
Hcy, μmol/l	10.7 (9.7; 12.7)	9.2 (8.5; 10.2)	0.02
RBC-GSH, μmol/l	744.7±298.4	1001.1±286.2	0.0005
RBC-GSSG, μmol/l	108.6 (104.7; 145.2)	81.3 (72.9; 103.0)	0.008
RBC-GSSG/RBC-GSH	0.16 (0.15; 0.22)	0.09 (0.08; 0.13)	0.0001
Folic acid in RBC-s, ng/ml	250.6 (243.3; 322.3)	211.4 (183.3; 269.3)	0.17

5.2.1.2. Relationship between blood pressure, homocysteine, vitamins and glutathione

In the hypertensive subjects, RBC-GSH concentration showed a negative correlation with SBP ($r = -0.44$, $p<0.004$, Figure 2), creatinine ($r = -0.43$, $p<0.002$), protein ($r = -0.41$, $p<0.004$) and RBC folic acid ($r = -0.30$, $p<0.04$). No correlation was detected between RBC-GSH and Hcy, ($r = 0.25$, $p<0.09$, Figure 3). For the controls, RBC-GSH correlated negatively and significantly with Hcy ($r = -0.41$, $p<0.03$, Figure 2), RBC folic acid ($r = -0.49$, $p<0.047$) and creatinine ($r = -0.45$, $p<0.02$).

The results of multiple regression analysis for the hypertensive patients are given in Table 5. Hcy, hemoglobin and protein were positively and significantly correlated with RBC-GSH level, SBP and creatinine were negatively correlated with RBC-GSH level.

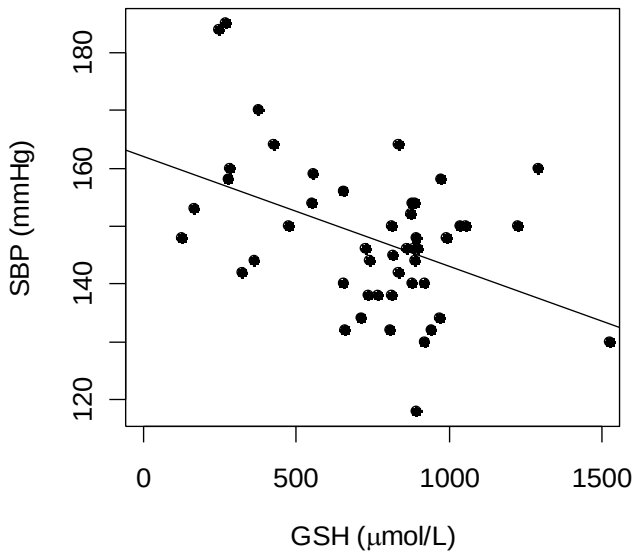

Figure 2. Correlation between between red blood cell reduced glutathione and systolic blood pressure in the patients with essential hypertension ($r = -0.44$, $p<0.004$).

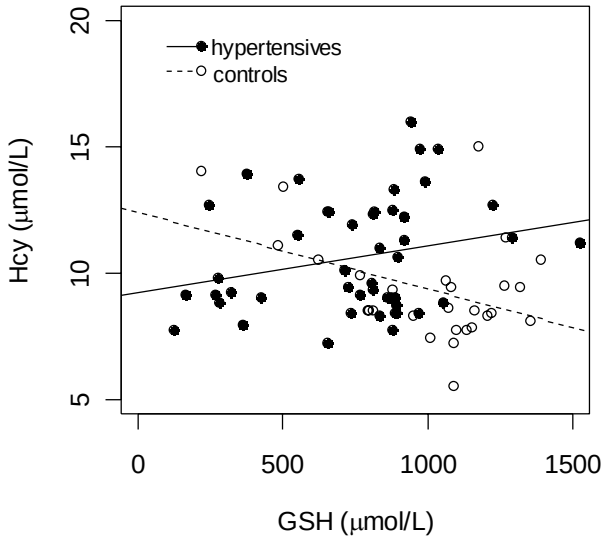

Figure 3. Correlations between red blood cell reduced glutathione and plasma homocysteine in the hypertensive patients and in the controls.

In the patients with essential hypertension no correlation was detected between RBC-GSH and Hcy ($r= 0.25$, $p<0.09$). In the controls, RBC-GSH correlated negatively with Hcy ($r= –0.41$, $p<0.03$).

Table 5. The results of multiple regression analysis predicting red blood cell reduced glutathione level in the hypertensive patients

Parameter	Regression coefficient	Standard error	p
SBP, mmHg	–6.85	2.83	0.02
Hcy, μmol/l	934.89	379.55	0.02
Hemoglobin, g/l	13.20	4.60	0.007
Creatinine, μmol/l	–1738.33	684.70	0.02
Protein, g/l	16.60	6.99	0.02

Dependent variable: RBC-GSH μmol/L ($r^2=0.50$, F statistic 7.81, $p<0.00001$).

5.2.1.3. Discussion

The results of this study confirmed that RBC-GSH level was significantly lower in the middle-aged untreated essential hypertension patients compared with the age-matched controls. This correlation was independent of age and BMI. This and the about twofold elevated glutathione redox ratio could indicate the dysbalanced/alterated cellular GSH system in the hypertensive patients. Our data support the findings of several previous studies. Firstly, it was shown that hypertension is directly associated with the elevated RBC glutathione redox ratio both in gestational hypertension (Nemeth *et al*. 2001) and in juvenile hypertensive patients (Turi *et al.* 2003). Secondly, considering middle-aged persons, intracellular GSH, but not plasma GSH, is characterized by a markedly lower level (Hernanz *et al*. 2000).

In this study, including middle-aged persons, we found an inverse correlation between SBP and RBC-GSH. It is plausible that RBC-GSH may have relevance in the pathogenesis of hypertension. GSH depletion is known to result in perturbation of the NO system and causes severe hypertension in normal animals (Vaziri *et al*. 2000). Thiol supplementation with GSH, given by intravenous infusion, selectively improves human endothelial function by enhancing NO effects (Kugiyama *et al*. 1998, Prasad *et al.* 1999). Moreover, GSH infusion causes reduction of BP in adult hypertensive patients (Ceriello *et al.* 1991).

Several previous studies have suggested an impact of elevated Hcy on hypertension (Kanani *et al.* 1999, Sheu *et al.* 2000, Virdis *et al.* 2001). However, its precise role remains to be elucidated. We propose that RBC-GSH and plasma Hcy should be assessed together in middle-aged hypertensive patients. It is known that approximately half of GSH is derived via Hcy metabolism in the human body. Although univariate analysis did not reveal a linear significant correlation between RBC-GSH and Hcy, in multiple regression analysis Hcy level was significantly correlated with RBC-GSH for the hypertensive patients. In accordance with previous studies (Kanani *et al.* 1999, Sheu *et al.* 2000, Virdis *et al.* 2001), this study demonstrated significantly higher plasma Hcy concentration. At the same time, the elevated Hcy level was not explainable by the effect caused by RBC folic acid deficit. An elevated level of Hcy leads to decreased GSH content, probably through different pathways. It has been reported that Hcy may induce intracellular suppression of the glutathione related antioxidant defence system, and possibly also reduce Hcy-derived-GSH synthesis (Mosharov *et al.* 2000). In the control group, a negative linear correlation was observed between RBC-GSH and Hcy. This is in accordance with *in vitro* studies, where increasing Hcy concentrations reduced intracellular GSH concentrations in endothelial cells (Hultberg *et al.* 1997).

5.3. The effect of antihypertensive treatment on homocysteine, vitamins and cellular oxidative stress in patients with essential hypertension (Paper III)

5.3.1. Baseline characteristics of the hypertensive patients and the healthy controls

Forty-nine patients with untreated hypertension (43 men, 6 women) were compared to 32 normotensive controls (27 men, 5 women). Baseline characteristics of the hypertensive patients and the healthy controls are shown in Table 6.

Table 6. Baseline characteristics of the hypertensive patients and the healthy controls.

Variable	Patients N=49	Controls N=32	p
Age, y	52.5±7.0	51.0±5.5	0.30
BMI, kg/m^2	27.0±2.1	24.5±2.7	0.0001
Men/Women	43/6	27/5	0.60
SBP, mmHg	151.1±10.3	117.9±9.6	0.0001
DBP, mmHg	96.3±6.6	76.4±7.4	0.0001
Serum creatinine, μmol/l	90.8±10.6	91.0±11.9	0.92
Plasma Hcy, μmol/l	10.1 (9.7; 11.1)	8.4 (7.9; 9.2)	0.001
Serum folic acid, ng/ml	5.6±2.8	5.4±1.9	0.65
Folic acid in RBC-s, ng/ml	221.9 (215.0; 309.5)	187.2 (161.3; 246.8)	0.20
Serum Vitamin B_{12}, pg/ml	305.9 (290.4; 353.6)	282.3 (241.4; 381.3)	0.1
RBC-GSH, μmol/l	924.6±168.2	1061.9±280.9	0.01
RBC-GSSG, μmol/l	121.1 (116.1; 160.0)	78.0 (71.0; 97.0)	0.0002
RBC-GSSG/RBC-GSH	0.13 (0.12; 0.18)	0.09 (0.07; 0.10)	0.0001

At baseline in addition to significantly higher BP values and BMI, the hypertensive patients had higher plasma Hcy and cellular OxS markers RBC-GSSG and RBC-GSSG/RBC-GSH than the controls. The groups were similar with regard to serum creatinine, vitamin B_{12} and both cellular and serum folic acid.

Three patients did not complete the study: two patients (one from either study group) left the study because of personal reasons unrelated to the study, and one patient in the amlodipine group discontinued treatment at week 6 due to skin reaction, yielding 46 patients for analysis. Twenty-four patients (22 men/ 2 women, mean age 53.9±6.9 yrs, BMI 27.0±2.1 kg/m^2, duration of

hypertension 16.0 yrs with the range from 0.5 to 35 yrs) were treated with candesartan and 22 patients (19 men/ 3 women, mean age 50.9±6.6 yrs, BMI 27.0±2.1 kg/m^2, duration of hypertension 12.4 yrs with the range from 0.5 to 44 yrs) were treated with amlodipine. There were no differences in the demographic data between the groups.

5.3.2. Changes in homocysteine, glutathione, vitamins and blood pressure during antihypertensive treatment

The changes in BP and biochemical variables, measured at baseline, at week 2 and at week 16 in the patients treated with candesartan (N=24) or amlodipine (N=22) are shown in Table 7. Throughout the study, the treatment groups did not differ with regard to the studied parameters. Both drugs caused a highly significant decrease both in mean SBP and DBP ($p<0.0001$) with no significant differences between the drugs. The decrease was most pronounced during the first two weeks. The further decrease in BP in the course of the treatment was insignificant. Doubling of the dose was required for 12 patients in the candesartan group and in for 9 patients in the amlodipine group. In comparison with baseline, a significant fall was detected in serum creatinine ($p<0.002$) and RBC-GSSG ($p<0.03$) levels at week 16 in both groups. The RBC-GSSG/RBC-GSH ratio decreased insignificantly ($p=0.054$) during the treatment. Plasma Hcy tended to increase in both treatment groups but these changes were not significant.

The changes in SBP were associated with the changes in DBP but were not associated with the changes in the biochemical variables. The decrease in DBP was associated with the decrease in SBP and RBC-GSSG level (relative toxic compound) and with the increase in cellular folic acid (Table 8).

Table 7. Changes in blood pressure and the biochemical variables during antihypertensive treatment with candesartan or amlodipine.

Variable	Candesartan baseline	Candesartan week 2	Candesartan week 16	Amlodipine Baseline	Amlodipine week 2	Amlodipine week 16
SBP, mmHg	150.3±12.3	135.2 ± 8.9	132.2 ± 7.8*	151.6±8.4	133.8 ± 9.5	131.2 ± 9.1*
DBP, mmHg	96.6±6.0	88.1 ± 7.1	86.3 ± 5.8*	96.5±6.4	85.6 ± 8.0	84.6 ± 5.1*
Creatinine, μmol/l	89.3±9.6	90.9 ± 9.7	84.0 ± 10.2*	91.6±10.9	90.5 ± 14.4	86.7 ± 14.3*
Hcy, μmol/l	10.3 (9.5; 11.6)	10.8 (9.1; 13.7)	10.7 (9.0; 14.3)	10.1(9.4; 11.3)	10.5 (9.6; 12.1)	10.6 (9.5; 12.6)
Serum folic acid, ng/ml	5.9±2.9	6.0±3.3	6.0±2.5	5.3±2.8	5.8±2.8	5.1±2.3
Folic acid in RBC, ng/ml	233.4 (202.7; 360.2)	181.1 (152.5; 275.1)	224.3 (197.1; 299.3)	209.9 (179.2; 310.5)	200.9 (173.6; 269.7)	186.2 (161.5; 242.0)
Serum vitamin $B_{12,}$ pg/ml	294.4 (261.0; 376.8)	269.3 (230.9; 351.5)	294.6 (264.7; 362.9)	330.2 (296.4, 354.1)	312.4 (274.0; 391.8)	322.1 (289.1; 391.2)
RBC-GSH, μmol/l	942.7±197.4	986.5±216.8	866.8±145.7	886.3±120.7	960.0±203.7	931.4±256.5
RBC-GSSG, μmol/l	119.9 (107.4; 150.5)	114.8 (102.2; 148.2)	98.4 (84.1; 150.4)*	119.1 (102.2; 180.8)	114.6 (100.4; 159.2)	96.4 (81.8; 158.0)*
RBC-GSSG/ RBC-GSH	0.13 (0.11; 0.16)	0.11 (0.10; 0.16)	0.11 (0.09; 0.18)	0.14 (0.11; 0.21)	0.12 (0.10; 0.18)	0.11 (0.08; 0.19)

* denotes a significant time trend. $p<0.05$ was considered significant.

Table 8. The results of multiple linear regression analysis predicting the changes in diastolic blood pressure during antihypertensive treatment with candesartan or amlodipine.

Parameter	Regression coefficient	Standard error	p
Changes in folic acid in RBC, ng/ml	–0.02	0.009	0.01
Changes in RBC-GSSG, μmol/L	0.03	0.01	0.05
Changes in SBP, mmHg	0.29	0.08	0.001
Treatment: candesartan – 1, amlodipine – 0.	–0.41	1.77	0.82

Dependent variable: changes in DBP (r^2=0.42, F statistic 7.20, p<0.0002).

5.3.3. Associations between the changes in homocysteine, glutathione and vitamins

A significant increase in plasma Hcy level (Figure 4) compared with the baseline values was detected in 12 (26%) patients: 5 in the candesartan group and 7 in the amlodipine group. These changes were not related to the antihypertensive efficacy of the drugs or to the need to increase the dose. Nor did the patients both with or without an increase in Hcy level differ with regard to age (54.4±6.4 vs. 51.1±6.9 yrs), gender (11 men/ 1 women vs. 28 men/ 5 women), BMI (27.4±1.6 vs. 26.7±2.2 kg/m^2) or duration of hypertension (6.6 (4.1; 17.1) vs. 8.5 (10.9; 20.7) yrs, respectively). In the patients with increase in Hcy level, folic acid decreased significantly in the serum at week 2 (5.2±2.5→4.7±1.9 ng/ml, p<0.03) and in RBC at week 16 (at baseline 267.3 (201.3; 391.5)→200.9 (165.2; 257.5) at week 16, p<0.01).

In the patients without an increase in Hcy level, no changes were detected in the biochemical variables at week 2. Compared with the baseline, RBC-GSSG/RBC-GSH (0.12 (0.11; 0.16) → 0.09 (0.08; 0.15), p=0.051) had slightly decreased; RBC-GSSG (111.0 (101.9; 144.4) → 98.9 (75.8; 122.0), p<0.02) and creatinine (88.2±8.5 → 83.0±12.4 μmol/l, p<0.006) had significantly decreased by the end of the study. Creatinine had decreased insignificantly also in the patients with an increase in Hcy level (94.5±11.9 → 91.7±10.3) but remained significantly (p<0.05) higher both at baseline and at the end of the study, compared with the patients without adverse changes in Hcy level.

When logistic regression analysis was fit for a variable with a value of 1 when Hcy had increased by at least 2 μmol/l and with a value of zero in all other cases, only the decrease in serum folic acid appeared to be a significant predictor (OR = 0.71 per unit with 95% CI 0.50–0.99) independently of the drug used or the changes in creatinine levels.

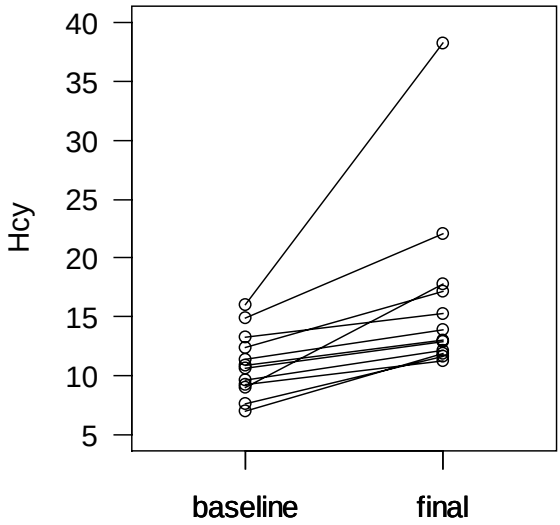

Figure 4. Individual changes in plasma homocysteine level at baseline and at the end of the study in patients with ≥ 2 μmol/l increase in homocysteine level.

5.3.4. Discussion

The primary goal of antihypertensive treatment is not merely to decrease increased BP, but also to prevent clinical complications. Any additional information about the responses of a particular patient to such treatment will contribute to the achieving of this goal.

Antihypertensive treatment with either candesartan or amlodipine did not alter Hcy concentration but it decreased the grade of cellular OxS in most individuals studied. To date, the data of the effect of antihypertensive treatment Hcy have been inconsistent, even conflicting. Sharabi *et al.* found in their observational study that treatment with antihypertensive drugs did not affect plasma Hcy concentrations, but patients who were receiving β-blockers, ACE inhibitors, diuretics and nitrates tended to have lower concentrations of plasma Hcy. Patients treated with α-blockers or calcium channel blockers tended to have higher Hcy values than untreated patients (1999). However, it was not stated whether the patients received monotherapy or a drug combination. In a randomised study in which captopril and hydrochlorthiazide were compared, 4 weeks of treatment with the ACE inhibitor captopril induced an insignificant (0.8 μmol/l) increase in Hcy concentration (Westphal *et al.* 2003).

An important finding of our study was that one subgroup of the study population revealed adverse alterations with regard to Hcy concentrations during antihypertensive treatment with either candesartan or amlodipine. Some

patients (26%) exhibited an increase in Hcy concentration, with a concurrent decrease in folic acid, first in the serum, and thereafter in RBCs by week 16. In the remaining 74% of hypertensive patients, the concentrations of Hcy and folic acid were not altered and a concomitant decrease occurred in cellular OxS.

Hcy metabolism and folic acid metabolism are closely related: folic acid deficit is the most common water-soluble vitamin deficiency in Europe, and a decrease in folic acid concentration leads to an increase in Hcy concentration (Stanger *et al.* 2003). In the present study no dietary restrictions were imposed on the patients, however, a limitation is that we did not record the dietary habits of the study subjects. Whether the decrease in folic acid concentration in some patients was the result of a limited use of fresh fruits and vegetables, or whether it was associated with antihypertensive medication is difficult to establish.

The effect of candesartan on Hcy or GSH in uncomplicated non-diabetic hypertensive patients has not been studied before. There exist no data as to the relationship between treatment with amlodipine and the concomitant changes in Hcy. In a randomised study of hypertensive patients with type 2 diabetes, no statistically significant changes were detected in Hcy after 1 month (–0.3 μmol/l) or 12 months (–0.9 μmol/l) of treatment with candesartan (Derosa *et al.* 2003). In the Framingham Offspring Study, the individuals who used antihypertensive medication had higher plasma Hcy concentrations than those who were not taking such medications. The increase in Hcy was not likely to have resulted from impaired renal function because the association was completely unaffected by the adjustment for serum creatinine concentrations (Jacques *et al.* 2001). The same is partially valid for our study. The patients with an increase in Hcy concentration had significantly higher creatinine values than those in whom such changes in Hcy were not recorded. However, creatinine decreased in both Hcy groups. It should be reminded that all studied patients had normal creatinine values before entering the study.

Both drugs were also similar regarding their renal effect. A significant decrease in serum creatinine occurred in both study groups. An experimental study demonstrated that that inhibition of AT II by candesartan had protective effects on glomerular damage, which extended beyond the hemodynamics and involved down-modulation of glomerular inflammation, reduction of mesangial cell proliferation and a decrease in chemokine expression (Perez de Lema *et al.* 2003). This seems to be a class effect, because in a randomised trial another AT_1 receptor blocker, losartan, also appeared to be renoprotective irrespective of its antihypertensive action (Iino *et al.* 2003). In a randomised placebo-controlled study in normotensive renal transplant recipients, amlodipine reduced serum creatinine only 8 weeks after treatment (Venkat Raman *et al.* 1999). In animal experiments, the favourable effects of amlodipine have also been attributed to an increase in eNOS activity (Tojo *et al.* 1996), which was accompanied by an improvement in the parameters of the microvasculature (Tojo *et al.* 1996, Kobayashi *et al.* 1999).

The decrease in OxS that we observed during antihypertensive treatment is consistent with the findings of several previous studies. A randomised placebo-controlled study demonstrated a significant reduction in OxS, determined by malondialdehyde in the case of candesartan (Koh *et al.* 2003) and by products of lipid peroxidation, free radicals and hydroperoxides in addition to total antioxidant capacity in the case of amlodipine (Digiesi *et al.* 2000), during antihypertensive treatment.

To date, scarce and even conflicting data have been reported about the changes in cellular GSH concentration during antihypertensive treatment, although an association has been established between hypertension and cellular GSH (Nemeth *et al.* 2001, Donmez *et al.* 2002, Turi et al. 2003). It was shown in a randomised study that long-term treatment (6 months) with enalapril reduces GSH concentration significantly, whereas no change was noted in patients treated with captopril (Golik *et al.* 1995). The impact of antihypertensive treatment with AT_1 blockers in humans has been evaluated in a randomised study of losartan, in which GSH increased significantly (Donmez *et al.* 2002). However, such studies have not been undertaken with candesartan. No human studies have been conducted on changes in cellular GSH occurring during antihypertensive treatment with amlodipine. In our study, GSH did not change in any of the groups studied.

The glutathione redox status is crucial in maintaining cellular function and viability (Jefferies *et al.* 2003). Several case-control studies have reported an approximately twofold higher glutathione redox ratio in hypertensive patients compared with normotensive controls, indicating an imbalanced/altered cellular glutathione system (Nemeth *et al.* 2001, Donmez *et al.* 2002, Turi *et al.* 2003). This finding was also a feature of our study. Comparison of the hypertensive patients at baseline with the normotensive controls showed that the former had a disturbed intracellular antioxidative status besides to increased plasma Hcy concentration. In the present study, favourable changes in the glutathione redox ratio and in GSSG concentration were detected in both treatment groups. In the hypertensive individuals, GSSG concentrations almost attained the values found in the normotensive controls. The decrease in GSSG concentration was associated with the decrease in DBP but not with the decrease in SBP. It should be noted that when changes in Hcy concentration according to our criteria were taken into account favourable changes in the cellular OxS markers were significant only in the patients in whom Hcy concentration did not increase.

Both candesartan and amlodipine were effective in decreasing BP in the patients with mild to moderate essential hypertension. This is consistent with the findings of a previous randomised study where both of these drugs were highly effective in controlling BP in patients with essential hypertension (Kloner *et al.* 2001). Furthermore, it should be noted that neither the efficacy of antihypertensive treatment nor the need to double the treatment dose was related to the adverse changes in plasma Hcy or folic acid concentrations. Whether such alterations were attributable to the genetic polymorphism of enzymes,

important for Hcy or GSH metabolism, or whether there exist some other involved mechanisms is not clear.

Another limitation of our study is that we did not include a placebo arm. Therefore, there may arise the question whether the changes in the biochemical parameters were indeed attributable to antihypertensive treatment. It is known that Hcy has low intraindividual variability (Stanger *et al.* 2003), and the same is valid for GSH and GSSG (intra-assay precision for GSH and GSSG is 7.7%, total precision 9.4%). All our patients were clinically stable and the majority of them had been untreated for several years. Thus we presume that the changes in the studied parameters were not caused by factors other than the treatment used.

5.4. The effect of antihypertensive treatment on lipid peroxidation, serum lipids and the impact of the change in homocysteine level on these markers (Paper IV)

5.4.1. Serum lipids and lipid peroxidation in hypertensive patients and changes in these markers during antihypertensive therapy

The study variables for the hypertensive patients at baseline and during treatment are shown in Table 9. Throughout the study, the treatment groups did not differ with regard to the parameters studied. According to multilevel linear regression analysis, serum TG and T-Chol levels did not change significantly throughout the study in either treatment group. A substantial and significant decrease ($p<0.0001$) occurred in OxLDL level reaching almost the upper kit reference value (Figure 5). The elevation of mean CD (Table 9) did not exceed the population-based upper reference limit for CD (45 μmol/l, data on the file). LDL-BDC and LDL-BDC-/LDL (Table 9) did not change significantly, remaining within reference limits (10.1–29.9 μmol/l for LDL-BDC and 3.1–7.9 μmol/mmol for LDL-BDC/LDL).

The changes in OxLDL and CD were not correlated with the changes in BP. The changes in LDL-BDC were positively associated with the changes in DBP ($r=0.30$, $p=0.045$). The changes in the LDL-BDC/LDL ratio were positively correlated with the changes in DBP ($r=0.39$, $p<0.01$), and there was positive correlation with borderline statistical significance with the changes in SBP ($r=0.28$, $p=0.058$). After adjusting for the treatment used, the correlations between the changes in LDL-BDC/LDL ratio and the changes in SBP and DBP remained significant.

Table 9. Changes in serum lipids and lipid peroxidation markers during antihypertensive treatment with candesartan or amlodipine.

Variable	Candesartan baseline	Candesartan week 2	Candesartan week 16	Amlodipine Baseline	Amlodipine week 2	Amlodipine week 16
T-Chol, mmol/l	5.4 ± 0.7	5.5 ± 1.0	5.3 ± 0.9	5.5 ± 0.8	5.5 ± 0.8	5.4 ± 0.9
LDL, mmol/l	3.6 ± 0.7	3.6 ± 0.8	3.6 ± 0.6	3.5 ± 0.8	3.4 ± 0.8	3.5 ± 0.9
HDL, mmol/l	1.3 ± 0.3	1.4 ± 0.2	1.4 ± 0.3	1.5 ± 0.4	1.6 ± 0.4	1.5 ± 0.4
TG, mmol/l	1.2 (1.0; 1.5)	1.3 (1.2; 1.6)	1.3 (1.1; 1.9)	1.1 (0.9; 1.7)	1.1 (0.8; 1.9)	1.2 (1.0; 1.8)
LDL-BDC, μmol/l	18.8 (16.9; 22.6)	18.8 (16.9; 22.6)	18.9 (17.2; 22.8)	18.8 (16.3; 27.1)	17.3 (14.4; 2.9)	17.6 (15.2; 24.7)
CD, μmol/l	33.2 (30.5; 41.6)	35.9 (33.2; 41.2)	39.8 (36.5; 46.4)*	33.7 (29.1; 47.1)	34.6 (28.6; 47.5)	41.3 (36.9; 50.2)*
LDL-BCD/LDL, μmol/mmol	5.4 (4.9; 6.4)	5.9 (5.3, 7.2)	5.4 (4.8; 6.6)	5.6 (4.8; 8.2)	5.2 (4.3; 7.0)	5.2 (4.5; 7.4)

* Denotes a significant difference from the baseline value. $p<0.05$ was considered significant.

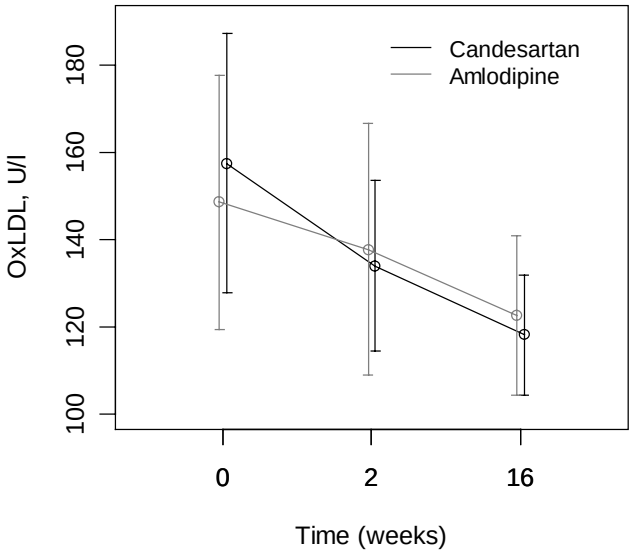

Figure 5. Oxidized low-density lipoprotein (mean with 95% CI) in the hypertensive patients treated with candesartan or amlodipine at different time points (in both treatment groups, p for trend <0.0001).

5.4.2. Associations between the changes in homocysteine, lipids and the lipid peroxidation markers

Changes in the lipid peroxidation markers and serum lipids as depending on the changes in Hcy level are shown in Table 10.

At baseline, the patients with an increase in Hcy level had significantly higher LDL-BDC and LDL-BDC/LDL levels. In the patients with an increase in Hcy level, OxLDL decreased but this change was statistically insignificant.

In the patients without an increase in Hcy level, OxLDL level decreased significantly, falling below the upper kit reference limit. In these patients mean CD level increased statistically significantly, not exceeding endemic normal. Groups were similar with regard to other study variables.

Table 10. Changes in the lipid peroxidation markers and serum lipids as depending on the changes in Hcy level during antihypertensive treatment

Variable		Increase in Hcy ≥ 2 μmol/l	No change or decrease in Hcy
OxLDL, U/l	baseline	163.0 ± 88.0	149.7 ± 60.0
	week 16	132.4 ± 38.0	115.9 ± 35.6 *
CD, μmol/l	baseline	35.6 (26.2; 58.4)	32.7 (30.8; 39.6)
	week 16	41.5 (33.4; 55.6)	40.2 (37.7; 45.8)*
LDL-BDC, μmol/l	baseline	25.4 (18.8; 35.9)	17.1 (16.0; 21.1)#
	week 16	22.7 (17.4; 32.5)	17.3 (16.0; 21.1)
LDL-BDC/LDL,	baseline	7.3 (5.6; 9.9)	5.0 (4.6; 6.4)#
μmol/mmol	week 16	6.6 (5.2; 9.3)	5.0 (4.6; 6.3)
Chol, mmol/l	baseline	5.6 ± 0.9	5.4 ± 0.7
	week 16	5.5 ± 1.0	5.3 ± 0.9
LDL, mmol/l	baseline	3.5 ± 0.7	3.5 ± 0.7
	week 16	3.5 ± 0.8	3.5 ± 0.8
HDL, mmol/l	baseline	1.4 ± 0.4	1.4 ± 0.3
	week 16	1.4 ± 0.4	1.5 ± 0.3
TG, mmol/l	baseline	1.5 (1.1; 2.5)	1.0 (1.0; 1.2)
	week 16	1.4 (0.9; 2.6)	1.1 (1.1; 1.5)

* denotes a significant difference from the baseline value within the study group.
# denotes a significant differences between the study groups. The level of significance was defined as $p<0.05$.

5.3.3. Discussion

The main finding of the present study was that OxLDL levels decreased substantially and to an equal degree in both treatment groups, reaching almost the upper normal reference limit (117 U/l). In subgroup analysis, regarding the change in Hcy level, this favourable change occurred only in the patients without an increase in Hcy level. Another finding was that neither LDL-BDC and LDL nor their ratio (LDL-BDC/LDL) changed during antihypertensive treatment with candesartan or amlodipine. This is the first study to study the impact of candesartan and amlodipine on LDL-BDC level. Similarly, the effect of candesartan on CD has not been studied before. The ratio of LDL-BDC to LDL is thought to characterize the degree of LDL oxidation (Brizzi *et al.* 2004) and hence indicating stability of the LDL particle. The fact that LDL-BDC, LDL-BDC/LDL and CD were within endemic norm throughout the study indicates that both studied drugs are neutral in this regard. This is consistent with previous findings where both candesartan and amlodipine were reported to be similar to placebo with regard to plasma lipid levels (McClellan and Goa

1998, Haria and Wagstaff 1995). It shows that the positive effects of either drug did not involve the predominating lipid peroxidation-targeted influence on OxLDL levels.

Our study confirms the high antihypertensive efficacy of both candesartan and amlodipine, well known from several previous reports (Haria and Wagstaff 1995, McClellan and Goa 1998, Kloner *et al.* 2001). The decrease in OxLDL levels was not correlated with the changes in BP irrespective of the drug used. It has been reported that the use of AT_1 receptor blocker losartan prevents or retards atherosclerosis beyond reduction in BP (Dahlöf *et al.* 2002). Dohi *et al.* showed that candesartan reduces OxS and inflammation in patients with essential hypertension independently of its effects on BP (2003). It has been demonstrated in hypertensive patients with type II diabetes that use of amlodipine is associated with the slowing down of progression of carotid atherosclerosis irrespective of BP changes (Pitt *et al.* 2000). In the current study, irrespective of the drug used, the decrease in BP was associated with the decrease in the LDL-BDC/LDL ratio suggesting that lowering of BP may have LDL particle stabilizing properties. Although the patients with an increase in Hcy level showed a significantly higher LDL-BDC and LDL-BDC/LDL at baseline, further increase was absent.

In the patients without significant increase in Hcy level, CD increased statistically significantly, however these changes have no clinical value as they remained within the reference values for CD.

For both studied drugs (candesartan and amlodipine), several mechanisms are suggested explaining reduction in OxS-related hypertension. Candesartan attenuated the cell-injurious effects of OxLDL (Li *et al.* 2000), restored NO availability and decreased production of ROS in vascular endothelial cells (Desideri *et al.* 2003) as well as lowered OxLDL level in VSMC (Watanabe *et al.* 2001). In hypertensive patients candesartan reduced lipid peroxidation as measured by malondialdehyde (Koh *et al.* 2003).

It is reported that the effects of amlodipine may be mediated in part by the prostanoid endothelium-derived factor and NO, via preservation of endogenous antioxidant activity, via VSMC membrane stabilization, and via endothelial cell protection (Mason 2002). It has been shown in experimental studies that amlodipine is able to suppress oxidisability of LDL *in vitro* (Chen *et al.* 1997) and to inhibit binding of OxLDL lipids to model membranes (Phillips *et al.* 2003). All these findings support the suggestion that although candesartan and amlodipine reduce significantly OxLDL level, evidently, mechanisms other than direct lipid peroxidation suppression may have a stronger impact in the human body.

6. CONCLUSIONS

1. Average plasma homocysteine level is in patients with acute coronary syndrome higher than in controls. Increased homocysteine level, defined as 11 μmol/l (relative hyperhomocysteinemia), has an impact on a new acute coronary event in normotensive patients. In the whole study group as well as in hypertensive patients, hyperhomocysteinemia seems to have no clear role in development of new acute coronary event.

2. Patients with hypertension have significantly higher both homocysteine and intracellular glutathione-related oxidative stress. The relationship between homocysteine, reduced glutathione and systolic blood pressure suggests that both biochemical markers are associated with essential hypertension.

3. Effective antihypertensive treatment with either candesartan or amlodipine does not alter mean homocysteine concentration but it decreases grade of cellular oxidative stress in most persons studied. Proceeding from the possible adverse alterations in homocysteine concentration in about one quarter of the studied hypertensive patients, this study points to the need for an individual approach when antihypertensive treatment is being prescribed.

4. Both candesartan and amlodipine are efficient drugs for reducing oxidized LDL level, remaining neutral with regard to serum lipids. A significant decrease in oxidized LDL level occurs only in patients without increase in homocysteine level. Unchanged level of baseline diene conjugation in circulating LDL particles suggests that mechanisms other than direct lipid peroxidation suppression may have a stronger impact on the human body.

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SUMMARY IN ESTONIAN

HOMOTSÜSTEIIN JA HÜPERTENSIOON: homotsüsteiini ja hüpertensiooni seosed ravitud ja ravimata kaasuva südame isheemiatõvega ja isheemiatõveta hüpertensiooniga patsientidel

Hüpertensioon on oluline, sõltumatu ja modifitseeritav ateroskleroosi riskifaktor (Kannel *et al.* 1986). Sõltumatuks ja oluliseks ateroskleroosi riskifaktoriks peetakse ka hüperhomotsüsteineemiat (Boushey *et al.* 1995, Graham *et al.* 1997).

Südame isheemiatõvega patsientide uuringutes on loetud homotsüsteiini taset kõrgenenuks erinevate väärtuste juures, alates 9 μmol/l (Nygard *et al.* 1997a) kuni vahemikus 11–15 μmol/l (Stampfer *et al.* 1992, Graham *et al.* 1997, Stubbs *et al.* 2000). Ägeda koronaarse sündroomi tekke ja homotsüsteiini taseme vahelise seose osas on andmed vastukäivad. On leitud nii seose puudumist (Voutilainen *et al.* 2000) kui ka esmase südameinfarkti ja ajuinfarkti riski tõusu kõrgemate homotsüsteiini väärtuste juures (Bots *et al.* 1999, Aronow *et al.* 2000).

Seni on vastuseta küsimused homotsüsteiini ja hüpertensiooni seostest nii kaasuva südame isheemiatõvega kui ka ilma isheemiatõveta patsientidel ning on ebaselge, missugusel homotsüsteiini tasemel need seosed ilmnevad.

Hüpertensiooni ja homotsüsteiini seoseid tuleb uurida arvestades klassikaliste riskifaktorite (vere lipiidid, kehakaal), homotsüsteiini metabolismis oluliste kofaktorite (vitamiinid B_6, B_{12} ja foolhape) ja ka ülemäärase oksüdatiivse stressi osakaalu. Oksüdatiivse stressi hindamisel ei piisa ainult ühe markeri määramisest (Dotan *et al.* 2004), vaid tuleb mõõta nii antioksüdantseid, nt. glutatioon, kui ka prooksüdantseid markereid, nt. oksüdeeritud madala tihedusega lipoproteiide. Mehhanismide hulgas, mille kaudu hüpertensioon viib lõpporganite kahjustuse ja ateroskleroosini, peetakse oluliseks nii pikaajalist väljendunud oksüdatiivset stressi (Alexander 1995, Russo *et al.* 1998) kui ka plasma homotsüsteiini taseme kõrgenemist (Kanani *et al.* 1999, Virdis *et al.* 2001). On näidatud, et homotsüsteiini endoteeli kahjustav toime on seotud kõrgenenud oksüdatiivse stressiga (Kanani *et al.* 1999, Virdis *et al*. 2001). Homotsüsteiini taseme kõrgenemine soodustab ka lipiidset peroksüdatsiooni vabade radikaalide produktsiooni suurenemise kaudu (Splaver *et al.* 2004).

Homotsüsteiin ja rakusisene võimsaim antioksüdant glutatioon on tihedalt seotud. Koekultuuril on näidatud, et ligikaudu pool glutatioonist toodetakse homotsüsteiinist tekkiva tsüsteiini baasil. Homotsüsteiini taseme kõrgenemine on tihti seotud foolhappe ja vitamiinide B_6 ja B_{12} madala tasemega (Stanger *et al.* 2003). Kuivõrd homotsüsteiini ja glutatiooni seoseid on uuritud eelkõige koekultuuridel, pole teada, kas need kehtivad inimorganismis.

Antihüpertensiivsed ravimid erinevad oma toimelt homotsüsteiini, glutatiooni ja lipiidse peroksüdatsiooni tasemele. Võimsa rakusisese protektori, redutseeritud glutatiooni, langus või plasma homotsüsteiini taseme tõus on

osade antihüpertensiivsete ravimite ebasoodsateks kõrvalmõjudeks. Seni teadaolevad andmed antihüpertensiivse ravi mõjust homotsüsteiini tasemele on vastukäivad: kirjeldatud on nii muutuse puudumist ja homotsüsteiini taseme langust (Korkmaz *et al.* 2003, Westphal *et al.* 2003) kui ka homotsüsteiin taseme tõusu (Westphal *et al.* 2003).

Nii angiotensiin II esimest tüüpi retseptorite (AT_1) antagonistid kui kaltsiumikanalite blokaatorid kuuluvad hüpertensiooni ravis esmavaliku preparaatide hulka (Cifkova *et al.* 2003). Mõlema ravimrühma esindajatel võib olla ka endoteeli funktsiooni parandav ja ilmselt ka teatud antioksüdatiivne toime (Digiesi *et al.* 2000, Koh *et al.* 2003). Antihüpertensiivsete ravimite puhul ei ole homotsüsteiini ja glutatiooni taseme muutusi samaaegselt seni määratud. Selles, kuidas võivad AT_1 retseptorantagonist kandesartaan ja kaltsiumikanalite blokaator amlodipiin mõjutada homotsüsteiini ja tema metabolismiga seotud faktoreid, on veel mitmeid vastamata küsimusi.

Uurimuse eesmärgid

1. Uurida ägeda koronaarse sündroomiga patsientidel hüperhomotsüsteineemia levimust, seost teiste kardiovaskulaarsete riskifaktoritega ning hüperhomotsüsteineemia ja hüpertensiooni koosmõju korduva ägeda koronaarse sündroomi tekke suhtes.

2. Uurida plasma homotsüsteiini ja rakusisese glutatiooni omavahelisi seoseid ning mõlema markeri seoseid vererõhuga et teha kindlaks, kas koekultuuril leitud seosed homotsüsteiini ja glutatiooni vahel kehtivad ka inimorganismis.

3. Hinnata antihüpertensiivse ravi AT_1 retseptorantagonisti kandesartaani ja kaltsiumikanalite blokaatori amlodipiini mõju plasma homotsüsteiini ja tema metabolismis oluliste vitamiinida tasemele, lipiidse peroksüdatsiooni markeritele ning rakusisese glutatiooni ja tema redoks-suhte tasemele.

Uuritavad ja meetodid

Uuriti 107 ägeda koronaarse sündroomiga patsienti ja 65 essentsiaalse arteriaalse hüpertensiooniga patsienti. Kontrollrühma moodustasid 76 normaalse vererõhuga tervet isikut.

Ägeda koronaarse sündroomiga patsientidest jäeti uuringust välja 11 isikut neerude funktsiooni languse või kaasuvate pahaloomuliste kasvajate tõttu. Uuritud patsientidest diagnoositi esmast müokardiinfarkti 54, korduvat 17 ja ebastabiilset stenokardiat 25 patsiendil.

Kerge kuni keskmise raskusega hüpertensiooniga (süstoolne vererõhk 140–179 mmHg, diastoolne vererõhk 90–109 mmHg) patsientidel (59 meest, 6 naist,

vanus 40–65 aastat) ei olnud südame isheemiatõve sümptomeid (anamneesi, EKG ja koormustesti alusel), hüperkolesteroleemiat, diabeeti ega teisi olulisi kaasuvaid haigusi. Anamneesi, läbivaatuse ja uuringute alusel (sh neeruarterite doppler-uuring) välistati sekundaarse hüpertensiooni võimalus. Neid patsiente, kes eelpooltoodud kriteeriumitele ei vastanud (N=34), uuringusse ei kaasatud.

Ägeda koronaarse sündroomiga patsientidel määrati plasma homotsüsteiini tase.

Esimeses hüpertensiooniga patsientide grupis määrati 48 patsiendil (neist 16-l määrati uuringu parameetrid ainult ravi eelselt, 32 said hiljem uuringu ravimeid) plasma homotsüsteiini ning rakusisese oksüdeeritud ja redutseeritud glutatiooni tase ning uuriti homotsüsteiini ja rakusisese glutatiooni omavahelisi seoseid, samuti mõlema näitaja seoseid vererõhuga. Teises haigete grupis (49 patsienti) raviti randomiseeritud topeltpimedas topelt-kaetud uuringus patsiente 16 nädalat kandesartaaniga 8–16 mg päevas või amlodipiiniga 5–10 mg päevas. Uuriti kandesartaani ja amlodipiini mõju plasma homotsüsteiinile, tema metabolismis olulistele vitamiinidele, lipiidse peroksüdatsiooni näitajatele ja rakusisese glutatiooni tasemele. Retrospektiivselt jaotasime hüpertensiooniga patsientide rühma kaheks vastavalt plasma homotsüsteiini taseme tõusule ≥2 μmol/l võrra 16. ravinädalal võrreldes esialgsega või sellise muutuse puudumisele. Lugesime homotsüsteiini taseme tõusu vähemalt 2 μmol/l võrra oluliseks, kuna enamuses seni avaldatud uuringutes on 2 μmol/l vahemik, mis eristab hüpertensiooniga patsiente tervetest (Nemeth *et al.* 2001, Turi *et al.* 2003). Saksamaa, Austria ja Šveitsi Homotsüsteiini Ühingute 2003. aastal avaldatud ühissoovituses eristab see vahemik soovitavaid homotsüsteiini taseme ülempiire tervete ning kõrgenenud kardiovaskulaarse riski/ olemasoleva kardiovaskulaarse haigusega isikute osas (Stanger *et al.* 2003). Lisaks keskmiste võrdlemisele nii erinevate ajapunktide kui rühmade vahel, on arvutatud ka kõigi korduvalt mõõdetud parameetrite osas muutused ravi alguse ja lõpu vahel ning on analüüsitud nende muutuste omavahelisi seoseid.

Ensüümimmuunmeetodil määrati homotsüsteiini ja oksüdeeritud LDL (oksüdatiivse stressiga seotud ateroskleroosi ja põletiku marker) tase. Lipiidse peroksüdatsiooni näitajatena määrati seerumis spektrofotomeetriliselt konjugeeritud dieenide (lipiidide peroksüdatsiooni marker) ja tsirkuleerivates LDL partiklites baasdieenkonjugaatide (LDL peroksüdeerituse marker) tase.

Klassikalisel ensüümmeetodil määrati erütrotsüütides oksüdeeritud glutatiooni (oksüdatiivne stressor) ja redutseeritud glutatiooni (antioksüdant ja redutseeriv jõud) tase. Glutatiooni redoks-suhe (rakusisese oksüdatiivse stressi näitaja) on saadud oksüdeeritud glutatiooni väärtuse jagamisel redutseeritud glutatiooni väärtusega. Vitamiin B_{12} ja foolhape määrati kemiluminestsentsmeetodiga.

Uurimuse peamised tulemused

Homotsüsteiini tase oli ägeda koronaarse sündroomiga patsientidel oluliselt kõrgem kui kontrollgrupil ja seostus normotensiivsetel patsientidel uue ägeda koronaarsündmuse tekkega. Hüpertooniatõvega patsientidel seosed homotsüsteiini ja uue ägeda koronaarse sündroomi tekke vahel puudusid. Suure tõenäosusega on kaasuv hüpertooniatõbi ise nii tugev riskifaktor, et homotsüsteiini mõju ei tulnud esile.

Ravimata ja oluliste muude kaasuvate haigusteta hüpertensiooniga patsientide võrdlemisel kontrollrühmaga selgus, et patsientidel oli oluliselt kõrgem homotsüsteiini, oksüdeeritud glutatiooni tase ja glutatiooni redoks-suhe ning madalam redutseeritud glutatiooni tase. Erütrotsüütides ja seerumis määratud foolhappe ning vitamiin B_{12} taseme alusel patsiendid tervetest ei erinenud. Seega homotsüsteiini taseme kõrgenemise põhjuseks ei ole tema metabolismis oluliste vitamiinide defitsiit.

Hüpertensiooniga patsientidel esines negatiivne seos redutseeritud glutatiooni, süstoolse vererõhu ja erütrotsüütides määratud foolhappe vahel. Leitud negatiivne seos redutseeritud glutatiooni ja süstoolse vererõhu vahel viitab sellele, et glutatiooni taseme langus võib omada olulist rolli hüpertensiooni patogeneesis. Lineaarset seost glutatiooni ja homotsüsteiini vahel ei olnud, kuid regressioonanalüüsil seostus redutseeritud glutatioon positiivselt homotsüsteiiniga ja negatiivselt süstoolse vererõhuga. Kontrollrühma uuritavatel leidsime negatiivse lineaarse seose redutseeritud glutatiooni ja homotsüsteiini ning rakusisese foolhappe taseme vahel. Rakusisese redutseeritud glutatiooni taset mõjutavad nii süstoolne vererõhk kui ka homotsüsteiin, mis viitab tugeva metaboolse seose olemasolule homotsüsteiini ja redutseeritud glutatiooni vahel ka inimorganismis.

Antihüpertensiivne ravi kandesartaani või amlodipiiniga alandas oluliselt vererõhku, kuid ei mõjutanud oluliselt keskmist plasma homotsüsteiini taset. Mõlemas ravimirühmas langes oluliselt oksüdeeritud glutatiooni tase; glutatiooni redoks-suhte osas esines langustendents. Vitamiinide tase oluliselt ei muutunud.

Mõlemal uuritud ravimil oli soodne toime oksüdeeritud madala tihedusega lipoproteiinide tasemele, mis langes praktiliselt kiti ülemise normväärtuseni. Konjugeeritud dieenide taseme mõningane tõus ei ületanud normi ülemist piiri. Baasdieenkonjugaatide tase tsirkuleerivates madala tihedusega lipoproteiidides ei muutunud. Seega oksüdeeritud madala tihedusega lipoproteiidide taseme oluline langus võib olla seotud muude mehhanismidega kui otsene lipiidse peroksüdatsiooni vähendamine.

Süstoolse vererõhu langus seostus diastoolse vererõhu langusega, kuid ei sõltunud biokeemiliste näitajate muutustest. Diastoolse vererõhu langus seostus nii süstoolse vererõhu kui ka oksüdeeritud glutatiooni taseme langusega ning erütrotsüütides määratud foolhappe tõusuga. Oksüdeeritud madala tihedusega lipoproteiinide langus vererõhu muutustega ei seostunud.

Kuigi keskmine homotsüsteiini tase ei muutunud, esines homotsüsteiini taseme kõrgenemine 2 μmol/l võrreldes esialgse väärtusega 12 patsiendil (5 kandesartaani ja 7 amlodipiini rühmas). Neil patsientidel kaasnes ka oluline foolhappe taseme langus ning puudusid soodsad nihked nii rakusisese kui ka plasma oksüdatiivse stressi näitajate osas. Ülejäänud patsientidel, kellel homotsüsteiini tase oluliselt ei muutunud või langes, kaasnes ka oluline oksüdeeritud glutatiooni ja glutatiooni redoks-suhte ning plasma oksüdeeritud madala tihedusega lipoproteiidide taseme langus. Vererõhu muutuste osas homotsüsteiini taseme alusel jaotatud patsientide rühmad omavahel ei erinenud.

Kuna esines patsientide rühm (26%), kellel soodsad muutused oksüdatiivse stressi näitajate osas puudusid, viitab meie uuring vajadusele jälgida hüpertensiooniga patsiente antihüpertensiivse ravi ajal homotsüsteiini ja foolhappe taseme osas.

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PUBLICATIONS

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Teadustegevus

Põhiliseks uurimisvaldkonnaks on olnud homotsüsteiini ja oksüdatiivse stressi seosed hüpertooniatõvega patsientidel nende näitajate muutumine antihüpertensiivse raviga. 9 teaduslikku artiklit, 16 ettekannet rahvusvahelistel konverentsidel.

Eesti Kardioloogide Seltsi ja Eesti Hüpertensiooni Ühingu liige.