Homocysteine

in cardiovascular disease with
special reference to longitudinal changes

Johan Hultdin

Umeå 2005
Anything that happens, happens.

Anything that, in happening, causes something else to happen, causes something else to happen.

Anything that, in happening, causes itself to happen again, happens again.

It doesn’t necessarily do it in chronological order, though.

Douglas Adams
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# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>CBS</td>
<td>Cystathione β-synthase</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence Interval</td>
</tr>
<tr>
<td>CT</td>
<td>Computerised Tomography</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular Filtration Rate</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Pressure Liquid Chromatography</td>
</tr>
<tr>
<td>MONICA</td>
<td>Monitoring of Trends and Determinants in Cardiovascular Disease</td>
</tr>
<tr>
<td>MTHFR</td>
<td>Methylene tetrahydrofolate reductase</td>
</tr>
<tr>
<td>OR</td>
<td>Odds Ratio</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction Fragment Length Polymorphism</td>
</tr>
<tr>
<td>SAH</td>
<td>S-Adenosylhomocysteine</td>
</tr>
<tr>
<td>SAM</td>
<td>S-Adenosylmethionine</td>
</tr>
<tr>
<td>SD</td>
<td>Standard Deviation</td>
</tr>
<tr>
<td>tHcy</td>
<td>Total Plasma Homocysteine</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofolate</td>
</tr>
<tr>
<td>VIP</td>
<td>Västerbotten Intervention Project</td>
</tr>
<tr>
<td>Vitamin B2</td>
<td>Riboflavin</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>Pyridoxine</td>
</tr>
<tr>
<td>Vitamin B9</td>
<td>Folate</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>Cobalamin</td>
</tr>
</tbody>
</table>
ABSTRACT

Abnormalities in homocysteine metabolism have been suggested as risk factors for stroke and myocardial infarction. In retrospective studies, elevated levels of total plasma homocysteine (tHcy) and/or methylenetetrahydrofolate reductase (MTHFR) 677C>T polymorphism have indicated an increase in risk. However, the fewer prospective studies have not been as conclusive. To further explore this, tHcy was studied in four prospective settings.

The first was a prospective nested case-referent cohort within the Västerbotten Intervention Program (VIP) and WHO MONICA project on 312 ischemic and 60 haemorrhagic first-ever strokes. The aim was to study tHcy and its main genetic determinant MTHFR. Risk for haemorrhagic stroke increased exponentially through tHcy quartiles, independent of hypertension and BMI, and increased for MTHFR 677 CT and TT. MTHFR 1298A>C appeared to be protective. In multivariate models, after adjustment for tHcy, BMI and hypertension, both tHcy and MTHFR remained as independent predictors for hemorrhagic stroke. Neither tHcy, nor the two MTHFR polymorphisms were significant predictors for ischemic strokes.

The second was a prospective long-term follow-up study within the VIP and MONICA cohorts to determine whether a first-ever myocardial infarction (AMI) causes increased levels of tHcy. Fifty cases developing AMI after the first screening participated in a second screening (mean follow-up 8.5 years) with 56 matched referents. Increase in tHcy did not differ between cases and referents. tHcy was related to AMI at follow-up, but not at baseline and no longer significant after adjusting for creatinine and albumin.

The third was a method study to determine if cystatin C, creatinine, albumin and other lipoprotein risk markers of cardiovascular disease could be analysed in Stabilyte™ plasma stored at -80°C. It was found to be suitable for all analyses tested and using this tube would simplify sampling for epidemiological studies.

The fourth study was a prospective longitudinal long-term study of 735 subjects (340 men and 395 women, age 25-64 at first screening), participating in two MONICA screenings nine years apart, who donated blood in Stabilyte™ tubes to study change over time in tHcy and its determinants. We confirmed the age dependency in a cross sectional setting. In contrast, if followed longitudinally over time, no change in tHcy or in the prevalence of hyperhomocysteinemia was found. Cystatin C and creatinine increased, and albumin decreased. In multivariate models baseline levels of albumin, creatinine, cystatin C, and to some extent hs-CRP, were predictors of tHcy at follow-up but gender differences were seen. Age was not a major determinant of change in tHcy over nine years.

In conclusion, tHcy and MTHFR are risk factors for first-ever haemorrhagic, but not ischemic stroke in a prospective setting. A first myocardial infarction does not cause an increase in tHcy. No long-term changes were seen in tHcy over a nine-year period in neither men, nor in women.

Key words: homocysteine, methylenetetrahydrofolate reductase (MTHFR), first-ever stroke, first-ever myocardial infarction, longitudinal, prospective, risk factor.
LIST OF ORIGINAL PAPERS


II. Hultdin J, Thøgersen AM, Jansson JH, Nilsson TK, Weinehall L and Hallmans G. 
Elevated plasma homocysteine: cause or consequence of myocardial infarction? 

III. Hultdin J and Nilsson TK. Analysis of cystatin C, creatinine, albumin, lipids and lipoprotein concentrations in serum and acidified citrate plasma (Stabilyte) tubes compared. 
Clinical Chemistry and Laboratory Medicine 2004;42(8):978-81.


Paper II reprinted with permission from Journal of Internal Medicine and paper III with permission from Clinical Chemistry and Laboratory Medicine.
INTRODUCTION

Homocysteine was first identified in patients in 1962. These individuals had homocystinuria, an inborn error of metabolism. In this disease homocysteine levels are very high and homocysteine is excreted in the kidneys and forms the disulfide homocystin in urine. The enzyme defect was identified in cystathionine β-synthase by Mudd et al. This group of patients showed some common clinical features, such as premature atherosclerosis, stroke, and other forms of thromboembolism, as well as osteoporosis and mental retardation. Over 50% of the patients had recurrent cardiovascular events and approximately 25% died before the age of 30. In the late 1960s homocysteine was first detected in plasma of these subjects. More sensitive techniques were introduced and in the mid-1980s methods had become sensitive enough for clinical applications. After this, there has been a remarkable increase in the number of articles published on homocysteine for every year (Figure 1), with 9974 references found on PubMed in the years 1950-2004 with the search term “homocysteine” (search performed 2005-03-31). Searching for “MTHFR” yielded 1857 references and the combination “homocysteine and MTHFR” yielded 944 hits. Limiting the searches to humans resulted in 6406, 1690 and 860 references respectively. Many studies have focussed on homocysteine and cardiovascular diseases, but other diseases such as cancer, dementia, birth defects, spontaneous abortions, osteoporosis and a large number of other diseases have also been studied. The main indication for testing homocysteine in Scandinavia has been to determine deficiency of vitamin B12 and folate. In Umeå total homocysteine was introduced as a routine test in 1992 with a steady increase in volume since then (Figure 2), and a concurrent slight decrease in the number of tests for B12 and folate ordered. In 2004 more than 26,000 homocysteine analyses (from our catchment area of 256,875 inhabitants 2004-12-31) were done for health care indications.
Figure 1. All references and human studies on “homocysteine” found on PubMed for the years 1950-2004.

Figure 2. Number of plasma/serum tests for homocysteine and B12 per year (Folate is of the same magnitude as B12 and thus not presented in the figure). As the numbers of B12 and folate tests ordered are virtually identical, only the data for B12 is presented. The number of requested Erc-Folate tests has been below 200 every year and has now petered out. After 2000 only four tests were ordered. This analysis can no longer be ordered from our laboratory.
The homocysteine metabolism is a complex process that involves the metabolism of methionine and folate. The metabolism includes a methionine cycle and a very closely linked folate cycle (Figure 3).

In the methionine cycle, methionine is taken up from proteins. Homocysteine is formed from methionine as an intermediary amino acid. Methionine synthase mediates the remethylation to methionine. The enzymatic reaction is dependent on methylcobalamin (vitamin B12) as a cofactor and 5-methyltetrahydrofolate as a methyl donor. Methionine synthase is present in all mammalian cells. If there is a functional deficiency of vitamin B12 and/or folate, the remethylation will be impaired and homocysteine accumulates. There is a second remethylation pathway, utilizing betaine as a methyl donor. This second pathway is only present in the liver and kidneys in some species and is not sufficient to keep up the remethylation if there is a deficiency of vitamin B12 and/or folate. If remethylation is impaired, the result is decreased levels of methionine.

Methionine is metabolized to S-adenosylmethionine (SAM), which is the methyl donor in practically all methylation reactions. Methyl groups need to be transported from folate through remethylation of homocysteine and conversion to SAM for methylation to occur. The major part of the methyl groups are consumed in the synthesis of creatinine. SAM also methylates DNA, proteins, phospholipids, neurotransmitters and is needed for the synthesis of the polyamines spermine and spermidine. After leaving the methyl group, SAM is converted to S-adenosylhomocysteine (SAH), which is then converted to homocysteine.

Homocysteine may be removed from the system via two enzymatic reactions in the transsulfuration pathway to cysteine. The first enzyme is cystathionine β-synthase, a heme-containing enzyme which is dependent on vitamin B6 as cofactor. The second enzyme, the vitamin B6 dependent cystathionine γ-lyase produces cysteine. This transsulfuration pathway is present in the liver, kidneys, small intestine and pancreas. These tissues have a high turnover of glutathione and there is a link between transsulfuration and production of glutathione.

In the folate cycle, methylenetetrahydrofolate reductase (MTHFR) is key enzyme for routing of 5,10-methylenetetrahydrofolate to either DNA-synthesis or to 5-methyltetrahydrofolate, the folate form needed for the remethylation of homocysteine to methionine.

The enzymes of the pathway are listed in Table 1 and are numbered according to Figure 3. The metabolism of methionine and folate is regulated by SAM. A high level of SAM indicates high methylation capacity and acts via negative feedback on MTHFR,
reducing the remethylation by reducing 5-methyltetrahydrofolate. SAM also increases the activity of cystathionine β-synthase, thus removing methylation capacity, i.e. homocysteine.

Figure 3. The methionine metabolic cycle is at the right and the folate cycle at the left. The numbers refers to enzymes, see table 1. MTHFR (no 9) has a key role in diverting folate to methylation to the right, or to DNA-synthesis at the left through the synthesis of thymidine from uracile. Examples of products in need for methylation for synthesis / function are seen in the box.

Homocysteine in plasma

Homocysteine has a very reactive sulphhydryl group. Only a small amount of homocysteine, less than two percent, is found in free form in plasma. The major part, over 80 % is bound to plasma proteins, where albumin binds the most of homocysteine in plasma. The oxidized disulphide form of homocysteine (homocystine) and mixed disulphide homocysteine-cysteine accounts for approximately 10-15 %. In a blood sample all free homocysteine is rapidly bound. Swift handling on ice and addition of acid is needed for the determination of free homocysteine, which makes it impractical for routine use. Analytical methods determining homocysteine usually measure total homocysteine (tHcy) after reduction of the disulphide bonds. Different method may vary in efficacy of this reduction, resulting in differences in the homocysteine levels determined.
Table 1. The enzymes of the methionine and folate cycles. The numbers refer to the positions in Figure 2.

<table>
<thead>
<tr>
<th>No</th>
<th>Enzyme</th>
<th>E.C. #</th>
<th>Trivial name</th>
<th>Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Methionine adenosyltransferase</td>
<td>2.5.1.6</td>
<td>AdoMed synthase</td>
<td>MAT</td>
</tr>
<tr>
<td>2</td>
<td>S-Adenosylmethionine-dependent methyltransferases</td>
<td>2.1.1</td>
<td>Methyltransferase</td>
<td>MT</td>
</tr>
<tr>
<td>3</td>
<td>S-Adenosylhomocysteine hydrolase</td>
<td>3.3.1.1.1</td>
<td>Adenosylhomocysteinease</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>5-Methyltetrahydrofolate: homocysteine methyltransferase</td>
<td>2.1.1.13</td>
<td>Methionine synthase</td>
<td>MS</td>
</tr>
<tr>
<td>5</td>
<td>Betaine:homocysteine methyltransferase</td>
<td>2.1.1.5</td>
<td>-</td>
<td>BHMT</td>
</tr>
<tr>
<td>6</td>
<td>Cystathionine β-synthase</td>
<td>4.2.1.22</td>
<td>-</td>
<td>CBS</td>
</tr>
<tr>
<td>7</td>
<td>Cystathionine γ-lyase</td>
<td>4.4.1.1.</td>
<td>Cystathionase</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>Serine hydroxyl-methyltransferase</td>
<td>2.1.2.1</td>
<td>-</td>
<td>SHMT</td>
</tr>
<tr>
<td>9</td>
<td>5,10-Methylene-tetrahydrofolate reductase</td>
<td>1.1.92.1</td>
<td>-</td>
<td>MTHFR</td>
</tr>
<tr>
<td>10</td>
<td>Thymidylate synthase</td>
<td>2.1.1.45</td>
<td>-</td>
<td>TS</td>
</tr>
<tr>
<td>11</td>
<td>Tetrahydrofolate dehydrogenase</td>
<td>1.5.1.3</td>
<td>Dihydrofolate reductase</td>
<td>DHFR</td>
</tr>
<tr>
<td>12</td>
<td>S-Adenosylmethionine decarboxylase</td>
<td>4.1.1.50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td>Mehiomyl-tRNA synthetase</td>
<td>6.1.1.10</td>
<td>-</td>
<td>AARS</td>
</tr>
<tr>
<td>14</td>
<td>Paraoxonase</td>
<td>3.1.1.2</td>
<td>-</td>
<td>PON1</td>
</tr>
</tbody>
</table>

Polymorphisms influencing methionine and folate metabolism

There is a high number of polymorphisms in the metabolism of methionine and folate. They have effects on enzyme activity or receptor functions. Some polymorphisms are directly involved in the metabolism in Figure 3. Some act on other steps, such as uptake and transport of folate from the intestine to receptor mediated uptake in cells, transport and delivery of vitamin B12, or degradation of homocysteine. For a short overview see Table 2. Apart from these polymorphisms there is a high number of rare mutations resulting in inborn errors of metabolism not listed here. The methylenetetrahydrofolate gene is the most studied gene in this metabolism.
<table>
<thead>
<tr>
<th>Gene, polymorphism</th>
<th>Function</th>
<th>Genotype</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>MTHFR 677C&gt;T and 1298A&gt;C OMIM *607093</td>
<td>Formation of 5-methyltetrahydrofolate.</td>
<td>8.2 % 677TT homozygotes 11.4 % 1298CC homozygotes</td>
<td>677C: 0.71 1298A: 0.68</td>
</tr>
<tr>
<td>Methionine synthase MS 2756A&gt;G OMIM *156570</td>
<td>Mediates the remethylation of homocysteine to methionine</td>
<td>AA: 61 % AG: 29 % GG: 10 %</td>
<td>A: 0.76 G: 0.24</td>
</tr>
<tr>
<td>MTRR 66A&gt;G OMIM *602568</td>
<td>Reactivation of methionine synthase</td>
<td>AA: 28 % AG: 49 % GG: 23 %</td>
<td>A: 0.52 G: 0.48</td>
</tr>
<tr>
<td>BHMT 742G&gt;A OMIM *602888</td>
<td>Remethylation of homocysteine to methionine</td>
<td>GG: 49 % GA: 39 % AA: 12 %</td>
<td>G: 0.68 A:0.32</td>
</tr>
<tr>
<td>CBS 844ins68 OMIM *236200</td>
<td>Converts homocysteine to cystathionine and has effects on methylation capacity.</td>
<td>No insertion: 90 % Heterozygotes: 10 %</td>
<td>No insertion: 0.95 Insertion: 0.05</td>
</tr>
<tr>
<td>PON1- LEU55MET/108C&gt;T and GLN192ARG OMIM *168820:</td>
<td>Functions as a thiolactonase of homocysteine and may lead to the formation of homocysteine thiolactone which may induce protein alteration.</td>
<td>LEU/LEU: 34 % LEU/MET: 53 % MET/MET: 13 % GLN/GLN: 48 % GLN/ARG: 30 % ARG/ARG 22 %</td>
<td>LEU:0.60 MET:0.40 GLN:0.63 ARG:0.37</td>
</tr>
</tbody>
</table>

Continued on next page
Table 2. Continued.

<table>
<thead>
<tr>
<th>Gene, polymorphism</th>
<th>Function</th>
<th>Genotype</th>
<th>Allele frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transcobalamin II TCII C259R, 775G&gt;C</td>
<td>Mediates the uptake of B12 in the cells via the transcobalamin receptor.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMIM *275350</td>
<td></td>
<td>775GG: 30 %</td>
<td>G: 0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>775GC: 50 %</td>
<td>C: 0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>775CC: 20 %</td>
<td></td>
</tr>
<tr>
<td>Glutamate carboxypeptidase II GCP II</td>
<td>Zinc dependent enzyme, degrades polyglutamates to monoglutamates in the intestine.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1571C&gt;T GCP III homologue gene</td>
<td>Uptake of folate in the food.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMIM *600934</td>
<td></td>
<td>CC: 89.6 %</td>
<td>C: 0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT: 10.1 %</td>
<td>T: 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT: 0.3 %</td>
<td></td>
</tr>
<tr>
<td>Reduced folate carrier I RFC-1 80A&gt;G</td>
<td>Mediates the uptake of folate in the cells.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OMIM *600424</td>
<td></td>
<td>AA: 27.2 %</td>
<td>A: 0.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AG: 44.7 %</td>
<td>G: 0.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG: 28.0 %</td>
<td></td>
</tr>
</tbody>
</table>

Methylenetetrahydrofolate reductase (MTHFR)

Of the enzyme polymorphisms with an effect on homocysteine levels, the MTHFR polymorphism has been subject to the most research. The first and very rare mutations found are related to inborn errors of metabolism. The enzyme consists of a tetramer, non-covalently bound. Vitamin B2 is needed for the binding, in deficiency the tetramer falls apart to two dimers. In the dimer, one part hosts the catalytic region and the other the regulatory region. SAM has negative feedback on MTHFR, when there is a high methylation capacity, the MTHFR activity will be reduced.

In 1995 a common polymorphism was reported, MTHFR 677C>T. The mutation induces thermolability of the enzyme, and is in the catalytic region. In a study with site directed mutagenesis, enzyme activity of the MTHFR carrying the 677T, enzyme activity is reduced to 45 %, and homozygotes have higher levels of homocysteine. It has been shown that normal enzyme activity may be seen if enough 5,10-methylenetetrahydrofolate is present. Approximately 3-5 × the normal amount of plasma folate is needed for normal enzyme function in homozygotes. One study reported that at plasma folate levels below 15,4 nmol/L homocysteine was higher in homozygotes compared to heterozygotes and subjects without this mutation. Most methods for
determining folate in plasma or serum have a lower reference limit at 6-7 nmol/L in populations where folate fortification of the food does not occur. Most individuals (around 75 %) with a homocysteine level above 40 µmol/L have been reported to be homozygous for the MTHFR 677C>T polymorphism 23.

The second common polymorphism, MTHFR 1298A>C, was reported in 1998 not inducing thermolability 24. This polymorphism is as common as the 677C>T polymorphism with approximately 10 % homozygotes reported. In a study with site directed mutagenesis, enzyme activity of the MTHFR carrying 1298C (having a glutamate to alanine substitution) is reduced to 68 %, but if this amino acid substitution was introduced into an enzyme that already carried the 677T (inducing the alanine to valine substitution) the activity was only reduced from 45 % to 41 %, i.e. hardly changed at all 20. These in vitro findings support the findings that the plasma homocysteine are hardly affected in subjects carrying the 1298A>C mutation 20,24,25. This polymorphism is in the regulatory region, where the negative feedback of SAM is supposed to take place. It is possible that the negative feedback may be impaired in the 1298C form. Thus MTHFR will in this case keep supplying the system with methyl groups even when SAM levels are high.

MTHFR has a key role for folate metabolism as it diverts 5,10-methylenetetrahydrofolate towards methylation or DNA synthesis depending on folate status and methyl group demand (Figure 4).

![Dietary folate cycle](image)

**Figure 4.** The dietary folate forms enter the folate cycle and are diverted towards methylation or DNA synthesis depending on the availability of methylation capacity (negative feedback) and catalytic function of the MTHFR enzyme depending mainly on the presence of absence of the 677C>T polymorphism. THF = tetrahydrofolate

The prevalence of homozygotes for the MTHFR 677C>T polymorphism reported among newborns varies a lot between different populations, from 2.7 to 32.2 % (Table 3) 26. The highest frequencies are often found in areas with high folate intake. Interestingly there is a Spanish study from 1998 stating that homozygote frequency was 13 % in the age group 20-40 years and 26 % in subjects younger than 20 years.
No common genetic selection could change genotype frequency in this short time. However, this big difference coincides with a campaign starting 20 years earlier to increase intake of vitamin supplements during pregnancy in Spain. As it is known that miscarriages are more common in folate deficiency, and MTHFR 677TT subjects are more vulnerable, an improved folate status could prevent spontaneous abortions in 677TT foetuses which could possibly have an effect on genotype frequency.

Table 3. MTHFR 677TT genotype in percent and T-allele frequency in different populations. Adapted from Wilcken et al 2003. P-values are shown for those studies where the polymorphism was not in Hardy-Weinberg equilibrium.

<table>
<thead>
<tr>
<th>Country</th>
<th>MTHFR 677TT (%)</th>
<th>Allele frequency (T)</th>
<th>Hardy-Weinberg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Europe</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Italy</td>
<td>10.6 – 26.4</td>
<td>0.266 – 46.0</td>
<td>P &lt; 0.05 in 1/3 of regions studied</td>
</tr>
<tr>
<td>Spain</td>
<td>11.8 – 13.7</td>
<td>0.333 – 0.336</td>
<td></td>
</tr>
<tr>
<td>France</td>
<td>11.8</td>
<td>0.357</td>
<td></td>
</tr>
<tr>
<td>The Netherlands</td>
<td>6.4</td>
<td>0.274</td>
<td></td>
</tr>
<tr>
<td>Finland</td>
<td>4.0</td>
<td>0.251</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Hungary</td>
<td>11.1</td>
<td>0.337</td>
<td></td>
</tr>
<tr>
<td>Russia</td>
<td>7.0</td>
<td>0.269</td>
<td></td>
</tr>
<tr>
<td><strong>Middle East</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Israel</td>
<td>8.6</td>
<td>0.257</td>
<td></td>
</tr>
<tr>
<td><strong>China</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>North</td>
<td>19.8</td>
<td>0.442</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>South</td>
<td>8.1</td>
<td>0.347</td>
<td></td>
</tr>
<tr>
<td><strong>Australia</strong></td>
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<td></td>
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<tr>
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<tr>
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<tr>
<td>Others</td>
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<td>0.217</td>
<td></td>
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</tbody>
</table>

*P*-values based on $\chi^2$. 

19
Age-related prevalence was reported in a Japanese study where homozygotes were found in 19% of subjects < 55 years, 14% in subjects 55-79 years and only 7% in the oldest group. In the two youngest groups the polymorphism was in Hardy-Weinberg equilibrium, but not in the oldest group, suggesting selection. However, other studies have not found differences depending on age groups, among them a Swedish study which found no difference between new-born (10%) and subjects aged 80-108 years of age (9.5%) \(^{28}\). They also made a meta-analysis adding three other studies and found no effect on longevity. In a Danish study on centenarians no differences in homozygote frequency was seen \(^{29}\).

The prevalence of homozygotes for MTHFR 1298A>C does not differ so much in different populations, ranging from to 9% in the Dutch \(^{24}\) and Canadian reports \(^{25}\), 12.5% in a French study \(^{30}\), and 14.2% in an Irish population \(^{31}\).

The MTHFR 677C>T and 1298A>C polymorphism are in trans configuration. Only a few studies have reported on sporadic cases MTHFR 677C>T and 1298A>C occurring in cis, i.e. on the same DNA strand. Finding the polymorphisms in cis may indicate genotyping problems, possibly in the RFLP analysis and these cases must be verified by other techniques.

**Determinants of homocysteine**

There are a number of diseases and life-style factors that may influence levels of homocysteine. For a review see \(^{9}\).

The most important determinants of homocysteine are folate and vitamin B12, in that order. Deficiency of folate can be defined as low levels of plasma folate or functional deficiency of folate due to MTHFR 677TT with levels in the lower part of the reference interval, in both cases an elevated homocysteine level is the result. As vitamin B12 is transported in blood by transcobalamin II, which comprises approximately 20-30% of B12 in plasma with a short half-life in blood, and by haptocorrins. Only transcobalamin II bound B12 is taken up by receptors by the cells. Haptocorrin bound B12 has a long half-life and must be recirculated by the liver through the bile for new uptake in the distal ileum. As haptocorrin levels may vary, functional B12 deficiency may occur at high, normal or low levels of B12 in plasma. Generally levels that are < 300 pmol/L may imply deficiency. Most other conditions act via B12 and folate deficiency. Vitamin B6 is not a determinant of fasting or non-fasting homocysteine, except for rare cases. Hyperhomocysteinemia as a result of B6 deficiency is restricted to elevated levels after a methionine loading test.
All diseases interfering with the uptake of B12 or folate may lead to elevated levels of homocysteine – atrophic gastritis, after gastrectomy of gastric bypass, pancreatic diseases, malabsorption in the small intestine, celiac disease, inflammatory intestinal disease are examples. Autoantibodies towards intrinsic factor or parietal cells resulting in B12 deficiency may be seen in diseases with associated with an autoimmune component, such as diabetes mellitus type II and thyroid disease. Liver disease may lead to B12 deficiency due to reduced hepato-biliary recirculation. If a liver disease is a result of heavy alcohol consumption, deficiency due to malabsorption or reduced intake may be the reason. Direct effects on metabolism have also been discussed 32.

There are also drugs interfering with B12 and folate, such as the folate antagonists methotrexate and trimetoprim. Most antiepileptic drugs interfere with folate uptake or metabolism. All drugs reducing gastric acid reduces uptake of B12 from food (but not from supplements) and may result in B12 deficiency if used for many years.

Life-style factors have an effect on homocysteine. Smoking has been associated with elevated levels in epidemiologic studies. An increase of 0.5 % in men and 1.0 % in women per cigarette smoked a day has been observed in the large Norwegian Hordaland study 33. Comparable results were seen in the New York University Women’s Health Study 34. This would lead to an increase of 0.75 µmol/L in men and 1.5 µmol/L in women if ten cigarettes are smoked daily calculated from a starting level of 15 µmol/L. This effect is of such a small magnitude that it is generally not useful at an individual level. Smokers are often considered to have lower levels of B-vitamins due to reduced intake of fruit and vegetables 35-37, but nicotine has also been implied to interfere with enzymes in the methionine and folate metabolism, such as methionine synthase 38. The effects of smoking have been reported to be highest in MTHFR 677TT 39.

Coffee consumption is also a determinant at an epidemiological level resulting in an increase in homocysteine level 40-42 especially in subjects with MTHFR 677TT 43. There has also been found an effect of caffeine itself on homocysteine, but of less magnitude than that of coffee.

Subjects with a sedentary life-style have been found to have higher homocysteine levels compared to those more active 33.

Age has been found to be a determinant for homocysteine levels. This is based on several cross sectional studies 33,44-46. The NHANES III study showed that homocysteine was higher in older individuals in three ethnic groups, based on 3766 males and 4819 females 47. In the Framingham cohort geometric mean was found to be 11 % higher in men aged ≥ 65 years compared to men < 45 years. In women the difference was 23 % 42. These results were based on 920 men and 1040 women, the blood samples were taken in the years before folate fortification in the
US. In a recent meta-analysis with data from nine European studies with 12541 men and 12948 women the prevalences of hyperhomocysteinemia (defined as >15 μmol/L) were 8.5 %, 4.7 % and 5.9 % in age groups 20-40, 40-60 and older than 60 years respectively 48. Only one long-term longitudinal study has been published on homocysteine by the Bergen group studying change in tHcy in the same subjects six years apart 132.

In women slightly lower levels are seen compared to men, at least before menopause. The gender difference has been estimated to be approximately 2 μmol/L but the difference decreases with age 9. However, the increase with age has been found to be similar in men and women 48. After menopause homocysteine levels has been reported to be higher than before. All these studies were cross sectional cohort studies, not longitudinal. Thus the picture is confusing and an observation of a female cohort followed longitudinally is desirable.

**Homocysteine and renal function**

Subject with homocystinuria were found to have large amounts homocystin (the oxidised form of homocysteine) in the urine. In renal disease, homocysteine levels are elevated. There are two theories on this association. The first is that elevated homocysteine results in impaired renal function. The second is that renal disease causes increased levels of homocysteine. Although glomerular filtration rate (GFR) is correlated with homocysteine, only a small fraction is filtered and excreted in the urine, usually less than 1 %. Another possibility is that homocysteine would be taken up by the renal tubules and metabolised through the transsulphuration pathway to cysteine. However, this has not been found in kidneys with normal function 50,51.

The most commonly used marker of renal function is plasma creatinine, which besides from being a marker of GFR and dependent on the diet, also is dependent on homocysteine metabolism for its synthesis as methyl groups are needed. As methyl groups are consumed, methionine is converted to SAM which donates the methyl group. A high consumption of methyl groups increases the levels of homocysteine. The bulk of methyl groups produced are spent on synthesis of creatinine. Supplementation with creatine decreases the demand for methylation and thus decreases homocysteine 52,53. Thus, the relation between creatinine and homocysteine is more complicated than the relation with the novel marker cystatin C, plasma levels of which mainly are dependent on GFR 54-56.
Homocysteine and cardiovascular disease

Many studies have been published during the last 15 years investigating elevated levels of homocysteine and cardiovascular disease. Already in the 1960’s individuals with the rare metabolic disease homocystinuria in most cases had cardiovascular disease and stroke before the age of 20.

Recent meta-analyses suggest that hyperhomocysteinemia is a risk factor for myocardial infarction and stroke, particularly ischemic stroke. In the Homocysteine Studies Collaboration meta-analysis, 30 studies published between January 1966-January 1999 were included, all studies providing data files for re-analysis of data. This resulted in 5073 events of ischemic heart disease and 1133 cases of stroke. Elevated tHcy was a moderate independent predictor. A three µmol/L lower level of tHcy associated with reduced risk - odds ratio 0.89 (0.83-0.96) for ischemic heart disease and 0.81 (0.69-0.95) for stroke. Only two prospective studies have been published so far on haemorrhagic stroke, both reporting tHcy as a primary risk factor for haemorrhagic stroke. In the Rotterdam study, which was actually not a primary risk factor study, tHcy was significantly associated with risk of stroke after adjustment for age and gender in the 12 hemorrhagic stroke cases which developed during the 2.7 years of follow-up. The recent larger Japanese study, which was a primary risk factor study, the follow-up time was not reported. In the 52 cases with hemorrhagic stroke, the multivariate odds ratio of tHcy was 2.36 (95% CI 0.59-9.48). The possible role of MTHFR was not discussed. Stroke cases in Japan are generally older than in Sweden, different mechanisms may be involved.

A meta-analysis of the MTHFR 677C>T polymorphism including 40 studies published before June 2001 reviewed 11162 cases and 12758 controls. An increase in risk of coronary heart disease was seen, especially in combination with low folate levels, odds ratio 1.16 (1.05-1.28). Another meta-analysis examined 72 retrospective studies in which MTHFR 677C>T genotype was analysed in cases and referents, and 20 prospective studies in which tHcy was determined. MTHFR genotype was transformed to a tHcy level in the retrospective studies and risk was assessed by an increment of three µmol/L. In this study tHcy was associated with increase in risk in the prospective studies on ischemic heart disease, and for stroke. Similar results were seen for MTHFR genotype. The similar results in studies on tHcy and MTHFR 677C>T polymorphism has sometimes been interpreted as proof of tHcy as a causal risk factor. A meta-analysis including only prospective studies on cardiovascular diseases reported 14 studies with an average relative risk of 1.49 (95% CI: 1.31-1.70) for cardiac events, and 1.37 (95% CI: 0.99-
1.99) for ischemic stroke. Many more meta-analyses have been performed on tHcy, and MTHFR in cardiovascular disease.

The prospective studies are not as common as the more numerous retrospective studies, and the results in the prospective studies are usually less convincing.

Homocysteine, a causal risk factor for cardiovascular disease?

Homocysteine lowering treatment, especially with vitamin B6 proved beneficial in reducing events in homocystinuric patients. Those who responded with a decrease in homocysteine from over 300 µmol/L to around 70 µmol/L had a drastic decrease in morbidity. A scientific dilemma is the fact that in studies in different populations homocysteine has been a risk marker already at levels within the most common reference interval (<15 µmol/L) and in the general population the risk increase has been found to be continuous even within the reference interval, thus there is no threshold for increased risk.

A parameter that is to be connected to disease can be assigned to one of three different levels. The first level is risk marker and is an attribute or an exposure connected with an increased risk for disease without a proven causality. Often this connection is only statistical. The next level is risk determinant; a causal connection might exist but is not proven. The last level is risk factor and this is a risk determinant, which is probably casually connected to increased disease risk, and it can modify disease risk. In some studies a casual effect is implied but this needs to be explored in randomized trials demonstrating that lowering homocysteine also reduces the incidence of atherothrombotic diseases, this would render homocysteine a causal risk factor for cardiovascular disease.

Only a few studies on homocysteine-lowering treatment and effects on cardiovascular disease have been published. The first study was on progress of atherosclerotic plaque in the carotids. Treatment of patients with homocysteine > 14 µmol/L with 2,5 mg folic acid, 0.25 mg B12 and 25 mg B6 showed an effect on progress and the plaque area decreased by 59% in 2001-2002 there were two interesting papers from a Swiss group. They studied the effects of restenosis and major adverse events after coronary angioplasty. They gave 1 mg folic acid, 0.4 mg B12 and 10 mg B6 during 6 months and compared with placebo. Homocysteine decreased in the vitamin group, which also had a significant decrease in the frequency of restenosis after 6 months of treatment. In a follow-up paper, after six months of treatment, they continued to observe the group for another 6 months after cessation of
vitamin treatment \(^{72}\). Now there were effects on adverse events defined as death, nonfatal myocardial infarction, and need for repeated revascularization. The group that had been treated with vitamins had a significantly lower frequency of major adverse events after one year, after balloon angioplasty. This was seen in spite of the fact that no vitamins were taken during the last six months. Probably the first 6 months of treatment was enough for an increase of the vitamin stores to ensure an enduring effect. In a similar double-blind, placebo-controlled randomized trial on folate-therapy and in-stent restenosis, Lange et al found an increased risk of restenosis in the group receiving folate \(^{73}\). In their study they used stent which now is used more often than balloon angioplasty. The increased risk was confined to the sub group of patients with tHcy < 15 µmol/L. The first study was performed in Switzerland \(^{71,72}\), the second in Germany and The Netherlands \(^{73}\). Studies like these are now more difficult to perform in the US due to the fortification of flour with folic acid. In the US fortification started voluntarily in July 1997 and was mandatory in January 1998. Canada began its mandatory fortification in November 1998. In Victoria, Australia, fortification began in 1996. A possible confounder is also the trend towards increasing consumption of prescribed and off the counter multivitamins. Another problem is that folic acid has a positive effect on blood vessels that might be independent of the homocysteine-lowering effect. These putative effects will be difficult to distinguish from the homocysteine-lowering effects \(^{74}\).

A meta-analyse standardizing the effects of vitamin effects on standardized homocysteine levels has shown that folic acid reduces homocysteine by 25% \(^{75}\). There are similar effects of folic acid in the doses 0.5-5 mg. For a mean dose of 0.5 mg B12 daily an additional lowering effect of 7% is seen. Vitamin B6 does not seem to have an homocysteine-lowering effect in the mean dose of 16.5 mg. A number of prevention studies are on the way but no answer has been given on the crucial point, if homocysteine is a causal risk factor or not \(^{74,76}\). There is a database by The U.S. National Institutes of Health (NIH), through its National Library of Medicine (NLM), called ClinicalTrials.gov (www.clinicaltrials.gov) \(^{77}\). Entering folic acid results in six hits (Table 4). As seen there are at present six ongoing trials reported to this database (March 2005). It may become compulsory to report clinical trials to this database in order to be accepted for publication. In fact, the journal *Blood* has already taken this decision. For a review on more trials and some more detail on design, see Table 5.
Table 4. Ongoing trials on folic acid reported to ClinicalTrials.org (www.clinicaltrials.gov)

<table>
<thead>
<tr>
<th>Status</th>
<th>Study</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Recruiting</td>
<td>Randomized Study of Folic Acid Therapy for Hyperhomocysteinemia in Patients with End Stage Renal Disease Receiving Hemodialysis</td>
<td>End Stage Renal Disease; Hyperhomocysteinemia</td>
</tr>
<tr>
<td>2. Recruiting</td>
<td>Oral Cleft Prevention Program</td>
<td>Cleft Lip; Cleft Palate; Congenital Defects; Pregnancy</td>
</tr>
<tr>
<td>3. Recruiting</td>
<td>Folate-Depleted Diet Compared With Folate-Supplemented Diet in Preventing Colorectal Cancer in Patients at High Risk for Colorectal Cancer</td>
<td>Colon Cancer; Rectal Cancer</td>
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<tr>
<td>4. Recruiting</td>
<td>Folic Acid for Vascular Outcome Reduction In Transplantation (FAVORIT)</td>
<td>Renal transplant recipients</td>
</tr>
<tr>
<td>5. Recruiting</td>
<td>VITATOPS: A Study of VITAmins TO Prevent Stroke</td>
<td>Stroke; Transient Ischemic Attack</td>
</tr>
<tr>
<td>6. Recruiting</td>
<td>VITAL - VITamins to slow ALzheimer's disease (Homocysteine Study)</td>
<td>Alzheimer's Disease</td>
</tr>
</tbody>
</table>

One trial published its results in 2004, the VISP trial. However, in the VISP prevention study, lowering homocysteine by vitamin treatment did not affect recurrent stroke or death in a two-year follow-up. That study may not have had statistical power to detect a risk reduction of 20-30%, which would still be clinically important. The treatment group received 2.5 mg folate acid, 0.4 mg of vitamin B12 and 25 mg vitamin B6. Folate fortification may have been a confounder, as well as the placebo pill which was a multivitamin tablet (0.02 mg folic acid, 0.06 mg B12 and 0.2 mg B6). It is therefore not possible to rule out homocysteine as a risk factor for ischemic stroke. More ongoing trials will reach completion in the next five to ten years, but some will be underpowered or under-recruited. The Norwegian studies will probably report results within a year from now. Another problem is that folic acid
has a positive effect on blood vessels that might be independent of the homocysteine-lowering effect. These effects will be difficult to distinguish.

For the time being, tHcy must be considered to be a risk marker without a proven causal connection to cardiovascular disease, i.e. a risk determinant.

Table 5. Overview of some ongoing prevention trials.

Studies in the US will have to deal with interferences of folate fortification in flour. Sample sizes are the planned sizes. Adapted.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sample size</th>
<th>Endpoint</th>
<th>Treatment/placebo</th>
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<tr>
<td>USA: WACS</td>
<td>6-8000</td>
<td>Stroke and coronary artery disease in women</td>
<td>Folic acid 2.5 mg, B12 1 mg, B6 50 mg/placebo</td>
</tr>
<tr>
<td>Australia, Sydney:</td>
<td>10000</td>
<td>Coronary heart disease</td>
<td>Folic acid 0.2 mg/placebo</td>
</tr>
<tr>
<td>PACIFIC</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Norway: NORVIT         | 3000        | Myocardial infarction, stroke, angina pectoris, and mortality | 1. Folic acid 0.8 mg  
                           |             |                                               | 2. B6 40 mg  
                           |             |                                               | 3. All of the above  
                           |             |                                               | 4. Placebo      |
| Norway: Bergen Vitamin study | 2000  | See NORVIT                                   | See NORVIT                                            |
| England, Cambridge:    | 4000        | Myocardial infarction /instable angina        | Folic acid 5 mg / placebo                              |
| CHAOS-2                |             |                                               |                                                       |
| England, Oxford:       | 12000       | Coronary heart disease                        | Folic acid 2 mg, B12 1 mg, combined with simvastatin/placebo |
| SEARCH                 |             |                                               |                                                       |
Preanalytical factors

Blood sampling for homocysteine should be standardised. We use the routine of resting in sitting position for 15 minutes prior to blood sampling. Homocysteine has been shown to be decrease after 30 minutes at supine rest by 15.3% \(^8\) and 19 % \(^1\). This effect was dependent on the decrease of albumin at the same conditions: other possible effects of physiological variations of albumin on homocysteine have not been fully explored, especially not in a longitudinal setting.

A three minute tourniquet application before blood is drawn has been shown to increase homocysteine slightly by 2.8 % but this was small compared to the 8.1 % intra individual variation \(^8\). Our samples have usually been taken without venous stasis.

Pre-analytical handling of samples for homocysteine analysis is crucial. Homocysteine is formed in erythrocyte metabolism and released to plasma as long as the erythrocytes are viable. If plasma is not separated homocysteine levels increase in samples significantly before centrifugation, corresponding to an increase of 0.5 - 1 µmol/L per hour in subjects, irrespective of initial level \(^2\). After separation of the erythrocytes homocysteine is stable in plasma or serum for a long period of time, even at room temperature. To prevent the erythrocyte-mediated increase EDTA-containing tubes have been stored on ice prior to centrifugation. Swift centrifugation of the sample and removal of the erythrocytes is another widely used protocol. This is the recommended way of processing the blood samples donated in MONICA screenings and in the VIP programme in the Northern Sweden Medical Research Bank.

Different types of test tubes have been considered for sampling of homocysteine. When EDTA blood samples are immediately put on crushed ice, the maximum period before centrifugation is delayed to 4 hours \(^3\). Fluoride oxalate and sodium citrate can not stabilise homocysteine in blood after collection \(^4\). Homocysteine in sodium fluoride-containing tubes has been found to be stable for three hours at room temperature before centrifugation \(^5\). Due to these reports the optimal test tube for sampling has not yet emerged. In the 1980s, a novel specimen collection tube, which stabilised the activity of the fibrinolytic enzyme tPA by drastically reducing the pH of the blood plasma was introduced \(^6\). This was achieved by substituting the ordinary 0.13 mol/L neutral sodium citrate with 0.5 mol/L highly acidic citrate, pH 4.3, which lowers the reaction rate of enzymes with pH optima in the neutral range, thus preventing their interaction with protease inhibitors. Such tubes containing acidified citrate (Stabilyte\(^\text{TM}\)) have been found to stabilise homocysteine for at least 6 hours at room temperature without
centrifugation, stability up to eight hours has been reported. These studies adjusted homocysteine levels in Stabilyte™ tubes with the dilution factor in order to obtain levels similar to the traditional EDTA-tubes. In our lab we employed the factor 1.19. This additive does not interfere with the immunological methods evaluated (FPIA-method on IMx®, Abbott Laboratories, IL, USA) but a matrix effect has been observed to possibly interfere with derivatives used for the detection in some HPLC-methods for determining homocysteine. In addition, this tube is optimal for several fibrinolytic and coagulation measurements.

A common recommendation is that sampling for homocysteine should be done after an overnight fast. Homocysteine levels are slightly higher in the afternoon compared to the morning. For research use the best is to standardise blood sampling but for clinical purposes the variation during a day is very small, as a small meal will not influence homocysteine levels, allowing blood sampling throughout the day. It takes a very large protein-rich meal to increase homocysteine levels. It has also been suggested that homocysteine levels after a ten hour fast might be a less favourable metabolic situation than a non-fasting level.

Reference interval and standardisation

The most common reference interval used for detection of functional deficiency of vitamin B12 and/or folate is < 15 µmol/L for individuals over the age of twelve. For pregnant women and children up to twelve years it should be < 10 µmol/L. Different reference intervals have been discussed. In some diseases decision limits are used, usually at levels lower than the reference interval. The cut-offs mentioned above refer to a population not supplemented with B-vitamins. Some Scandinavian laboratories use cut-off levels derived from reference subjects on multivitamin therapy.

There is no international standard for homocysteine yet. This can make it difficult to compare results obtained with different methods. A crucial step in analysis of homocysteine is reducing bound homocysteine to free homocysteine. A weak reduction step results in lower levels. In Scandinavia we have an external quality assessment programme for total homocysteine, currently including between 75-80 laboratories, to reduce inter laboratory variability.
Methionine loading test

Methionine loading test is performed to evaluate the homocysteine metabolism. The subjects are given 0.1 g methionine per kg in a body weight in a test breakfast after an overnight fast. Homocysteine is measured before methionine ingestion and generally after 4-6 hours. Some researchers have evaluated shorter times, as short as two hours \(^97\), but this probably increases the within-person variability \(^98\). The test may be used as an indirect measure of vitamin B6 deficiency as the transsulfuration pathway of homocysteine is vitamin B6 dependent. Deficiency of vitamin B6 is rarely reflected by elevated levels of homocysteine. The test has also been used to find heterozygote carriers for the defect in cystathionine \(\beta\)-synthase resulting in homocystinuria.

The methionine loading test has in some studies been used to detect hyperhomocysteinemia defined as fasting homocysteine above a cut-off level and/or the post-load level or post-load increase above a cut-off level. Some researchers define the cut-off as a post-load level \(5 \times\) the fasting level. In our laboratory we defined the upper reference interval for the four hour post-load increase as < 31 \(\mu\)mol/L (calculated as the homocysteine four hours after methionine ingestion – fasting homocysteine).

We earlier studied stroke in 80 consecutive stroke patients aged 18-44 years old and evaluated them with methionine loading test three months into the convalescent phase \(^99\). Fasting homocysteine levels did not differ between the groups but stroke subjects had significantly higher post-load increase (\(p = 0.02\)). In a recent study of young stroke subjects < 45 years of age the methionine loading test revealed hyperhomocysteinemia in subject not detected by fasting homocysteine \(^100-102\). In another case-control study we performed on young men and women with coronary artery disease but without myocardial infarction, no difference was seen either for fasting or post-load homocysteine (data not published).

Several studies have evaluated the methionine loading test as a tool to find hyperhomocysteinemia and found that the test only adds a few subjects not already found with fasting homocysteine \(^103,104\) and thus they do not recommend the test for routine use.

Due to aspects above, we have not included methionine loading tests in the ongoing cohort studies.
OBJECTIVES OF THE STUDY

To test whether homocysteine is related to risk of first-ever stroke and to evaluate the role of the MTHFR polymorphisms in this putative association in a prospective nested case-referent study.

To evaluate in a nested case-referent study whether if followed prospectively, homocysteine levels are higher in case subjects before and/or after development a first myocardial infarction, when compared with referent subjects. It was also to determine whether the association between homocysteine and myocardial infarction was greater at follow-up than at baseline.

To evaluate the suitability of Stabilyte™ plasma as a medium for analysis of determinants for homocysteine, such as albumin, creatinine and cystatin C, as well as for traditional lipids and lipoprotein cardiovascular risk factors.

To characterise in a prospective setting the long-term changes of homocysteine in a cohort followed longitudinally over nine years, and of sufficient size to allow us to control for major determinants such as age, gender and renal function.
MATERIAL AND METHODS

ETHICS

The study protocols for paper I, II and IV were approved by the Research Ethics Committee of Umeå University, Umeå and the data handling procedures by the National Computer Data Inspection Board. For paper III we used paired de-identified serum and Stabilyte™ plasma samples for in an in vitro method comparison study.

STUDY COHORTS

MONICA screenings

The MONICA project (Multinational Monitoring of Trends and Determinants in Cardiovascular Disease) was initiated in the late 1970’s when it was evident that the secular trends in cardiovascular mortality differed between industrialized countries. Between 1982 and 1986 38 populations in 26 countries on four continents were entered into the project. They were followed for at least ten years. The first survey in Northern Sweden was in 1986. Further surveys have been undertaken 1990, 1994 1999 and 2004. The surveys consist of 2000–2500 randomly selected subjects of 25–74-year-old inhabitants of Västerbotten and Norrbotten counties with a target population of 316,015 in 1999 (total population 516,300 inhabitants). The subjects are invited to participate in health surveys. The samples are stratified by age and sex, age groups are 25-23, 35-44, 45-54 and 55-64 years. The mean participation rate in the four surveys was 77.2%.

The Västerbotten Intervention Programme (VIP)

The VIP is an ongoing community intervention programme. It was launched in 1985 and the objectives are prevention of cardiovascular disease and diabetes in the Västerbotten County. It has a population-oriented strategy focussing on health promotion activities, combined with an individually-oriented strategy. Every citizen is invited to
participate in a health survey at their primary health care centre the year they turn 30, 40, 50 and 60 years old. The design is similar as for the MONICA population surveys. The overall participation rate in the VIP has been approximately 60%. Comparisons of social characteristics between participants and nonparticipants have shown little evidence of selection bias. In this study the 1992 and 1993 VIP screenings were compared with the 1990 Population and Housing Census with respect to total income, socio-economic group, employment and education. The health selection was evaluated by comparing results of body mass index, total cholesterol, systolic and diastolic blood pressure, and daily smoking with the 1990 and 1994 MONICA screenings. Between 1 January 1985 and 31 September 2003, about 88,150 health examinations have been performed in the VIP health surveys and of these 13,000 were individuals that were examined a second time after ten years. Thus, 75,000 unique subjects have been examined. For 2004 it is estimated that 6000 health examinations were performed, 50% of which were re-examinations after ten years.

Case-finding

The case finding of suspected events of acute myocardial infarction and acute stroke was based on hospital discharge records and death certificates. Information from necropsies was included when available. All surviving patients were informed and asked to contact the MONICA secretariat if they did not give consent to be in the register. Approximately 0.6% of stroke survivors and 0.4% of survivors of myocardial infarction did not concur and these subjects were entered without identification. The process is described in detail by Stegmayr et al. Uniform criteria for stroke and acute myocardial infarction are used.

Study cohort on stroke, paper I

This was a prospective nested case-referent study, based on the MONICA and VIP cohorts. For each case, five matched referents without known cardiovascular disease or cancer were selected from the two cohorts. Matching was by sex, age (±2 years), cohort (MONICA or VIP), date (±1 year) of health survey, and geographical area. Each control subject received a questionnaire with items on any cardiovascular event that had occurred since the baseline survey. As a first choice, the first two referents for whom samples were available and who had consented to genotyping were used in the analyses. If any of these did
not respond, the third (or occasionally the fourth) referent selected was used.

All cases (in-hospital and out-of-hospital) with acute stroke (in the age group 25–74 years) were included in the registries using WHO criteria and MONICA methodology. Possible stroke events were identified through screening of hospital discharge records, general practitioners’ reports and death certificates, with ICD-9 codes 430–438 corresponding to ICD-10 codes I60–69. The stroke subtypes are defined in Table 6. Subjects with earlier stroke, acute myocardial infarction or subarachnoidal haemorrhage were excluded. Of the 473 first-ever stroke cases, 458 (97%) were examined with either a computed tomography / magnetic resonance (CT/MR) scan or, if the stroke was fatal, had been subjected to an autopsy. Of these, 387 had ischemic stroke and 71 had hemorrhagic stroke. The 15 cases with unclassified stroke were not included in this study. No cases with Subarachnoid haemorrhage were included.

Table 6. Definition of stroke subtypes.

<table>
<thead>
<tr>
<th>Stroke subtype (ICD-9)</th>
<th>Definitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subarachnoid haemorrhage (430)</td>
<td>bloodstained cerebrospinal fluid and an aneurysm or an arteriovenous malformation found on angiography or positive finding on CT scan or necropsy;</td>
</tr>
<tr>
<td>Intracerebral haemorrhage (431)</td>
<td>positive finding on CT scan or necropsy;</td>
</tr>
<tr>
<td>Brain infarction (434)</td>
<td>no signs of haemorrhage on CT scan or at necropsy;</td>
</tr>
<tr>
<td>Unspecified stroke (436)</td>
<td>not investigated by CT scan or necropsy.</td>
</tr>
</tbody>
</table>

Study cohort on myocardial infarction, paper II

This was a prospective case–referent study nested within two subcohorts MONICA and VIP. A diagnosis of myocardial infarction was confirmed if the event met WHO MONICA criteria, definition 1. The study was based on a study of 78 participants developing a first myocardial infarction previously. In that study by Thøgersen et al, neither homozygosity for MTHFR 677C>T or total homocysteine levels were associated with a first myocardial infarction. Of these 78 cases, 50 had their fasting homocysteine repeated at follow-up. The first health surveys for the participants in the present study occurred between April 1988 and November 1993, and the follow-up examinations were performed between July 1997 and February 2001. The time interval between the two screening episodes was 8.4 ± 1.8 years for cases and 8.7 ± 1.7 for referents (ns). Cases were matched with one referent subject for
age (±2 years), sex, date (±1 year), type (MONICA or VIP) of health survey and geographical region. Three cases had no matching control subjects in the follow-up examination, and seven cases had two matching referent subjects. Two additional referent subjects were used without corresponding cases. Thus, for the conditional logistic regression analysis, 40 cases with one referent and seven cases with two referents were included. Of the 28 cases not participating in the follow-up survey, 24 had died in the interim, one had severe dementia, one had terminal cancer, one had relocated outside the catchments area and one was lost to follow-up. There was no difference in mean baseline homocysteine concentrations between the 50 cases included in the study and the 28 cases not included in the follow-up.

Cohort for method evaluation, paper III

For this method evaluation we used de-identified paired blood samples from 49 consecutive patients referred for investigation of thrombotic disorders to the department of clinical chemistry in Umeå University Hospital. Sampling was performed after an overnight fast.

Longitudinal study cohort, paper IV

This study utilised a population-based biobank from the MONICA cohort, including randomly selected 25- to 64-year-old subjects living in northern Sweden, invited to participate in health surveys for cardiovascular disease in 1990 and 1999. To secure a longitudinal cohort for this study, all 2000 subjects invited in 1990 were re-invited to participate in 1999. Survey procedures were according to the MONICA methodology. Briefly, we included 510 randomly selected subjects for whom blood samples were available from both the 1990 and 1999 screenings, 25-64 years at baseline; age at follow-up was 34-73 years.

BLOOD SAMPLING

As part of the MONICA and VIP programs, participants are invited to donate a fasting blood sample to be stored at the Medical Biobank in Umeå for future research. For further information see the official website of the Medical Biobank (http://www.umu.se/phmed/naringsforskning/forskning/Medical_Biobank/indexB.htm). The aim was to obtain samples after a minimum of four hours of fasting: 65.5 % of the samples were collected after a minimum of 8 hours fasting, 31.2 % after 4-8 hours, and 3.4 % after less than 4
hours. Plasma levels of folate, vitamin B12, and homocysteine for these fasting durations did not differ significantly (Kruskall-Wallis, $p = 0.196$, $0.201$, and $0.125$, respectively). For this peripheral venous blood is drawn, without using stasis, into evacuated glass tubes one containing EDTA and one heparin, total volume 20 mL. In the MONICA surveys 1990 and 1999 samples were drawn into evacuated test tubes containing acidified citrate (Stabilyte™ tubes, Biopool AB, Umeå, Sweden). The Stabilyte™ tubes contain 0.5 mL of 0.5 mol/L citrate buffer, pH 4.3, and the tube draws 4.5 mL of blood, which results in a final pH of about 5.9 in plasma.

A two-hour oral glucose tolerance test with 75 g glucose dissolved in 300 mL water ingested within five minutes is also performed in most VIP subjects and in 65% randomly selected in the MONICA survey.

For the determination of glucose blood was drawn into sodium fluoride-containing tubes in the MONICA surveys when blood is sent to the hospital laboratory. In the VIP project capillary blood is drawn and analysed at site. Tubes without additives were used for the determination of total cholesterol, triglycerides and HDL-cholesterol.

Plasma or serum was obtained by centrifugation at 1500g for 15 minutes. Plasma was aliquoted, and stored frozen at $-80^\circ$ C until analysis, as were aliquots of buffy coat and erythrocyte fractions. For paper I heparin plasma was used for our analyses and for paper II EDTA-plasma was used. In paper IV Stabilyte™ plasma was used.

In paper III peripheral venous blood samples were drawn into evacuated test tubes containing acidified citrate (Stabilyte™ tubes, Biopool AB, Umeå, Sweden) and into SST serum tubes (Becton Dickinson, Franklin Lakes, NJ, USA). All samples were centrifuged within 20 min of collection. Plasma and serum were frozen at $-20^\circ$ C and within a few weeks transferred to $-80^\circ$ C. The paired acidic citrate and serum specimens had been stored for a median time of 8 months (range 2-12 months). The samples were de-identified and analysed at the same time.

In paper IV, peripheral venous blood samples were drawn into evacuated Stabilyte™ tubes as in paper III. Samples were obtained after a minimum of four hours of fasting. For serum samples blood was drawn without stasis into evacuated glass tubes without additives. Plasma or serum was obtained by centrifugation at 1500g for 15 minutes. Plasma was aliquoted and stored frozen at $-80^\circ$ C until analysis.
BIOCHEMICAL ANALYSES

Serum analyses

In the MONICA cohort, total cholesterol, triglycerides and HDL-cholesterol were analyzed at a hospital laboratory. In the VIP cohort total cholesterol was measured with an enzymatic method using Reflotron bench-top analyzers (Boehringer Mannheim GmbH Diagnostica, Mannheim, Germany).

Albumin, Cystatin C and creatinine in paper III were analysed with the same methods as for plasma, see below.

Plasma analyses

In paper I we used HPLC with electrochemical detection for determination of total plasma homocysteine. For the remainder, a fluorescence polarization immunoassay on an IMx® unit (Abbott Laboratories, IL, USA) was employed. Folate and vitamin B12 were analyzed by Quantaphase II® radioassay (BioRad Diagnostic Group, CA, USA). Coefficients of variation for B12 and folate were under 7.5%.

Plasma specimens were analyzed in triplets of 1 case and 2 referents, with the position of the cases varied at random within each case-referent triplet to avoid systemic bias and inter assay variability. The investigators and laboratory staff were blinded to case and referent status.

In paper II and IV total homocysteine was measured using a fluorescence polarization immunoassay on an IMx® (Abbott Laboratories, Abbott Park, IL, USA) 111. Inter- and intra-assay coefficients of variation (CV) were 1.31 and 1.42 % and 2.10 % and 1.00 % at mean levels of 12.8 and 25.7 µmol/L respectively.

In paper II and IV plasma creatinine and plasma albumin concentrations were determined using the Vitros DT60 II dry chemistry system (Ortho-Clinical Diagnostics, Raritan, NJ, USA). With this Vitros method, no decimals are given for plasma albumin. In paper IV we used a Hitachi 911 multianalyser (Roche, Mannheim, Germany). Creatinine (Crea plus, enzymatic method) was analysed with kits from Roche/Boehringer (Mannheim, Germany). Albumin was analysed with immunoturbidimetry and cystatin C by immunoparticle turbidimetry, both with reagents from DAKO, Copenhagen, Denmark.

In paper III and IV, plasma samples were analysed on a Hitachi 911 multianalyser (Roche, Mannheim, Germany). Total cholesterol,
triglycerides, and creatinine (Crea plus, enzymatic method) were analysed using kits from Roche/Boehringer (Mannheim, Germany). Albumin and lipoprotein(a) were analysed with immunoturbidimetry and cystatin C by immunoparticle turbidimetry, all with reagents from DAKO, Copenhagen, Denmark. LDL- and HDL-cholesterol were measured by direct, homogeneous assays based on detergent treatment of the serum or plasma (N-geneous™ HDL-c and N-geneous™ LDL reagents, respectively, from Genzyme Corporation, Cambridge, MA, USA). In paper III serum samples were analysed with the same methods at the same time as the paired plasma samples.

**POLYMORPHISM ANALYSIS**

DNA was prepared from buffy coat by the chloroform/phenol method and provided by the Medical Biobank, Umeå.

MTHFR genotyping was performed at two occasions. For the first 328 subjects, RFLP analysis was employed according to Frosst et al 19 for 677C>T and Weisberg et al 25 for 1298>A/C. DNA was delivered in tubes. In the remaining 812 subjects, genotyping was performed by the TaqMan allelic discrimination method, using Minor Groove Binder (MGB) probes 112,113. This was also used in a few subjects analyzed previously, for whom the RFLP method had yielded unlikely results suggestive of the presence of chromosomes with two mutations on the same strand. For this TaqMan method 10 ng of DNA was pipetted in 96 well microtiter plates, allowed to dry and stored at room temperature until analysed. TaqMan assays and reagents were from Applied Biosystems (Foster City, CA, USA). PCR reactions were performed at the Center for Genome Research, Umeå University, on GeneAmp PCR system 9700, PCR programs were according to the manufacturer (Assay-on-Demand, ABI). PCR products were analyzed on the ABI PRISM 7900HT Sequence Detection System using the same principle as real-time PCR but we utilised end-point reading only. The results are presented visually as in Figure 5 and in a data file containing a proposed diagnostic statement.

**BLOOD PRESSURE AND ANTHROPOMETRY**

Blood pressure is measured twice in every subject after five minutes rest in the sitting position with Hawksley’s random zero sphygmanometer 114. The mean values of the determination are used for the studies. Length and weight is registered for calculation of Body Mass
Index (BMI). Waist-hip ratio is determined in a standing position with the feed closely together (12-15 cm). More details can be found at the Monica website (http://www.ktl.fi/monica/index.html) and in the paper by Stegmayr et al.\textsuperscript{105}

![Figure 5. Example of distribution of the MTHFR 677C>T genotypes when assayed with MGB-probes.](image)

**QUESTIONNAIRES AND DIETARY ASSESSMENT**

A thorough questionnaire on different aspects of lifestyle, social background, drug consumption, heredity and history of cardiovascular diseases, social support, demand and control in work, unemployment, migration, and items for women including questions on hormonal therapy and menopause was used. A validation of the quality of these data has been published.\textsuperscript{115} For dietary data a food frequency questionnaire (FFQ) was used, available for approximately half of the cohort in paper I. Of these, 85% had used The Northern Sweden 84-item food-frequency questionnaire which has been described and validated earlier.\textsuperscript{116} Daily intakes of folate and vitamin B12 were calculated with the aid of specific portion questions in the FFQ, standard portion sizes (Vikttabell), the Swedish Food Tables (Swedish food composition table.), and the internet-based Swedish Food Database (http://www.slv.se, 2003), the latter three from The Swedish National Food Administration. The folate content of various foods was updated according to recent analyses. The procedure has been described earlier.\textsuperscript{117}

**STATISTICS**

SPSS version 11.0 and 12.0 (Chicago, IL, USA) was used for statistical analyses and graphical presentation. In paper III we also used Analyze-It Clinical Laboratory, v 1.67, an add-in software module for Excel (Analyze-It Ltd., Leeds, UK, www.analyseit.com). For more details, see respective paper.
RESULTS AND DISCUSSION

HOMOCYSTEINE AND MTHFR IN RELATION TO STROKE (PAPER I)

The average time from recruitment to the stroke event was over four years. For both ischemic and hemorrhagic stroke there were significant baseline differences between cases and referents with respect to BMI, systolic and diastolic blood pressure, hypertension, and HDL-cholesterol. In Table 6 in paper I the distribution of all subjects according to the combined genotypes for 677C>T and 1298A>C is shown. No combined heterozygotes were found in this study. Thus, the mutated MTHFR 677T and 1298C alleles were always in trans position in this population. We have by now studied 3500 subjects (1100 in Örebro, Sweden and 2400 in Umeå, Sweden) without finding a single individual with the 677T and 1298A alleles in cis (unpublished data).

The distribution of homocysteine levels in all subjects demonstrated the expected relation with the MTHFR 677C>T genotypes, with the highest homocysteine levels seen in homozygotes for 677TT among referents. No differences were in homocysteine were seen for MTHFR 1298A>C locus.

Ischemic stroke

For ischemic stroke vs. referents total cholesterol, triglycerides, and smoking differed significantly. Plasma total homocysteine (tHcy) did not differ between ischemic stroke cases and referents ($P = 0.908$). When lag-time to stroke was analysed in ischemic stroke cases no difference was seen for quartiles of tHcy in for Kaplan-Meier survival plot, see figure 6.

The genotype distributions of the MTHFR 677C>T and 1298 A>C polymorphisms in referents and ischemic stroke subjects did not differ from that predicted by the Hardy-Weinberg equilibrium. Neither MTHFR 677C>T nor 1298A>C were significant predictors for ischemic stroke in all subjects, or in the male or female subgroups.
Hemorrhagic stroke cases had higher median tHcy levels than referents ($P = 0.001$). Table 2 in paper I shows odds ratios for stroke for quartiles of tHcy. Risk for haemorrhagic stroke increased exponentially in the full study group and in the sub-group of men. Even in the smaller sub-group of women the $P$-value for trend was significant. In the multivariate model, including BMI and hypertension, a graded increase in risk for haemorrhagic stroke with increasing tHcy quartile was seen, including a significant $P$ for trend.

The odds ratios for haemorrhagic stroke for the MTHFR 677 CT and TT genotypes were 2.23 (1.10-4.55) and 2.78 (0.94-8.25) respectively (Table 4). The $P$ for trend was statistically significant ($P = 0.022$). The mutated 1298C allele was associated with a significantly lower risk of haemorrhagic stroke than the normal allele 1298A when adjusted for BMI and hypertension. The genotype distributions of the 677C>T polymorphism in referents and haemorrhagic stroke subjects did not differ from that predicted by the Hardy-Weinberg equilibrium. In contrast, the 1298A>C distribution deviated significantly from the

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Figure 6. Kaplan Meier survival plot for plasma tHcy quartiles on the lag-time to ischemic stroke.

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Haemorrhagic stroke
Lag-time to stroke was analysed in haemorrhagic stroke in tHcy quartiles and tertiles with Kaplan-Meier survival plots (Figure 7). When quartiles and tertiles of tHcy are compared, it is apparent that some subjects in the lowest quartile of tHcy get stroke earlier than subjects in the higher quartiles. This effect would not be easily seen if analysing tHcy only by tertiles. Since the number of such subjects with short lag-time is quite low this does not contradict our main finding that it is an increased tHcy that is a risk factor for haemorrhagic stroke. According to earlier studies on acute myocardial infarction incidence in young age by Hamsten et al.\textsuperscript{118,119}, subjects who had contracted a disease early in life often have a particular set of risk factors, especially high levels of plasminogen activator inhibitor type 1, or lipoprotein disturbances. In their studies they found that lipoproteins were associated with diffuse coronary atheromatosis at a young age, but not to rapid progression to more advanced coronary lesions\textsuperscript{118,119}. This progression appeared to be largely determined by other mechanisms. These findings on the hazard of rapid stroke onset in the low tHcy quartile are corroborated by the inverse finding for plasma folate, where it is the highest quartile of folate which shows the shortest lag-time to stroke (Figure 8).

Haplotype analysis

Haplotypes were constructed based on the genotype distributions of the two MTHFR loci. The prevalences are shown in Table 6 of paper I. In haemorrhagic stroke, an over-representation of haplotype H1 (677T—1298A) and under-representation of haplotype H2 (677C—1298C) was observed. To calculate the odds ratios for stroke, each subject’s haplotype variable was assigned the value 0, 1 or 2, depending on whether the subject had 0, 1 or 2 copies of the respective haplotype, and entered as independent quantitative variables in logistic regression models. The H0 haplotype was unrelated to stroke. The H1 haplotype was positively associated with haemorrhagic stroke, OR 1.81 (95% CI 1.09-3.00), \(P = 0.022\). The H2 haplotype was not significantly related to haemorrhagic stroke, OR 0.65 (0.37-1.14), \(P = 0.13\). The H1 and H2 haplotypes were not related to ischemic stroke. When tHcy was added to the model, the OR for H1 in haemorrhagic stroke decreased to 1.46 (0.85-2.5), \(P = 0.16\), while the magnitude of the OR for H2 for
hemorrhagic stroke was unchanged, 0.66 (0.35-1.22), $P = 0.18$. In a multivariate model with both H1 and H2 as independent variables, the respective OR’s were 1.68 (0.97-2.89; $P = 0.06$) and 0.80 (0.43-1.48; $P = 0.48$).

Figure 7. Kaplan Meier survival plot for plasma tHcy quartiles and tertiles on the lag-time to haemorrhagic stroke.
The relation of homocysteine versus folate in MTHFR genotypes and haplotypes

Among 677C>T and 1298A>C genotypes, as well in the H0 haplotype, there is a tendency towards increasing tHcy from the highest to the lowest quartile of intake of folate from food (Figure 9). For MTHFR 677C>T the well-known pattern of higher tHcy in 677TT throughout the quartiles of folate intake is seen, compared to the 677CC and TT genotypes. For 1298A>C and the H0 haplotype no such effect is seen, suggesting minimal influence on the relation between folate intake and tHcy. Quartiles of plasma folate were associated with tHcy levels as expected (Figure 10). The pattern was more consistent with this respect compared to the pattern for folate intake. Some minimal effect was seen for MTHFR 677C>T but the 1298A>C and haplotype H0 had no obvious effect modification. The results for the H1 and H2 haplotypes were practically identical to the MTHFR 677C>T and 1298A>C genotypes, data not shown.

As the two genotypes in MTHFR may interact, we analysed the effects of the 1298A>C genotypes in the sub groups of 677 CC and CT.
In MTHFR 677 CC subjects, a consistent pattern was seen with increasing tHcy from the highest to the lowest plasma folate quartile (Figure 11). The levels of tHcy were the same in all 1298 genotypes (Table 7), thus, the 1298A>C genotypes showed no gene dose effect within the 677CC subjects. In the MTHFR CT subjects, the 1298AA subjects had 1.4 µmol/L higher tHcy compared to 1298AC (Figure 11 and Table 7). This difference is comparable to the findings reported by Weisberg et al.\textsuperscript{20} As the mutations are in trans position, there are only two groups of 1298A>C and a gene dose effect can not be fully explored.

### Table 7. Effects of MTHFR 1298A>C on tHcy (mean ± sd) in MTHFR 677 CC and CT subjects.

<table>
<thead>
<tr>
<th>MTHFR 677C&gt;T</th>
<th>MTHFR 1298A&gt;C (n)</th>
<th>tHcy, µmol/L</th>
<th>P (Anova)</th>
</tr>
</thead>
<tbody>
<tr>
<td>677 CC</td>
<td>1298 AA (144)</td>
<td>11.3 ± 4.3</td>
<td>0.768</td>
</tr>
<tr>
<td></td>
<td>1298 AC (254)</td>
<td>11.7 ± 4.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1298 CC (119)</td>
<td>11.5 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>677 CT</td>
<td>1298 AA (197)</td>
<td>11.4 ± 3.1</td>
<td>0.087</td>
</tr>
<tr>
<td></td>
<td>1298 AC (193)</td>
<td>12.8 ± 11.2</td>
<td></td>
</tr>
</tbody>
</table>
Figure 9. Error bar with mean and 95% for tHcy vs. MTHFR 677C>T, 1298A>C and haplotype H0, split by quartiles of folate intake from food.
Figure 10. Error bar with mean and 95 % for tHcy vs. MTHFR 677C>T, 1298A>C and haplotype H0, separated by quartiles of plasma folate.
Figure 11. Effects of MTHFR 1298A>C on tHcy in MTHFR 677 CC and CT subject.
HOMOCYSTEINE BEFORE AND AFTER A MYOCARDIAL INFARCTION (PAPER II)

The aims were to explore in a prospective, longitudinal nested case-referent study if a first myocardial infarction may lead to increased tHcy, which has been suggested in the literature. By doing this we would also be able to determine if the association between tHcy and myocardial infarction was greater at follow-up compared with baseline.

The numerous retrospective studies have more often found a positive relationship between tHcy and event, compared to the relatively fewer prospective studies. In a retrospective case-referent design blood is drawn from subjects in the convalescent phase. A possible confounder might be that the event in itself causes increased levels of tHcy.

The first myocardial infarction occurred after a median of 20 months after baseline screening. THcy at baseline was correlated to tHcy at follow-up (Spearman r = 0.633, P < 0.001).

The mean increase in tHcy was 1.0 ± 2.8 vs. 1.1 ± 2.6 µmol/L in the 50 cases vs. the 56 referents respectively. These data are not compatible with the hypothesis that tHcy increases secondarily to a first myocardial infarction.

Creatinine increased significantly in both subgroups during the eight years follow-up, and albumin decreased significantly. In Figure 12, it is seen that a substantial number of cases and referents decreased in tHcy and in creatinine, despite an increase in mean values of tHcy and creatinine during the follow-up time of eight years. Albumin, in contrast, decreased in a majority but a substantial number of subjects had an increase in albumin during the follow-up period. Looking at these plots, it is obvious that increases over time are not uniform. These findings needed further exploration in a prospective longitudinal setting, which was done in paper IV.

When tHcy concentrations were dichotomized, the levels at follow-up were related to a first myocardial infarction, which was not longer significant after adjustment for creatinine. The need for adjustment for creatinine has been highlighted by others as well 120.

Several studies have addressed the changes in total plasma tHcy during the acute phase or short-term follow-up of acute myocardial infarction 121-125 and stroke 126-128. Such changes may depend on many pathophysiological acute phase changes such as volume redistribution, proinflammatory cytokines etc. No consistent pattern of change has been seen. However, all these studies have been on a short-term basis. In our study we found no effect of a first myocardial infarction on tHcy levels. Thus, the event was not found to be a confounder for tHcy levels if a retrospective design is used.
Figure 12. Scatter plots of Δcreatinine and Δalbumin (no decimals given for the Vitros method used, hence the pattern) vs. ΔHcy.
EVALUATION OF THE SUITABILITY OF THE STABILYTE™ TEST TUBE FOR ANALYSIS OF RISK FACTORS OF CARDIOVASCULAR DISEASE AND MARKERS OF GLOMERULAR FILTRATION RATE (PAPER III)

In a recent paper it has been pointed out that when comparing assay results in stored samples from different specimen collection devices, differences can due to at least three causes. The first is a matrix effect directly on the assay performance in frozen as well as in fresh samples. The second is a storage effect due to intrinsic instability of the analyte over time. The third is an interaction between the first two causes, which was tentatively called “storage × matrix” effect. This combined effect means that the magnitude of the storage instability effect may in reality differ markedly (or alternatively, may not differ at all) between samples stored in different matrices. With respect to the Stabilyte™ tube, the effect first has been well characterised in several previous studies while the second effect is typically related to physical factors such as temperature, and the study design we used in paper III, which is similar to most long-term storage stability studies, likely yields results addressing the third combined cause.

In our paired serum and Stabilyte™ plasma, stored for two to twelve months (median eight months), we found that total cholesterol and triglycerides showed a linear association across the studied range with a slope not significantly different from that predicted by the dilution effect (0.83-0.86 depending on the haematocrite of the individual subject) of the citrate additive. HDL- and LDL-cholesterol, as well as lipoprotein(a) also showed good linearity and a slope within the range predicted from the dilution.

Albumin and creatinine, which are major determinants of plasma total tHcy, also had slopes not significantly different from that predicted by the dilution. Cystatin C, a new and better marker of glomerular filtration, showed a different pattern. Surprisingly, in this case the slope was not significantly different from unity, and again there was no significant intercept. The difference plot for cystatin C in Figure 3 in paper III confirms that the values in serum and plasma do not differ significantly in this material.

In conclusion, the Stabilyte™ plasma was suitable for all the evaluated analytes. This tube would thus substantially simplify sampling for biobank purposes in epidemiological studies as it allows a number of analyses in the same tube. As for the finding of unity for cystatin C in
serum and Stabilyte plasma, this may indicate instability upon storage of cystatin C in serum, a finding noted by Finney et al.\textsuperscript{129}, although their storage time was shorter than ours. This possible instability has to be explored further in other studies.
In cross sectional analysis in the 1990 and 1999 screenings the expected age-dependent increases in several variables were confirmed. The gender- and age-dependences of several of these analytes were explored in Figure 1 and 2 in paper IV. Among males and females, an age-dependence was confirmed (Kruskal-Wallis $P$ for trend < 0.01), despite the fact that no effect of age was seen on a longitudinal base. Using Mann-Whitney test, tHcy was higher in men compared to women in all age groups, both at baseline and at follow-up.

$P$ for trend for age group was significant for albumin, hs-CRP, cystatin C and GFR, but not for creatinine, at cross sections at baseline, and at follow-up, in all subjects, and in men and women (Kruskal-Wallis test). Cystatin C showed a statistically significant age-dependence, similar for both genders. A consistent numerical difference of about 0.02 mg/L between the genders across the age groups was also seen. Plasma creatinine exhibited a pronounced gender difference. GFR was showed a gender difference. Plasma albumin decreases with age in both genders and this decrease appears to be somewhat steeper in women, as a gender difference within each age group is seen in the 1999 samples, but not in the 1990 samples. For hs-CRP no gender difference was seen within its general slight up-trend with age. All the putative determinants of tHcy were significantly different over the nine-year period: albumin, creatinine, cystatin C, GFR, and hs-CRP. However, there was no increase in tHcy over this period, neither in men, nor in women.

Some of the inconsistencies observed above between age- and gender differences when assessed by cross-sectional as opposed to longitudinal observations can be resolved through an analysis of the delta-variables; defined as the value in 1999 minus the value in 1990. Differences in changes due to gender were seen for Albumin, GFR and BMI. The distribution of values of the delta-variables includes significant proportions of subjects who decrease their values over times as well as increase them. This was true for tHcy as well as for its major putative determinants such as cystatin C, creatinine, GFR, albumin, hs-CRP and BMI. Spearman correlations between tHcy at baseline and at follow-up varied between 0.618 – 0.728 in all subjects and separated for gender and for age groups (all $P < 0.001$). This correlation is consistent with our findings in paper II 130, and of a reports from the Hordaland study by Ueland et al over six years, however, this did only report Spearman for tHcy and no absolute values as the study dealt with
changes in cysteine over six years \(^49\). In a Framingham study on Alzheimer, levels of homocysteine were analysed in 935 subjects at baseline and eight years before baseline \(^{131}\). They reported that a significant Pearson correlation, \(r = 0.47\) in the total group, they did not report the values before baseline in that study.

**Figure 13.** Age at inclusion for men and women plotted vs. the \(\Delta tHcy\) in \(\mu\)mol/L over nine years.
A linear regression model with $\Delta tHcy$ over nine years as dependent, and age at first sampling as independent showed that the slope of the regression function was 0.024 (95 % CI –0.13 - 0.062) in men and 0.017 (95 % CI –0.015 - 0.049) in women. Thus it was not significantly different from zero neither in men, nor in women (Figure 13). Our finding of absence of change was confirmed by this analysis in the age-span 25-64 years during the nine year follow-up.

Another way of analysing change is by comparing the prevalences of hyperhomocysteinemia (HHcy), conventionally defined as $tHcy > 15 \mu$mol/L for the different age groups. As all subjects grow nine years older the follow-up, the change in prevalence in the same subjects can be observed (Table 8). Cross section data shows a higher frequency of HHcy in the older subjects, consistent with higher $tHcy$ in higher age groups; this is seen both at baseline and at follow-up. In contrast, when following the same subjects as they become nine years older, no significant changes of $tHcy$ were found in the different age groups or in the total cohort. HHcy is used for decisions on treatment with vitamins to reduce vitamin deficiency and for decisions on folate fortification. Thus, the finding of the longitudinal change in the population are important.

**Table 8. Prevalence of hyperhomocysteinemia, HHcy, (defined conventionally as > 15 $\mu$mol/L) in the same subjects at baseline and at follow-up.**

<table>
<thead>
<tr>
<th>Age in 1990 (n)</th>
<th>HHcy, %</th>
<th>Age in 1999, n</th>
<th>HHcy, %</th>
<th>P-value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-34 (107)</td>
<td>4.7</td>
<td>34-43 (107)</td>
<td>6.5</td>
<td>0.317</td>
</tr>
<tr>
<td>35-44 (129)</td>
<td>7.8</td>
<td>44-53 (129)</td>
<td>8.5</td>
<td>0.739</td>
</tr>
<tr>
<td>45-54 (148)</td>
<td>12.8</td>
<td>54-63 (148)</td>
<td>11.5</td>
<td>0.695</td>
</tr>
<tr>
<td>55-64 (126)</td>
<td>19.0</td>
<td>64-73 (126)</td>
<td>24.6</td>
<td>0.090</td>
</tr>
<tr>
<td><strong>Total (510)</strong></td>
<td><strong>11.4</strong></td>
<td><strong>Total (510)</strong></td>
<td><strong>12.9</strong></td>
<td><strong>0.285</strong></td>
</tr>
</tbody>
</table>

$^1$Calculated by Wilcoxon signed ranks test.

Multivariate models with $tHcy$ in 1999 as dependent and measurements in 1990 as predictors are showed that there were gender differences with the $R^2$-values higher in women compared to men. Models were constructed with either cystatin C or creatinine. After adding $tHcy$ at baseline as a predictor of $tHcy$ values at follow-up, creatinine was not significant, but cystatin C remained significant in men. Some effect could be seen for hs-CRP in women. Gender and age at baseline were significant predictors.

We also constructed multivariate regression models with $\Delta tHcy$ as dependent and $\Delta$-variables as predictors. In women $\Delta$creatinine was a significant predictor, whereas $\Delta$albumin was a predictor for men in the model with creatinine but not with $\Delta$cystatin C. $\Delta$hs-CRP and $\Delta$cystatin
C were not predictors for $\Delta t\text{Hcy}$. In these models, age and gender were not significant in the models without including $t\text{Hcy}$ at baseline.

The literature on long-term changes in $t\text{Hcy}$ is sparse. We have only found one recent study reporting on longitudinal changes $^{132}$ They studied subjects aged 41-42 and 65-67 years old. No change in $t\text{Hcy}$ was seen in the total population. In their study, age could not be studied as a continuous variable. $t\text{Hcy}$ was analysed at two different time-points close in time to sampling. In our study analysis was performed simultaneously for all samples. Apart from this there are only a few reports. In a study of the relation of $t\text{Hcy}$ with cognitive impairment, $t\text{Hcy}$ was measured at base-line and at follow-up, follow-up time 2.3 years $^{133}$. Of the participants, 104 had a significant increase in their $t\text{Hcy}$ levels (8.87 vs. 10.4 $\mu$mol/L), and 76 had a significant decrease of $t\text{Hcy}$ levels (12.0 vs. 9.3 $\mu$mol/L). Retracing the total group (not reported in the paper) yields baseline and follow-up levels of 10.19 and 9.94 $\mu$mol/L, resulting in $\Delta t\text{Hcy} = 0.256$ $\mu$mol/L in the total group. In a study on regression dilution by Clarke et al $^{134}$, data was collected from four different studies where the respective study groups provided Clarke with results of $t\text{Hcy}$ from two time-points. The cohorts were the Rotterdam Scan Study $^{135}$ with 736 subjects and a follow-up time of three years, the Hordaland study $^{40}$, with 527 subjects and a follow-up time of six years, the Framingham study $^{136}$, 1055 subjects during eight years, and the United Kingdom Prospective Diabetes Study $^{137}$ with 430 subjects during twelve years. Using the mean $t\text{Hcy}$ levels at baseline and follow-up, the $\Delta t\text{Hcy}$ for the studies (not reported in the study) were estimated to 0.6, 0.2, 1.3 and 0.5 $\mu$mol/L respectively. No sub-group analysis for gender or different age groups was reported by Clarke et al $^{134}$. In paper IV no change in $t\text{Hcy}$ levels at baseline and at follow-up were seen after a relatively long follow-up period of nine years. This was consistent for both men and women. Age seems not to be a major determinant over this time-period in subjects 25-64 years at baseline.

In cross-sectional studies age has been found to be a determinant of $t\text{Hcy}$ $^{33,44-47}$. In contrast, one of our main findings were that $t\text{Hcy}$ does not increase uniformly in all subjects with time, but increases only in a subset of subjects, whereas in others it actually decreases over time. In multivariate analysis, age was significantly correlated with $t\text{Hcy}$ at follow-up. This could be due to the strong relation at baseline with $t\text{Hcy}$. Adding $t\text{Hcy}$ at baseline weakened the predictive value of age was, and it was no longer statistically significant in the subgroup of men in the model with both cystatin C and $t\text{Hcy}$ at baseline. In multivariate models with $\Delta$-variables, gender and age at baseline were no longer significant if $t\text{Hcy}$ at baseline was added to the model. Thus, age was not found to be a major determinant of change for in $t\text{Hcy}$ neither in men, nor in women in a longitudinal setting.
The multivariate models with $\Delta$-variables had lower predictive power ($R^2$) than did models with variables at inclusion. As $\Delta$-variables have a more pronounced coefficient of variation, we conclude that the predictive values are appreciable and note-worthy in these models as well as in the models based on inclusion variables.

The decrease in renal function over time has been one of the putative explanations why tHcy increases with age. This decrease in GFR, reflected by the subsequent increase in creatinine and cystatin C is counteracted by the decrease in albumin, the main carrier / transporter of tHcy. In multivariate analysis, creatinine at inclusion was a predictor for tHcy at follow-up; however the predictive power vanished when tHcy in 1990 was entered to the model. Cystatin C at inclusion was predictive for tHcy at follow-up in the models, adding tHcy at inclusion retained the predictive power in all subjects and in men, but the prediction was no longer significant in women. When analysing $\Delta$-variables as predictors of $\Delta$tHcy in models with and without tHcy at inclusion as a predictor, both $\Delta$creatinine and $\Delta$cystatin C were significant predictors among women but not among men, the reverse was seen for $\Delta$albumin. The tHcy thus seems to be more related to markers of GFR in women and to albumin in men.

Creatinine is also dependent on tHcy metabolism for its synthesis. Thus, the relation between creatinine and tHcy is more complicated than the relation of tHcy with cystatin C, which is a marker of GFR only. In multivariate models predicting tHcy at follow-up, those models with cystatin C at baseline had a higher predictive value than those with creatinine. In models with $\Delta$-variables predicting $\Delta$tHcy, $\Delta$albumin was a better marker than the renal markers in men. In women no markers were consistently significant.

We found lower tHcy levels in in women compared to men, the difference was 1.6 at baseline and 1.4 at follow-up, in accordance with earlier reports of a difference of approximately 2 $\mu$mol/L. However, it cross sectional studies have reported an increase with age was similar in men and women, similar to our findings. However, no change over time was seen on a longitudinal basis, neither in men, nor in women.

In conclusion, age-related differences in tHcy were seen in cross sectional analysis at baseline and at follow-up but no changes in individual tHcy levels were seen over a nine-year period, neither in men, nor in women. No increase in hyperhomocysteinemia could be seen on a longitudinal basis. These findings may have public health implications. Baseline levels of albumin, creatinine, cystatin C, and to some extent hs-CRP, were predictors of tHcy at follow-up but gender differences were seen. Adjusting for tHcy at baseline showed that cystatin C was an independent predictor but creatinine was not. Determinants of change in tHcy were change of albumin in men and change of creatinine in women.
CONCLUSIONS

Both total plasma homocysteine and the MTHFR 677 T allele may be risk factors for haemorrhagic stroke; this has not previously been reported in the same prospective study. In contrast, no association with ischemic stroke was found.

A first myocardial infarction does not appear to cause long-term increases in plasma total homocysteine concentrations compared to referents, in subjects followed longitudinally in a prospective setting. High concentrations of homocysteine at follow-up were associated with a first myocardial infarction, but no longer significant after adjusting for creatinine.

Age-related differences in tHcy were seen in cross sectional analysis at baseline and at follow-up, in contrast no net changes of individual tHcy levels were seen over a nine-year period, neither in men, nor in women.

No increase in the prevalence of hyperhomocysteinemia could be seen on a longitudinal basis during nine years.

Baseline levels of albumin, creatinine, cystatin C and hs-CRP were predictors of tHcy at follow-up but gender differences were seen. Adjusting for tHcy at baseline showed that cystatin C was an independent predictor but creatinine was not. Determinants of change in tHcy were change of albumin in men and change of creatinine in women.

Age seemed not to be a strong determinant of change in total homocysteine over time, but rather a marker of an increasing number of modifications in true mechanistic determinants, for instance life-style, metabolic disorders or other intercurrent diseases.

Stablyte™ plasma can be utilised for analysis of the traditional lipid risk factors and lipoproteins, as well as novel emerging risk factors in the fibrinolytic system, and homocysteine and its determinants, albumin and the renal markers creatinine and cystatin C. Using acidic citrated plasma as the single medium would thus substantially simplify sampling for epidemiological studies and biobank purposes.
IMPLICATIONS FOR THE FUTURE

Further studies are needed to explore the gene-nutrient interactions in folate- and methionine metabolism in relation to cardiovascular disease, as well as in other diseases.

There is still a need for prospective studies of homocysteine in relation to cardiovascular disease, cancer, cognitive function, dementia and other diseases.

The changes over time of homocysteine over time need to be further explored, in relation to changes in other biochemical and lifestyle determinants not covered in paper IV.

The long-term stability of cystatin C in frozen serum needs to be further explored.

The findings that neither total homocysteine, nor hyperhomocysteinemia increased during nine years may have public health implications.

The causality of homocysteine as a risk factor still needs to be clarified in on-going and future prevention studies. However, as these studies are biased by for example population-based fortification with folate, self-treatment with multivitamins, treatment with statins and other cholesterol-lowering drugs, prospective biobank studies will remain a valuable source of information on homocysteine related diseases.
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