General introduction
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Essentials of homocysteine and 1-carbon metabolism

Cardiovascular disease (CVD) is one of the most prominent causes of death in the modern world. Research has shown that a combination of nutrition, lifestyle and genetic predisposition influence our susceptibility to this disease. High blood pressure, high cholesterol and obesity are all well established risk factors for acquiring CVD. However, they do not account for all incidents. A relatively new risk factor is elevated plasma homocysteine (Hcy), a sulfur containing amino acid. Numerous clinical studies have established elevated plasma levels of this amino acid as an important risk factor for the development of CVD (1), independent from other known risk factors.(2) Even small changes in plasma Hcy levels are associated with detrimental effects. Increases of fasting plasma Hcy level as small as 3 µmol/L are associated with an increased incidence of ischemic heart disease (11%) and ischemic stroke (19%).(1;3) Additionally, there does not seem to be a threshold effect in this association. Whether an elevated level of Hcy is merely a signal (biomarker), or could trigger cardiovascular events itself has been the subject of many studies and speculations.

There are indications that Hcy could affect the vascular endothelium resulting in altered vasodilatation. Additionally, increased levels of Hcy could promote oxidative stress by reducing nitric oxide (NO) and inducing the production of reactive oxygen species (ROS).(4;5) However, the question as to whether such effects truly exist in-vivo and explain the association with CVD has not been answered satisfactorily.

For a better understanding of the role of Hcy in CVD, it will be necessary to comprehend the entire metabolic pathway Hcy is part of, which we will refer to as one-carbon metabolism (1C metabolism, Figure 1).

The main purpose of the 1C metabolism is to provide methyl groups (1C units) to virtually every methylation reaction in the body, and to provide formyl groups (1C units) for de novo purine synthesis. Methionine (Met) enters the body via our diet (proteins). In order to make the methyl group of Met available for donation to other molecules, an adenosyl group is added and S-adenosylmethionine (SAM) is formed. SAM can donate its methyl group to a wide variety of molecules (from small molecules, like guanodinoacetate, to large molecules, like proteins and DNA). The resulting molecule S-adenosylhomocysteine (SAH) is hydrolyzed to Hcy. After the donation of a
methyl group by 5-methyltetrahydrofolate (5-methylTHF) Met is reformed, and the cycle can start again.

**Figure 1.** Simplified scheme of one-carbon metabolism.

*CBS (cystathionine β-reductase), MAT (methionine adenosyl transferase), MTHFD (methylene tetrahydrofolate dehydrogenase), MTHFR (methylene tetrahydrofolate reductase), MS (methionine synthase), SAH (S-adenosylhomocysteine), SAM (S-adenosylmethionine), SAHH (SAH hydrolase), THF (tetrahydrofolate).*

**Why are the homocysteine-lowering trials negative?**

The most endeavored approach to lower Hcy has been supplementation with folate. Indeed, supplementation with folate lowers plasma Hcy about 15%.(6) However, the risk of cardiovascular events is unaffected.(6-9) Several factors could contribute to these disappointing results. First of all Hcy could be merely a biomarker, and not contribute to CVD itself, despite the experimental evidence that suggests otherwise. Some studies suggest that other 1C metabolites rather than Hcy itself trigger detrimental effects leading to CVD. One of the suggestions is SAH, which will be discussed later.(10) Secondly, the design of the intervention trials could contribute to their negative results:

- *Duration of the intervention*

  The development of an atherosclerotic plaque is the result of a process that takes several decades. Intervention studies on the other
hand, rarely exceed 5 years. If Hcy contributes to atherosclerosis in any but the final stages, the follow-up period of the trials may simply be too short. A meta-analysis investigating the effects of B–vitamins on stroke indeed suggested that when only studies with a duration exceeding 36 months are taken into the account, a significant risk reduction of 29% is observed.(11)

- **Parallel use of multivitamins / food fortification**

The use of multivitamins is so widespread nowadays, that several of the large Hcy lowering intervention trials were done in people taking some kind of supplementation. In addition, the fortification of food with B-vitamins is also very common (even in countries where it is not mandatory). It will be difficult to observe an effect when the B-vitamin status of the control group is improved before the trial even starts.

And thirdly, the most widely used form for supplementation and treatment of hyperhomocysteinemia is folic acid (FA). This is a synthetic, oxidized analogue of natural folates. Because it structurally resembles other folates very closely, but has no physiological activity on its own, it could interfere with binding to – and activity of- folate enzymes and transporters. This way, FA supplementation could potentially lead to unwelcomed side-effects like folate deprivation of certain cell types, and altered folate form distribution. Additionally, folate supplementation, in any form, could contribute to the growth and inflammatory activity of already existing atherosclerotic plaques.(12) A study investigating CVD in elderly people supports this notion by suggesting that elderly actually benefit from relative folate deprivation.(13)

**An alternative approach the Hcy paradox**

One way of addressing the apparent lack of positive results of Hcy lowering trials is to look closer to the place Hcy holds in the 1C metabolism (Figure 1). Knowing that high plasma Hcy predicts CVD, the question raises which factors could influence plasma Hcy levels? Part of this thesis is aimed to resolve this question, for which the following components of 1C metabolism are relevant:

1. The equilibrium between intracellular and extracellular compartments
2. The remethylation to methionine
The Hcy molecule holds a thiol (SH) group; a group that is readily oxidized to a disulfide in oxidizing milieus like blood. Therefore, in plasma, Hcy will be mainly present as a (mixed) disulfide, or be bound to cysteine residues in proteins. In contrast, the cytosol is a reducing milieu, were Hcy is mainly present as a monomer. This difference in redox potential causes differences in intra- and extracellular levels of free and bound Hcy.

1C metabolism occurs intracellularly. However, all studies have focused on lowering Hcy in plasma. Whether plasma and intracellular Hcy are correlated has not been sufficiently addressed. Therefore, first the equilibrium between plasma Hcy and intracellular Hcy needs to be established, followed by a study of what happens to intracellular levels of Hcy after supplementation with folate.

**Figure 2.** The remethylation of methionine.

DHFR (dihydrofolate reductase), dTMP (thymidinemonophosphate), dUMP (deoxyuridine monophosphate), MTHFD (methylene-tetrahydrofolate dehydrogenase), MTHFR (methylene-tetrahydrofolate reductase), MS (methionine synthase), SAH (S-adenosylhomocysteine), SAM (S-adenosylmethionine), SHMT (serine hydroxymethyltransferase), THF (tetrahydrofolate), TYMS (thymidylate synthase).
2. The remethylation to methionine

In the remethylation pathway, Hcy accepts a methyl group from 5-methylTHF, forming Met and tetrahydrofolate (THF). This reaction is catalyzed by methionine synthase (MS) and requires vitamin B12 as a co-factor. In order to regenerate 5-methylTHF, the THF molecule must undergo a series of reactions. The final (irreversible) reaction in this series is catalyzed by methylenetetrahydrofolate reductase (MTHFR). The activity of MTHFR is regulated by SAM: when SAM levels increase, MTHFR activity is lowered. MTHFR is present in virtually all cell types. For the remethylation of Hcy, an alternative pathway is available, in which betaine serves as a methyl donor. However, this pathway is mainly confined to the liver. Patients suffering from MTHFR enzyme deficiency show elevated plasma levels of Hcy and premature CVD.(16) A common MTHFR polymorphism (C677T, prevalence of the homozygous variant is approximately 12-15% in Caucasian populations (17)) is without severe clinical symptoms, however elevated plasma Hcy levels are observed, particularly if folate status is relatively low.(18)

3. The transsulfuration to cystathionine

The transsulfuration pathway comprises of two irreversible reactions. In the first step of this pathway Hcy and serine are condensed to cystathionine (Cysta) by the enzyme cystathionine β-synthase (CBS). Its activity is regulated by SAM. When SAM levels rise, CBS activity increases too. In the second, step cysteine is formed by cystathionine γ-lyase (CL). Both reactions require pyridoxal-5-phosphate (PLP, vitamin B6) as a co-factor. The transsulfuration pathway is also the link between Hcy and glutathione. The latter is the main regulator of the redox potential of the cell, among other functions.

The irreversible conversion of Hcy to Cysta is the only way to remove Hcy from the 1C metabolism. Under normal conditions, CBS in the liver is responsible for about 40-50% of the conversion of Hcy. Increasing dietary Met increases this percentage even more.(19) CBS however, is not expressed to the same extent in all cell types. Only, liver, kidney and pancreas show substantial CBS activity. The activity of CBS is low in endothelial cells. Whether this activity is high enough to influence Hcy levels in this cell type and could contribute to the risk of CVD is yet unknown.

Patients with an inactivating mutation in the CBS enzyme suffer from extremely high plasma levels of Hcy and premature CVD.(20) Individuals
heterozygous for CBS deficiency may have elevated Hcy, in particular after methionine loading. (21)

**Figure 3.** The transsulfuration of homocysteine.

*CBS (cystathionine β-reductase), CL (cystathionine γ-lyase), SAH (S-adenosylhomocysteine), SAM (S-adenosylmethionine), THF (tetrahydrofolate).*

The association between CBS gene variants and elevated plasma Hcy levels raises the question whether CBS could be a valid target for homocysteine-lowering therapies (for instance vitamin B6). A mutation in the C-terminal regulatory domain of the CBS gene is able to increase its activity and, hence, lower plasma Hcy. (22) This may provide an option for future Hcy-lowering therapies by activating CBS via attaching small molecules to this part of the enzyme.

Recently, both CBS and CL were shown to have an alternative role in the production of H₂S, a compound that mediates smooth muscle relaxation and subsequent vasodilatation. (23) Disturbance of this function could potentially contribute to risk of CVD.
4. The equilibrium with S-adenosylhomocysteine

The hydrolysis of SAH to Hcy is a reversible reaction, and is catalyzed by S-adenosylhomocysteine hydrolase (SAHH). Energetically, the reaction of Hcy to SAH is favored. (24) However, in vivo, the hydrolysis reaction will proceed when the products (Hcy and adenosine) are effectively metabolized. (24) When genetic or nutritional variations disturb/reduce effective removal, elevated levels of Hcy are expected to induce accumulation of SAH. In fact, elevated levels of Hcy from any cause are expected to increase SAH levels. Some studies suggest that SAH rather than Hcy is a risk factor for CVD (25). Both elevated Hcy and SAH are correlated with an increased incidence of CVD. However, after oral FA administration only plasma Hcy levels were lowered while plasma SAH levels remained unchanged. (26) Since the risk of CVD remains unchanged, this may support the hypothesis that SAH is the risk factor and not Hcy. It does, however, raise questions as to what happens to SAH levels intracellularly when homocysteine-lowering is effectuated. Again, the equilibrium between Hcy and SAH would suggest that SAH is lowered together with Hcy, but this remains to be shown.

![Figure 4. The equilibrium with S-adenosylhomocysteine.](image)

SAH (S-adenosylhomocysteine), SAM (S-adenosylmethionine), SAHH (SAH hydrolase), THF (tetrahydrofolate).

SAH has been shown to (product) inhibit methyltransferases, and could potentially influence the activity of a high number of transmethylation
reactions. (27) For instance, the methylation of DNA could be diminished, leading to DNA hypomethylation and subsequent altered gene expression. (28) A popular hypothesis is that altered gene expression triggers CVD. (29) However, patients suffering from adenosine kinase deficiency and subsequent increased levels of adenosine and SAH did not show diminished global DNA methylation. (30) And a patient suffering from SAHH deficiency even showed global hypermethylation of DNA. (31) In addition, none of the aforementioned patients showed premature CVD suggesting that elevated levels of SAH alone do not seem to trigger CVD via this route.

**The closed loop of 1C metabolism**

An important thing to keep in mind is that elevated plasma Hcy levels could result from a disturbance in any of the above mentioned pathways or even a combination of them, since they are all connected. The universal methyl donor SAM is able to control the activity of at least 3 key enzymes (methionineadenosyl transferase (MAT), CBS, and MTHFR) involved in 1C metabolism, all with the purpose of controlling its own availability. However, by influencing the activity of both CBS and MTHFR, SAM also controls the levels of Hcy. Therefore, Hcy should not be considered as a lone metabolite but as part of a tightly interregulated metabolism. Hence future (intervention) studies investigating the link between Hcy and CVD, as well as the effects of Hcy-lowering interventions should include other 1C metabolites as well. In addition, intracellular levels of the 1C metabolites could offer better insight into what is actually happening inside cells.

In order to answer such questions surrounding Hcy, new analytical tools are necessary. Analytical methods used for the determination of 1C metabolites in plasma usually lack the sensitivity to measure intracellular levels. Since considerable progress has been made with the introduction of increasingly sensitive LC-MS/MS systems, measurements of these levels are now within reach.
Aims of this thesis

As was outlined before, there are several possibilities as to why FA based Hcy-lowering intervention trials are failing. With the emerge of new sensitive analytical instruments, it has now become possible to develop methods that are able to accurately measure Hcy concentrations in a wide variety of compartments. Hence it became possible to elucidate a couple of the hypothesis for the lack of results of the Hcy-lowering trials:
- plasma Hcy concentrations are not an accurate reflection of Hcy levels in the various compartments (i.e. cerebrospinal fluid, cells, and tissues).
- FA therapy lowers plasma Hcy levels, however this effect does not extent to other compartments within the body.
- FA itself has detrimental effects that could cancel out the proposed beneficial effects.

Outline of the thesis

First the necessary tools to measure 1C metabolites levels and relevant enzyme activities and transport velocities were developed. They are incorporated in all chapters, and are the main focus of Chapter 2 and 3.
To study whether plasma Hcy values are an accurate reflection of concentrations in other compartment, plasma Hcy levels were compared to Hcy concentrations in peripheral blood mononuclear cells (PBMCs). To study the effect of 1C metabolism in cells in relation to CVD, the most optimal cell to study this in-vivo would be endothelial cells. However, obtaining this cell in-vivo is practically impossible. The only readily available human cells that possess the entire set of 1C metabolism enzymes are PBMCs. They are also exposed to the same environment (plasma) as endothelial cells. The correlation between human PBMC and plasma levels in studied in Chapter 4. The expression of 1C enzymes is not comparable for all cell types. Especially, CBS and MAT are differently expressed in liver. To investigate whether plasma homocysteine concentrations reflect liver levels, the correlation between plasma and rat liver 1C homocysteine was studied in Chapter 5. Distribution of Hcy between different compartments in the body (blood and cerebrospinal fluid) is studied in Chapter 6.
To study the effect FA therapy has intracellularly, PBMC Hcy levels and its relation to other 1C metabolites was studied in Chapter 4.
Finally, the possible detrimental effect FA might have on enzyme activities and transporter velocities was investigated in Chapter 7 and 8.
References


