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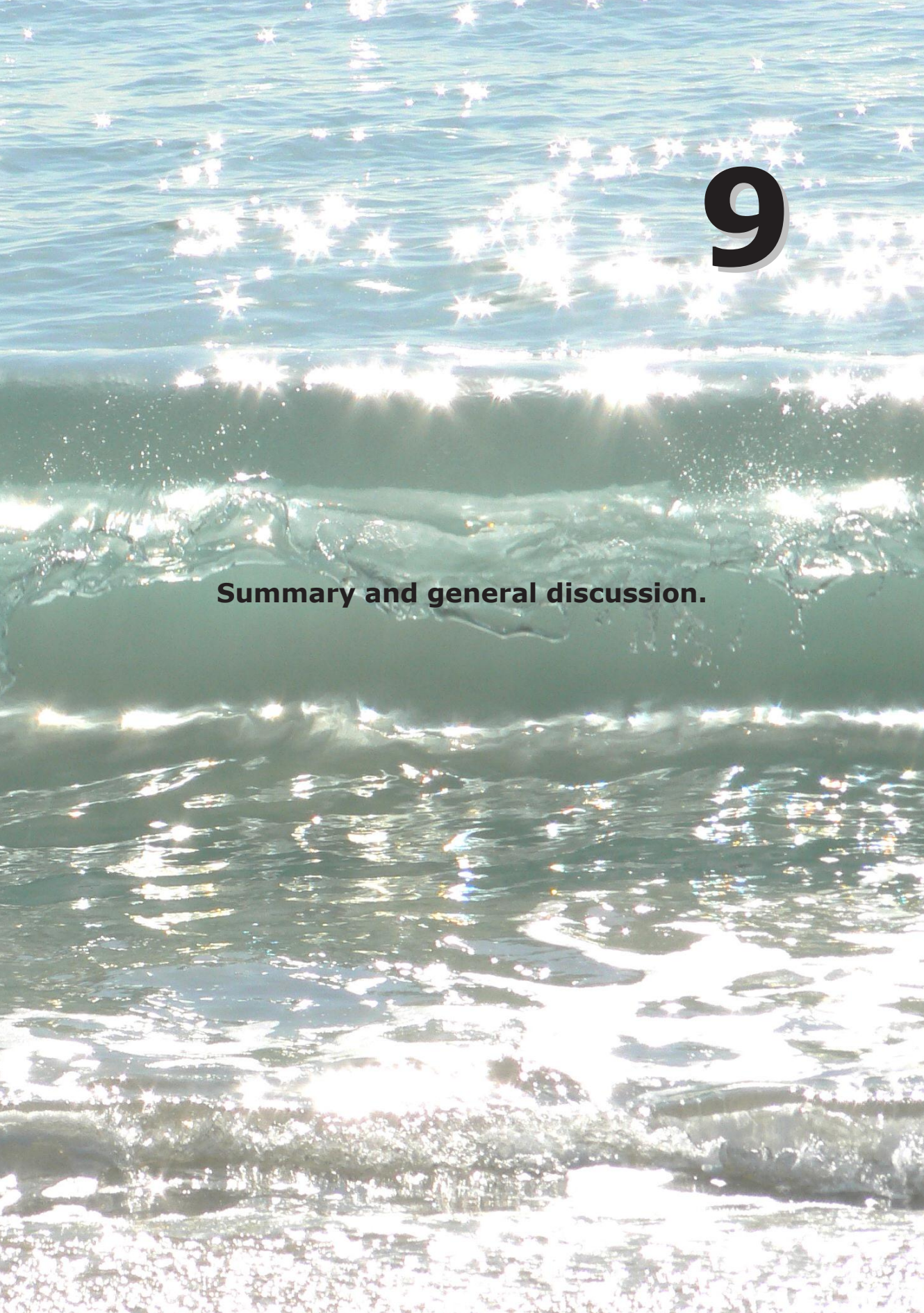

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Summary and general discussion.

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This thesis started with one simple fact: elevated levels of plasma Hcy are linked to an increased risk of CVD. The mechanism(s) behind this phenomenon remains unclear. Hcy might contribute to vascular damage itself (risk factor), or merely be a predictor (risk marker). Where *risk markers* can only predict a certain outcome, *risk factors* causally contribute to the development of the disorder.(1;2) Numerous papers have provided evidence to support the claim that Hcy is a *risk marker* for CVD.(3;4) Whether Hcy is in fact a *risk factor* remains more elusive. Mechanistic links to various phenomena like vascular tone, oxidative stress and apoptosis have been suggested.(5-7) However, there is no definite proof as to how Hcy might cause CVD. In the past, successful treatments have been developed without knowing whether the targeted compound was in fact a risk factor. For hyperhomocysteinemia, many intervention trials aimed at lowering plasma Hcy by means of vitamin B supplementation failed to lower the risk of a cardiovascular event.(8) There is even some evidence that an excess of B-vitamins might contribute to more rapid atherosclerotic lesion development.(9) However, the negative outcome of the intervention trails does not change the fact that elevated levels of plasma Hcy are linked to an increased risk of CVD, independent of known risk factors like blood pressure, smoking, diabetes and obesity.(10) Since the most straightforward interventions failed, the search should continue to discover how exactly plasma Hcy levels are linked to CVD. This thesis aimed for a better understanding of the 1C metabolism in order to find answers as to why Hcy in plasma is elevated, whether plasma levels are a realistic reflection of tissue values, and what happens to these parameters when supplemental folate is administered.

Development of the necessary analytical tools.

In order to answer many of the questions raised in the aims of this thesis, 1C metabolite levels in both plasma and intracellularly need to be accurately measured. Methods for the measurement of 1C metabolites in plasma already exist. However, in general these methods usually lack the sensitivity to measure 1C metabolites intracellularly. In addition, methods for the measurement of enzyme activities and transport velocities exist but they often use radioactive isotopes and consist of lengthy protocols. Therefore, new analytical methods are required. With the development of increasingly sensitive LC-MS/MS methods, these measurements are within reach.

Throughout this thesis (Chapter 2, 3, 4, 7 and 8), new analytical methods for the accurate measurement of 1C metabolites levels, relevant enzyme activities and transport velocities are described.

1C metabolism at baseline

Plasma 1C metabolite concentrations versus intracellular levels.
Since elevated levels of **plasma** Hcy were correlated to a higher incidence of cardiovascular disease, most intervention studies aimed at lowering this parameter. But is plasma Hcy an adequate reflection of what is happening intracellularly? Most proposed harmful effects of Hcy (i.e. altered methylation, oxidative stress, endothelial nitric oxide synthase (eNOS) inhibition, cell proliferation) occur intracellular. Therefore, it is essential to understand the effects of interventions on intracellular 1C metabolism. Could plasma Hcy levels provide an accurate reflection of these effects? In order to address this, it is important to realise that plasma Hcy levels reflect the sum of export from, and uptake into, cells/organs).(11) Most likely, many tissues contribute to the plasma Hcy equilibrium (the sum of export and uptake of cells/organs).(11) Since liver, kidney and pancreas are the only organs with an active transsulfuration pathway (irreversible conversion to cystathionine (Cysta)), these organs probably play a leading role in the metabolization of Hcy (12). However, the kidney does not significantly filter or convert Hcy.(13) This leaves the liver as the most plausible major contributor to Hcy conversion, and therefore plasma Hcy levels might be expected to mirror hepatic levels.(14) However, Chapter 5 showed only a weak correlation between plasma and liver Hcy. While some tissues may be responsible for levels of Hcy in plasma, plasma Hcy levels in turn, could contribute to the intracellular Hcy concentrations of specific cell types. Elevated levels of plasma Hcy have been shown to increase influx of Hcy into tissues like the heart.(11) Uptake would suggest a correlation between plasma and intracellular levels, and this was studied in Chapter 4. However, the correlation between plasma Hcy and intracellular PBMC Hcy levels was poor.
Since in this thesis was shown that plasma Hcy levels itself are not an adequate reflection of intracellular concentrations, the levels of other 1C metabolites, mainly SAM and SAH, might offer more information. However, Chapter 4 showed that the plasma levels of these other metabolites are also a poor reflection of intracellular concentrations. In conclusion, these 2 chapters indicate that plasma 1C metabolites do not accurately reflect intracellular 1C

metabolite levels. Therefore, measurement of plasma Hcy as a reflection of what happens in cells has important limitations.

Interrelations of 1C metabolites intracellularly.

Studies on the relations between intracellular 1C metabolites are the focus of Chapter 4 and 5. In this tightly regulated 1C metabolism, one would expect the levels of the individual metabolites to correlate to each other. Especially SAM levels should conceivably correlate to other 1C metabolites, since SAM acts as a central regulator of multiple 1C enzymes. In plasma, no such correlations were found.(15;16) This could be due to the fact that the chemical properties of SAM prevent easy passage across the membrane. SAM has the ability to regulate its own levels by affecting the activity of both CBS and MTHFR (high SAM levels activate CBS activity, while inhibiting MTHFR activity). By doing so, it also controls Hcy levels. Therefore, a tight intracellular correlation between SAM and Hcy would be expected. In both PBMCs and liver, a positive correlation between intracellular SAM and Hcy was indeed found, and shows the value of measuring intracellular 1C metabolites. Intracellular SAM levels are not predicted by the same 1C metabolites in PBMCs (Hcy and SAH) and liver (Hcy and Met). This may be due to the fact that 1C enzymes are expressed differently in both cell types. The correlation between SAM and SAH in PBMCs may be explained by negligible CBS activity in this type of cell. The correlation between SAM and Met in liver may be due to the different isoforms of MAT in this cell type.

From Chapter 4 and 5 could be concluded that, in contrast to plasma values, the intracellular levels of 1C metabolites are highly correlated to each other. This fact merits a consideration for future intervention studies: An intervention that would reduce Hcy intracellularly probably also would have an impact on the levels of other 1C metabolites. Whether such effects would render benefit remains to be elucidated.

Distribution between different compartments.

In Chapter 6, the distribution of 1C metabolites between blood and cerebrospinal fluid is studied. Cerebrospinal fluid (CSF) Hcy and 5-methylTHF concentrations correlated well with their plasma levels, suggesting that plasma levels of these 1C metabolites are indicative of what is available to the brain.

While a lot of studies have investigated correlations between 1C metabolites in plasma, CSF is less well studied in this respect. In CSF, Hcy was correlated to CSF 5-methylTHF and CSF SAH. The anticipated link with SAM levels was not observed. CSF SAH correlated to CSF 5-methylTHF and CSF Hcy. CSF SAM

did not correlate to any of the other CSF metabolites. Similar results were found in plasma before, suggesting that SAM is regulated mainly as an intracellular compound. Measuring SAM in extracellular matrices is unlikely to reflect what is happening in cells.

1C metabolism after FA supplementation

Extracellular 1C metabolite levels.
In order to lower plasma Hcy aiming to reduce the risk of CVD, large intervention studies have been performed, the vast majority of them using FA. What happens to plasma levels of Hcy and other 1C metabolites after FA supplementation has been extensively researched. Levels of plasma 5-methylTHF increased and FA appeared in plasma (17), and the anticipated lowering of plasma Hcy indeed occurred.(8) However, the 1C metabolites SAM and SAH concentrations did not alter.(18) The main result of the intervention trials was that the risk of a cardiovascular event was not reduced.(8)

Intracellular 1C metabolite levels.
What happens to intracellular 1C metabolite levels after FA supplementation has not been studied extensively. The main obstacle for this is to obtain a cell type that can be studied in humans. Chapter 4 describes what happens to PBMC 1C metabolite concentrations after eight weeks of 500 µg FA treatment. In contrast to plasma values, where an increase in folate and decrease in Hcy can be observed, none of the PBMC 1C metabolite concentrations altered significantly. This unexpected result can maybe provide a clue as to why folate intervention trials fail to lower risk of CVD. Another unexpected finding was that folate deprivation in rats (Chapter 5) leads to an unexpected *decrease* in liver Hcy levels. Could this be a reflection of low 1C metabolism fluxes? One thing that could be observed in this folate deprivation model, is the fact that folate deprivation seems to favour the synthesis of 5-methylTHF over non-methylTHF, which shows the importance of this piece of metabolism.

Interrelations of 1C metabolites.
In PBMCs the interrelations between 1C metabolites change after FA supplementation. PBMC SAM levels no longer correlate with PBMC Hcy or any of the other 1C metabolites. And only PBMC Hcy and PBMC SAH still correlate. Since CBS activity is very low in this cell type, this result may indicate that at higher folate concentrations, SAM loses its ability to inhibit MTHFR. In an in-vitro lymphoblast cell model this could indeed be demonstrated (at

physiological folate concentration SAM is able to inhibit MTHFR activity, while at higher folate concentrations this inhibition ability is lost). This shows that folate supplementation, next to beneficial effect (i.e. lowering of plasma Hcy) also could contribute to the disruption of the regulation of the 1C metabolism, which might not be beneficial. SAM is a methyl donor for a whole variety of reactions. Changing SAM levels could potentially affect the equilibrium of all these reactions. Therefore, SAM has the ability to strictly control its own levels by influencing the activities of CBS, MTHFR and MAT. Disruption of this ability could have a wide range of unexpected effects.

Possible unsolicited side-effects of FA supplementation.

Another approach to explaining why Hcy-lowering therapies fail to lower the risk of CVD, is to investigate whether the therapy itself, particularly FA, may be harmful. If so, any beneficial effect that may arise from lowering plasma Hcy could be cancelled out by harmful effects of the intervention. What harmful effects could theoretically be expected from FA supplementation? Some answers may lie in the chemical structure of FA. FA is a synthetic analogue of natural folates and resembles their structure very closely. One of the resulting disconcerting properties of FA is the ability to interfere with folate enzymes and transporters. FA was assumed to be completely converted to natural folates before entering the systemic circulation. However, several studies have already shown that unmetabolized FA is present in blood, following FA supplementation or even food fortification.(17;19) Moreover, FA has been found in for example liver cells from rats.(20) However, little is known about persistence of FA inside the cell before it is metabolized to 5-methylTHF. Bearing in mind that DHFR, the enzyme responsible for the initial conversion of FA, is expressed very sparsely and variably in humans in comparison to for example rats (21) it is very likely that FA can be found in human cells as well. Chapter 7 describes that in cultured lymphoblasts, FA is indeed still present 1 hour after removing FA from the medium.

Effect of FA on enzyme activity.

Chapter 7 describes that, in-vitro, FA causes inhibition of folate enzymes MTHFR and DHFR. MTHFR, however, does not seem to be inhibited severely, which appears to be in contrast to earlier work.(22) However, DHFR activity is reduced significantly. DHFR not only reduces FA to THF, but also facilitates

the conversion of BH_2 to BH_4.(23;24) This reaction must suffer to a larger extent from the presence of FA in the incubation mixture, as BH_2 has a lower affinity for this enzyme than DHF and FA. An in comparison 100 fold lower FA concentration already halves this conversion rate (Chapter 7). Lowered concentrations of BH_4 could for instance lead to impaired NO release.(25) One might expect DHFR activity to increase after FA supplementation (since FA needs DHFR for its reduction to DHF). In a lymphoblast cell experiment, however, addition of FA to medium instead of 5-methylTHF reduced DHFR activity. In addition to its inhibitory effect on folate enzymes by competing with the natural substrate, FA supplementation could also potentially interfere with the regulation of enzymes by other compounds. Chapter 4 indeed suggests that increased folates levels interfere with the ability of SAM to regulate MTHFR.

Effect of FA on transporter velocity.

In Chapter 8, we show that FA levels that have been measured in plasma following FA supplementation (17), can lower the uptake of the natural bioactive folate 5-methylTHF in primary human umbilical vein endothelial (HUVEC) cells by as much as 60%. This was previously reported in intestinal and epithelial cells.(26) Moreover, the expression of one of the transporters present in this cell-type, the proton coupled folate transporter (PCFT), was decreased. Since the amounts of FA in this study resemble the ones found in human plasma (10-20 nmol/L (17;27)), it is conceivable that similar observations could be done in-vivo. If indeed human endothelial cells would display reduced MTHF uptake after brief/intermittent exposure to FA, the consequence could be reduced intracellular folate concentrations. The importance of adequate folate status of endothelial cells was shown by a recent study where higher vascular 5MTHF concentrations were associated with lower vascular superoxide radical generation (oxidative stress).(28)

Effect of FA on intracellular folate concentrations.

As FA may interfere with (i.e. lower) both transporter velocities and enzyme activities, an altered intracellular folate concentration and distribution would be a conceivable consequence. In Chapter 7, lymphoblasts are cultured under different folate conditions, and indeed show approximately 80% lower folate levels in FA conditioned medium compared to 5-methylTHF. Similar observations were made in HUVECs in Chapter 8, which indicates that the folate concentrations of most cell types will be negatively affected by the presence of FA. In addition, the methylTHF – non-methylTHF ratio was increased. Apparently, the formation of 5-methlyTHF in liver is strongly

favoured over other folate isoforms. In Chapter 4, folates in PBMCs of healthy volunteers are measured. The folate content of this cell-type does not alter significantly after FA administration. This implies that either there is no effect of FA supplementation on PBMC folate levels or the inter-individual variability is too high to detect the relative small changes due to supplementation.

Conclusion

Regardless of negative outcomes of plasma Hcy lowering trials, the simple fact 'elevated levels of plasma Hcy are linked to an increased risk of CVD' is still true. Since FA based Hcy lowering therapies seem to have no effects, solving the puzzle will need a more elaborate approach than merely B-vitamin supplementation. A better understanding of the 1C metabolism (metabolites, enzyme activities and transporter velocities) and the effect FA has on this system is vital. This thesis contributes to these issues in the following way:

1 Hcy can not be regarded as a sole metabolite because it is part of a very tightly regulated network. Attempts to lower Hcy concentrations will unmistakably alter other 1C metabolite levels as well. Future therapies should take this into account.
2 Plasma Hcy is an equilibrium (sum of export and uptake by several cells and organs). However, as was shown in this thesis, plasma Hcy levels are not a reflection of its intracellular concentrations. Not even a correlation with the most likely contributor, the liver, was found. If plasma Hcy is not a reflection of intracellular levels, then what does plasma Hcy represent? Perhaps plasma Hcy is simply the reflection of the methylation potential of the cell. When 1C fluxes slow down (for instance due to lowered B-vitamin status or gene alterations), intracellular Hcy levels become too high and Hcy is transported out of the cell. This would make elevated plasma Hcy an indicator for diminished methylation capacity of the cell.
3 Elevated plasma Hcy levels arise from different origins. They encompass life style factors (like age, sex, and smoking), nutritional factors (B-vitamin deficiency, excess methionine intake), and genetic factors (polymorphisms like MTHFR 677C>T). Therefore, treating them as a homogeneous group might not be correct. Subgroups might benefit from specific therapies (like B-vitamin supplementation) while others don't. This could mask any beneficial effect the therapy might offer.

4 FA therapy itself might have harmful side effects, potentially cancelling out all beneficial effects of lowering plasma Hcy. This thesis showed that FA might indeed shows some unsolicited effects (i.e. disturbed regulatory properties of SAM, inhibition of enzyme activities, lowered conversion to BH_4, and folate transport). Lowered availability of 5-methylTHF may in turn lead to elevated Hcy levels, opposite to the original intention.

5 Plasma Hcy levels arise from different organs and tissues. There is a large diversity of 1C enzyme and transporter expression patterns between these organs and tissues. Therefore, individual responses to the designed therapy might differ. Maybe some cell types benefit from FA therapies while others don't. For the prevention of CVD due to elevated plasma Hcy levels, it is vital to understand the effect of FA therapy on specific cell types (like endothelial cells) and not the sum of all tissues.

Future perspectives

Results mentioned in this thesis shed new light on the questions: "are plasma Hcy levels a reflection of intracellular levels?" and "what effect does FA have on intracellular 1C metabolism?". A couple of new directions that could be endeavoured are:

Extrapolation of intracellular 1C metabolite level results to a patient group.

Now that the levels and correlations between intracellular 1C metabolite in healthy individuals are more clear, the next step would be to study intracellular 1C metabolites in patients suffering from different forms of hyperhomocysteinemia. In addition, the effect of FA and natural folate treatment in such patient groups would be of interest.

Change of supplementation / intervention

Hcy-lowering intervention trials have used the synthetic FA in doses of 0.5 to up to 40 mg/day (daily requirement is estimated to be 400 µg of natural, reduced folate).(8) Chapter 8 suggested that FA interferes with the uptake of the natural folate 5-methylTHF, and lowers intracellular 5-methylTHF concentrations in HUVECs. Therefore, FA could potentially lead to a reduction of natural folates in certain cell types, especially those cells lacking a transport system (i.e. FR) for FA. In addition, Chapter 7 showed that folate

enzymes can be partially inhibited by FA, which could lead to an altered distribution of folate forms. This, in turn, could shift priorities in the 1C metabolism. What results would be obtained when 5-methylTHF was used? Likewise, would the trial results have been different if the administered folate dose was more physiological? After all, Chapter 4 suggests that increased folate levels could interfere with normal regulatory pathways in the 1C metabolic cycle.

More appropriate animal model

Not all animals express 1C enzymes to the same extent as humans. For example the activity of DHFR is 25 times higher in rats than in humans. (21) To study the effect and persistence of this synthetic compound in cells, an animal model with comparable DHFR activity would be more suitable. In order to find a more suitable animal model, first 1C enzyme activities in several animals should be established or a mouse model with human DHFR could be constructed. Subsequently, the effect of different folate forms and concentration on 1C metabolism could be studied.

Fluxes instead of metabolites

As the chapters illustrate, the 1C metabolism is very complex. It certainly is more than the sum of all metabolites. Studying the fluxes of the different pathways in this cycle will offer more insight. The effect of FA on fluxes (i.e. increased remethylation and transmethylation) in the 1C metabolism has been addressed in previous studies.(29) However, the results of these studies are based on plasma metabolites. For a clear picture, intracellular fluxes in the different organelles need to be established too. The combination of metabolite concentration and fluxes might show more exactly what is happening in 1C metabolism in hyperhomocysteinemia when FA is administered.

How to treat hyperhomocystenemia for the time being?

A foolproof treatment for hyperhomocysteinemia still does not exist. This thesis provides some new clues for an improved treatment. First of all, when folates are chosen to lower plasma Hcy, the preferred treatment would be a low dose (i.e. < 400 µg daily) of a natural folate (i.e. 5-methylTHF or folinic acid). Secondly, it must be taken into account that hyperhomocysteinemia patients are not a homogenous group since hyperhomocysteinemia may arise from different sources. Some hyperhomocysteinemia patients might benefit from one type of treatment while others require something else.

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