PDEng – Chemical Product Design

ST6904 – Individual Design Project

Design of a treatment protocol to improve the health of B12 deficient patients

Diagnostic tools and corrective measures to analyse and improve cellular function of vitamin B12

Final Report

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Summary

The design project entitled “Design of a treatment protocol to improve the health of B12 deficient patients” is carried out as an individual design project of PDEng program in Chemical Product Design. This project is commenced for the B12 Institute, Rotterdam, which aims to improve the diagnosis and treatment of patients with vitamin B12 and folate deficiency. The experience of the B12 Institute showed that the current diagnostic and treatment protocol is not sufficient to improve the health of some severe patients.

This design project aims to provide knowledge beyond the classical theories of B12 deficiency, such that an extended protocol can be recommended to enhance the recovery and health of B12 deficient patients. The recommended protocol consists of diagnostic means and subsequent corrective actions.

The approach of the project is divided into 4 major steps:

1. study of the biochemistry of vitamin B12 cellular metabolism;
2. generation of hypotheses on disorders related to B12 deficiency according to the biochemistry study;
3. recommendation of biomarkers/diagnostic tools;
4. recommendation of corrective actions.

This report discusses the hypotheses of the vitamin B12 cellular inactivity, which extends the theory of the classical B12 deficiency. The hypotheses revolve around the failure of the B12 cellular activation due to enzymes defects and the activation cofactors deficiencies, as well as the failure of B12 co-enzymatic function due to oxidative stress. A series of vicious cycles between the overlooked causes and impacts in B12 inactivity is also described. The highlights of the hypotheses are summarized as follows:

- B12 deficiency causes folate cycle block, which leads to glycine deficiency,
- Glycine deficiency leads to glutathione deficiency and collagen deficiency,
- Glutathione deficiency causes an elevated oxidative stress, and vice versa,
- Collagen deficiency leads to intestinal bacterial dysbiosis,
- Intestinal bacterial dysbiosis causes the production of bacterial toxins, including formaldehyde,
- An excess of formaldehyde exacerbates oxidative stress and damages to the body, and may as well inactivate cellular B12.

However, we found that the current knowledge on the mechanisms explaining a lower B12 enzyme activity may still be insufficient to explain the whole condition and issues related to B12 cellular inactivity.

Finally, we designed a mini study to obtain evidence on a number of the hypotheses, especially those related to oxidative stress. New biomarkers for the extension of the diagnostic tools are explored. The new biomarkers are expected to provide better tools to explain the condition and symptoms of the patients. In addition, several supplements are recommended as corrective actions of the anticipated issues disclosed by the new biomarkers. The concepts and the results of the project are expected to provide new insights for the medical research and practice of B12 deficiency treatment, as well as to provide major improvement to the health of the patients.

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1 Introduction

1.1. Project Background

This project is an individual design project in TU Delft PDEng Chemical Product Design program together with the B12 Institute. B12 Institute is a non-profit health clinic practice for the diagnosis and treatment of B12 deficiency, situated in Zuidplein Rotterdam, the Netherlands. The B12 Institute aims to improve the diagnosis and treatment of patients with health issues related to vitamin B12 deficiency.

The standard diagnosis and treatment procedure of B12 deficiency is given by the Dutch federation of general practitioners, Nederlands Huisartsen Genootschap (NHG) [1]. The diagnosis is based on:

- the total serum B12,
- the blood level of other commonly related vitamins and metabolites (e.g. vitamin B1, B2, B6, homocysteine, and methylmalonic acid),
- the presence of (neurological and physiological) symptoms (see Appendix A),
- the medical and family health history.

Oral cyanocobalamin (CNCbl) is preferred as the first choice treatment of B12 deficiency suggested by NHG. However, the efficacy of oral CNCbl may be low due to digestive uptake limitations. Meanwhile, hydroxocobalamin (HOCbl) injection is given for patients with more severe symptoms to quickly normalize the B12 level. The loading dose is 1000 mcg hydroxocobalamin given every 3 days for 10 injections, while the maintenance dose is either 1000 mcg hydroxocobalamin every 2 months or 300 mcg hydroxocobalamin every month.

Patients at the B12 Institute mostly have neurological symptoms, thus receive a more stringent treatment than the abovementioned standard procedure. All patients at the B12 Institute receive a personalized treatment. For example, some patients receive at least 2 times of injection per week for 2 years. Within almost four years of experience treating more than 1800 patients, B12 Institute found several cases where patients did not experience the expected recovery outcome, with distinct experiences during and after treatment, such as:

- A quick normalization of total serum B12 level right after the first injections without a recovery from the symptoms; whereas a longer treatment will improve patients health eventually;
- Symptoms worsening in the early stage of the treatment; improvement/recovery does happen after a longer period of the treatment;
- Once the treatment is stopped, symptoms reoccur, leading sometimes a worse state;
- Symptoms reoccur during the maintenance dose (i.e. when the frequency and/or dose of treatment is decreased).

The phenomena above have not yet been fully explained by the current research knowledge about vitamin B12, therefore there is an urgent need of a deeper understanding of the B12 uptake and cellular metabolic aspects and based on that to create a better treatment protocol. The issues regarding the patients unpleasant experience during the treatment give rise to at least the following statements and questions:

- The standard diagnosis protocol lacks in tools to monitor the cellular function of B12;
- The standard treatment protocol may not fit all patients; patients with a prolonged B12 deficiency need a more stringent and a longer treatment period to fully recover, if able to, from the damages related to B12 deficiency;

- The health issues/symptoms experienced by patients may not be only rooted in the B12 deficiency, but may also be a result of underlying causes and undiagnosed consequences of untreated health problems;
- How to properly diagnose, analyse, and treat the (overlooked) root causes and consequences of B12 deficiency?

1.2. Classification Terms of Vitamin B12 Deficiency

The classical vitamin B12 deficiency is described as a below cut-off points of B12 serum level due to an autoimmune disorder in which the antibody attacks the intrinsic factor and/or the parietal cells (i.e. the production site of intrinsic factor). The Intrinsic factor is an important chaperone for the transport and uptake of B12 in the digestive system (further discussed in Section 4.2). Patients with pernicious anemia/B12 deficiency may develop macrocytic anemia, a condition where the red blood cells are enlarged and do not function well, marked by a high mean corpuscular volume (MCV). The lack or absence of intrinsic factor is supposedly balanced by a high dose of B12 intramuscular injection, which can bypass the transport of vitamin B12 directly to blood.

In contrast to the classical description, B12 Institute had found several cases of patients with intrinsic factor antibody with the absence of anemia. This particular condition is often marked by a low serum B12 with neurological and/or physiological symptoms (see Appendix A), but without anemia. Another commonly found condition in vitamin B12 deficiency is a low to normal serum B12 combined with a high level of methylmalonic acid and/or homocysteine, i.e. signs of non-functional cellular B12.

In addition, the case where the normalization of B12 serum level is not directly followed by resolved physiological symptoms could indicate at least two issues:

1. The damages caused by B12 deficiency or by other factors leading to B12 deficiency are very slowly reversed (e.g. damages in nerves system), and/or
2. The cellular function/activity of supplemented B12 is blocked by other unidentified issues.

The slow repair of B12 deficiency damages can eventually be resolved by giving continuous supply of injections to patients, such that they can slowly recover in a long term. However, if there are other issues impeding the cellular function of B12, the recovery of the patient might not be guaranteed. Therefore, the focus of this project is to investigate, improve, and maintain the cellular activity of vitamin B12 to ensure the health improvement of the patients. The aim of this project is to discover overlooked issues, i.e. causes and consequences, related to B12 cellular inactivity, such that the treatment can be optimized.

1.3. Project Goal, Approach, and Scope

The goal of this project is to design an extended treatment protocol for the B12 Institute to enhance the recovery and health of B12 deficient patients. There are two design issues included:

- To design an additional diagnosis protocol (i.e. markers) to identify disorders and analyse the cellular function of B12;
- To propose supportive corrective measures to improve the health of B12 deficient patients.

To achieve the goal and address the design issues, the project approach has four steps:

1. Identification of overlooked issues related to B12 cellular activity
    - Literature study on the biochemistry of B12 uptake and transport mechanisms.
    - Literature study on the cellular activation and activity of the related enzymes, and which factors may negatively influence these activities.

- Identification of potential bottlenecks within the flow of B12 delivery to cells and its functioning activity.

2. Generation of hypotheses of the non-classical causes and consequences of B12 deficiency
    - Comprehensive summary of the identified proposed bottlenecks in the transport, uptake, and activity of cellular B12.
3. Recommendation of diagnostic methods for the hypotheses
    - Suggestions of practical measurements or analytical methods to identify the proposed issues.
    - Search and contact with expertise in (medical) analytical equipment for practical access to the required assay/diagnosis protocol.
4. Recommendation of corrective actions
    - Suggestions of practical corrective actions to treat each relevant disorder/issue in patients. Supplements, diet, and daily exercise are examples of the recommended corrective actions.

There are several challenges in carrying out the project. First, there is limited background knowledge on the biology and biochemistry of the human body for the trainee. Therefore, a rigorous literature study is required in the early phase of the project. The project is limited to one year, while there are many interesting topics to discover; thus it is important to identify which topics are relevant to be prioritized. Hence, framing a project scope is important to determine which tasks to be included and which ones to be set out of the project. The scope of the project is given in Table 1.1. Finally, the limiting step in the project is expected to be on the step of making collaboration with the experts. Without the success of this step, it will be difficult to realize the design of the treatment protocol.

Table 1.1 Scope of the project

Inside scope	Out-of-scope
Study mechanisms of B12 transport and uptake, B12 cellular metabolism and degradation	Experimental work
Propose hypotheses of the causes and consequences of B12 cellular inactivity	Design of product and/or equipment
Identify biomarkers to support the approach of the hypotheses	Proof-of-concept
Contact potential collaborators in (medical) analytical methods	Material balance on the vitamin B12 related homeostasis
Explore corrective actions	
Conceptual design of a mini study of the new biomarkers	
List research questions for simultaneous and/or follow-up research work	

1.4. Stakeholders Needs

Analysis of key stakeholders and their needs is crucial to determine the direction of a project. Key stakeholders in this project are patients and doctors at B12 Institute. Therefore, their needs are considered of significance to be fulfilled, such that the work of the design is directed towards these needs (Table 1.2).

Table 1.2 Key stakeholders needs.

Doctors	Patients
Deeper understanding about disorders related to B12 (cellular) deficiency	Useful diagnostic procedures
Access to the required analytical tools	Precise and quick results
Monitoring tools	Proper treatment actions
	Non-invasive methods (optional)
	Monitoring tools
	Easy to understand information about their health
	Guidelines on proactive actions to improve their health

The most important need for doctors is to have a feasible access to the relevant analytical tools to draw the correct diagnosis and to monitor the health status of the patients. Indeed, doctors need other diagnostic tools in addition to the existing standardized serum B12 measurement. Therefore, issues related to B12 inactivity are explored to gain knowledge about potential biomarkers that can be used to analyse the health of the patients. Available analytical methods in the Netherlands are to be explored in order to provide doctors with the required analytical tools.

On the other hand, patients need proper and efficient treatment procedures (i.e. diagnosis and corrective actions) that will not waste their energy and money. In addition, patients need to be able to monitor their own progress in the treatment journey, as for most cases the recovery requires some time. While still suffering from the symptoms, the patients need to see their progress which is slow but sure, in order to lift their spirit. Non-invasive methods are highly preferred, but are optional to provide the best analytical and health monitoring means. Finally, patients need to be informed in the easiest way about their adverse health condition and on how to proactively improve their health on daily basis.

1.5. Project Organization

1.5.1. Design and Steering Team

The design and steering team consist of the CPD trainee and supervisors:

- Gabriela Hadiwinoto — *CPD trainee* — *TU Delft*
- Peter Daudey — *Design coach* — *TU Delft*
- Gabrie Meesters — *Scientific coach* — *TU Delft*
- Clara Plattel — *Principal and Mentor* — *B12 Institute*
- Kim Suijker — *Mentor* — *B12 Institute/Erasmus University*

1.5.2. Project Planning, Milestones, and Deliverables

The project duration is 1 year, starting from 17 February 2020 until 31 January 2021. Four major meetings as check points for milestones are held throughout the project i.e. Kick-off, Basis of Design, Intermediate, and Final. The timeline of the project check points is illustrated in Figure 1.1 and the milestones and deliverables of the project is given in Table 1.3.

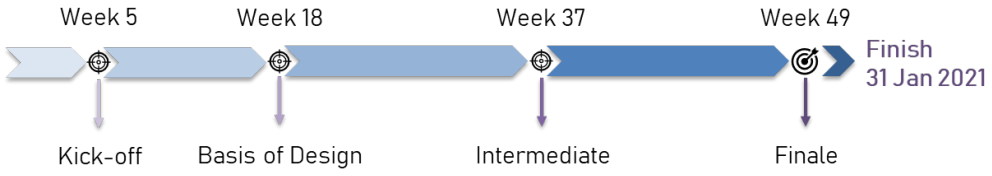


Figure 1.1 Project timeline and check points.

Table 1.3 Project milestones and deliverables.

Check point (date)	Milestones	Deliverables
Kick-off (2 April 2020)	• Project introduction, management, and planning • Preliminary design thoughts	• Kick-off presentation
Basis of Design (BOD) (19 June 2020)	• Relevant mechanisms details • Proposed topics of disorders • Reframed scopes	• BOD presentation • BOD report
Intermediate (30 October 2020)	• Proposed hypotheses list • Proposed approach to investigate hypotheses • Contact with relevant expertise	• Intermediate presentation • Intermediate report
Final (21 January 2021)	• Proposed corrective measures • A design of a mini study	• Final presentation • Final report • Final colloquium at TU Delft

2 Design Approach

2.1. Design Type and Driver

The determination of design type and driver is one of the earliest steps in generating a design concept to solve an issue. Design type and driver are the basis to take the proper approach and to select the design methodology. The design type of this project is platform derivative, which means that the design aims to add to the existing family of diagnostic tools and treatment products to improve the clinical practice of B12 deficiency treatment. This project does not aim to radically innovate products and analytical techniques, but rather to use the available technologies to support the analysis of B12 cellular activity, as non-invasive as possible. Therefore, the main design to be delivered in this project are the relevant biomarkers, with feasible analytical technologies, as well as a protocol with corrective actions using existing products in the market.

The design driver of this project is market pull, which means that the needs of the customers, i.e. patients and doctors, are put upfront as they are considered the most important. In other words, the design is directed to fulfil the customer needs. It is clear that the patients at the B12 Institute need a better treatment, while the doctors need access to better diagnostic tools to monitor and analyse the health status of the patients. The stakeholder needs have been discussed in Section 1.4.

2.2. Design Methodology

The design steps follow the approach described in Section 1.3. A schematic block diagram is given in Figure 2.1 to depict the steps in the methodology used for this project.

Generating design issues, framing the scopes, and determining the stakeholders needs are the first steps, followed by a rigorous literature study to obtain a deep understanding about the mechanism of B12 uptake and cellular function. Only by then, the potential bottleneck and issues in the mechanisms can be identified and translated into a list of hypotheses about the non-classical causes and consequences of B12 deficiency. In this particular step, chemical engineering logic (i.e. breaking down the body functions, depicting parts of the function as small reactors) is used to analyse relevant issues in the metabolism of B12. However, out of scope is the modelling of the biochemical fluxes and concentrations of biomarkers in a quantitative way.

The hypotheses are grouped according to the related aspects, and suggestions of diagnostic methods are generated for every hypotheses group. The information on potential biomarkers are collected from the literature to support the preliminary approach of the hypotheses. In parallel, corrective actions are explored to complete the whole package of the supporting treatment protocol for B12 deficient patients. Finally, the experts of relevant analytical measurements are to be contacted in the later stage to give access to the analytical tools required. In summary, the main deliverables of this project are the biochemistry studies, the hypotheses groups, as well as the recommended analytical and treatment, which can be considered for new direction for the research of B12 deficiency.

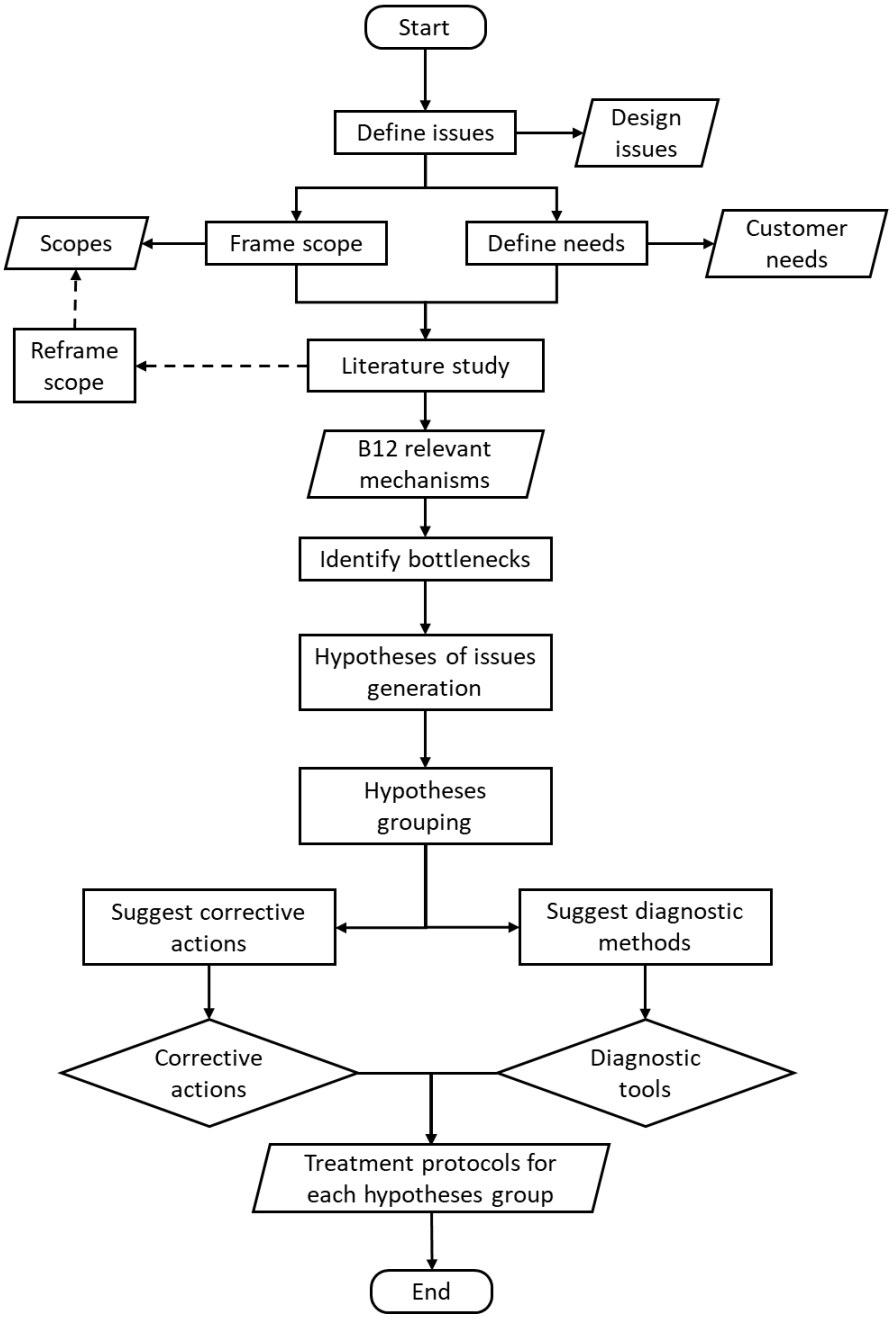


Figure 2.1 Design methodology steps.

3 Vitamin B12 Deficiency: General Knowledge and Loopholes

This chapter introduces the current knowledge of vitamin B12 (cobalamin) including:

1. the essential functions of cobalamin,
2. the causes of cobalamin deficiency,
3. the current diagnosis and treatment procedures,
4. the loopholes in the standard diagnosis and treatment procedures, and
5. the prevalence of cobalamin deficiency.

3.1. Cobalamin Essential Functions

Cobalamin is a water-soluble, essential vitamin that is involved in the metabolism in every cell of human body. Cobalamin is the largest and the most complex vitamin, and it is essential as it cannot be *de novo* synthesized in the human body, so that an adequate intake of cobalamin from food is required [2]. However, besides the intake of cobalamin, also the transport of cobalamin from food and the functioning of cobalamin in every cell in the body are very sophisticated processes that will be discussed in Section 4.2 and 4.3.

Cobalamin is known as an important nutrient to maintain the health of nerves and red blood cells. In the cells, cobalamin has the important role as coenzyme of the cytosolic methionine synthase (MS) enzyme and of the mitochondrial methylmalonyl-CoA mutase (MCM) enzyme. These essential cobalamin-dependent enzymes work in every cell for the biosynthesis of DNA and other important metabolites. Methionine synthase functions as key methylation enzyme in the one-carbon cycle, which includes both the methionine cycle and the folate cycle. Methionine cycle is the methylation center in which a methyl group is transferred from the folate cycle for the synthesis of DNA and other metabolites [3]. One well-known metabolite is phosphatidylethanolamine (PE), the precursor of phosphatidylcholine (PC) synthesis. Phosphatidylcholine, or lecithin, is a major myelin constituent [4]. The failure in the methionine cycle may cause DNA hypomethylation and failure in the myelin sheath regeneration [5]. Therefore, it is common for patients to suffer from nervous system damages, thus they experience neurological symptoms (see Appendix A). The pathway of the one-carbon cycle is discussed further in Section 4.4.1.

Cobalamin is also known to have an antioxidant property, such as the direct scavenging of reactive oxygen species (ROS) [6], [7].

A deficiency in cobalamin manifests in the accumulation of homocysteine due to one-carbon cycle block. Early studies identified homocysteine to have a pro-oxidant activity and to induce the activity of NADPH oxidase to produce ROS such as superoxide [8]. An elevated homocysteine is also observed to inhibit the activity of superoxide dismutase (SOD), an enzyme that functions to scavenge superoxide [9]. Oxidative stress and reactive oxygen species are further discussed in Section 5.1.

3.2. The Causes of Cobalamin Deficiency

The human body cannot synthesize cobalamin, hence cobalamin is an essential vitamin to be included in the diet. Cobalamin sources for the body come from dietary meat, egg, dairy, fish, and shellfish products [10]. The transport and uptake of cobalamin from food to every cell in the body is a complex pathway, involving numerous factors such as proteins carriers (i.e. haptocorrin, intrinsic factor, and transcobalamin-II) and (digestive) enzymes.

The study of cobalamin deficiency causes have always been focused on the inadequate intake and/or the poor intestinal uptake of dietary cobalamin. A more detailed discussion on the uptake of cobalamin is discussed in Section 4.2, and the classical cobalamin uptake issues are described in Section 4.2.1. As

described in Section 1.2, the most classically acknowledged cause of cobalamin deficiency is pernicious anaemia. Furthermore, rare genetic disorders which affect cobalamin protein carriers also take part in the classical cobalamin deficiency. Several other factors beyond the digestive uptake that determine the cobalamin functional deficiency are at least:

- the cellular transport and the cellular activation of cobalamin by enzymes (Section 4.3), and
- the trafficking of cobalamin to the designated enzymes (Section 4.4).

3.3. Cobalamin Deficiency Standard Diagnosis Protocol

The diagnosis of cobalamin deficiency as instructed by NHG is based on the total serum B12 level (Table 3.1), as well as the blood level of methylmalonic acid, homocysteine, and holotranscobalamin (holoTC) [1].

Table 3.1 Cut-off value of total serum B12 standard level [1]. LESA algorithm = an advice for laboratory diagnostic of NHG.

Standard level	**Cut-off value by NHG**	**Cut-off value by the LESA algorithm**
Low	< 148 pmol/l	< 150 pmol/l
Low-normal	148 – 221 pmol/l	150 – 250 pmol/l
Normal	> 221 pmol/l	> 250 pmol/l

Despite being the standard diagnostic criteria, the serum B12 level hardly correlates to the persistence of symptoms in patients. For example, the serum B12 level is normalized by the high dose of cobalamin injection, while the symptoms still persist and the patients remain ill. In this case, despite the normalized level of serum B12, the patients should not be considered "recovered" and the treatment should not be stopped before the symptoms are relieved. Instead, the patients need a longer treatment period and more injections. It is well-known that the damages caused by B12 deficiency are not quickly resolved and require some time to be reversed (e.g. nerves system damage), thus a continuous supply of B12 is necessary for a long-term recovery. In addition, there may be other root causes leading to B12 deficiency or to the complex of symptoms that is generally ascribed to B12 deficiency. The biggest challenge in the current B12 deficiency treatment is the monitoring of B12 cellular function, which makes it impossible for doctors to qualify the efficacy of the treatment of the patients.

3.4. Cobalamin Deficiency Standard Treatment

NHG suggests cyanocobalamin oral supplementation for the treatment of B12 deficiency. There are commercial oral cyanocobalamin tablets available in the market with doses up to 1000 µg. However, the digestive uptake of cobalamin needs intrinsic factor, which has an uptake limit of 2 µg/meal under physiologic condition [10]. So, oral supplementation is not the best treatment because the uptake is limited by intrinsic factor, especially for patients with pernicious anaemia that are already low in intrinsic factor.

Hydroxocobalamin intramuscular injections are given to quickly normalize the B12 blood level. The dosage of HOCbl injection is prescribed by Farmacotherapeutisch Kompas (FK) [11], as given in Table 3.2. HOCbl is favoured as the treatment for patients with rare impaired cobalamin metabolism proteins [12]. However, the superiority of HOCbl for B12 deficiency treatment remains to be elucidated.

Table 3.2 Hydroxocobalamin intramuscular injection treatment doses [11].

Patients condition	**Loading dose**	**Maintenance dose**
Obvious neurological symptoms	1000 µg 1 – 2 times per week for 2 years	
Less neurological symptoms	1000 µg x 10 every 3 days	1000 µg every 2 months, or 300 µg every month

3.5. The Prevalence of Cobalamin Deficiency in Undiagnosed Patients

Cobalamin deficiency is sometimes overlooked by doctors and physicians in the absence of anemia. However, a research done by B12 Institute on 161,548 undiagnosed patients[1] in Rotterdam showed three important results [13]:

- Cobalamin deficiency highly prevails across age groups, and
- Macrocytic anaemia prevails in 1.9% of low B12 case (serum B12 < 148 pmol/l)
- Macrocytic anaemia prevails in 15.6% of low folate case (< 6.8 nmol/l)

It is important to note that the population used in this study is the patients with suspicion and symptoms of B12 deficiency, therefore cannot be defined to represent the total population. A demographic illustration based on the result of this study is depicted in Figure 3.1. Macrocytic anemia is a condition where the red blood cells are unusually large and have low hemoglobin (mean corpuscular volume (MCV) > 100 femtoliter; Hb < 7.5 mmol/l for women, Hb < 8.5 mmol/l for men). The conclusion of the study is that macrocytic anemia is mainly explained by low folate rather than low B12. Although one cause of macrocytic anemia is cobalamin deficiency, it is absolutely inaccurate to eliminate cobalamin deficiency in the case of absence of anemia. In addition, the study showed that the prevalence of cobalamin deficiency amongst undiagnosed population is high, such that the current design work can be of importance to help unhealthy patients in the future.

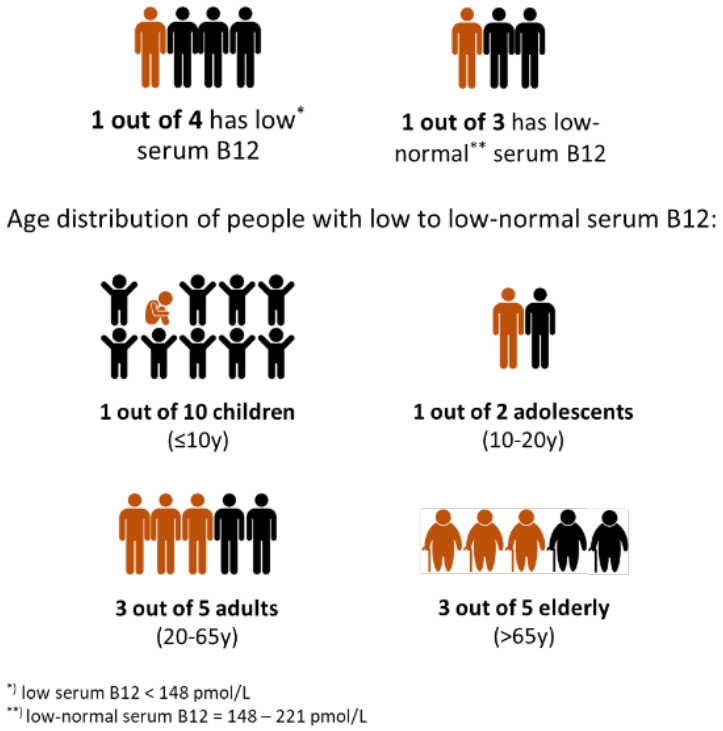


Figure 3.1 Results of study on cobalamin deficiency prevalence in undiagnosed patients population in Rotterdam (n = 161548, man = 32%) [13].

[1] Undiagnosed patients = patients with suspicion and symptoms of B12 deficiency.

4 Cobalamin Metabolic Pathways and Cellular Activity

This chapter includes:

1. the introduction to cobalamin chemistry,
2. the mechanism of uptake and transport to the site of actions,
3. the mechanism of cobalamin cellular activation, and
4. the mechanism of action in the designated site in the cell.

These mechanisms are important to be scrutinized, as one can expect a single defect/bottleneck within the flow can cause the failure of cobalamin function in the whole system.

4.1. The Chemistry of Cobalamin

The structure of cobalamin is presented in Figure 4.1A. Cobalamin comprises of a central cobalt atom, coordinated with four nitrogen atoms forming a corrin ring. One of the nitrogens bears a negative charge, while the side chain comprises of a 5,6-dimethylbenzimidazole group (DMB) attached to one of the carbons in the corrin ring [14]. The upper ligand (β-ligand) can either be absent or presented with various types of ligand, thus form different types of cobalamin. Some of the commonly known types of cobalamin are [10] (Figure 4.1B): (1) 5'-deoxyadenosylcobalamin (AdoCbl), (2) methylcobalamin (MeCbl), (3) hydroxocobalamin (HOCbl), (4) cyanocobalamin (CNCbl), and (5) sulfitocobalamin (SO_3Cbl).

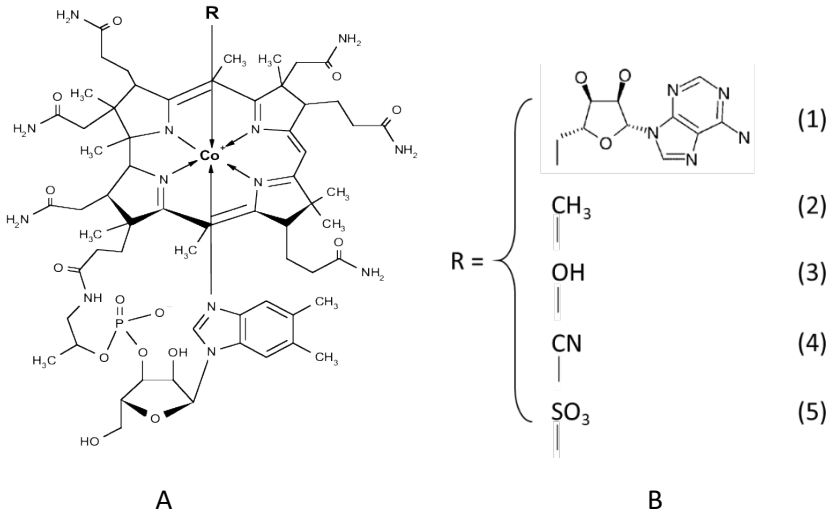
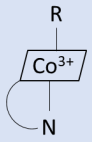
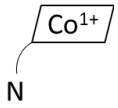
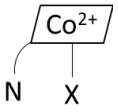
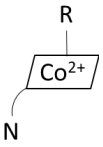
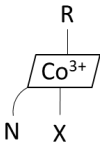


Figure 4.1 A) Cobalamin structure [15]*; B) Different types of Cobalamin upper ligand.*

The structure is called "base-on" when the DMB group is attached to the cobalt atom as the lower ligand (α-ligand), while it is called "base-off" when the DMB group is unattached from the central atom. In the base-off form, the α-ligand can be absent or replaced by an exogenous ligand [14]. The cobalt atom can be sustained in three oxidation states: Co(I), Co(II), and Co(III), depending on the presence or absence of axial ligands, controlled by the enzyme in which it is situated, and on the catalytic cycle. Cob(I)alamin has no axial ligand, cob(II)alamin has one axial ligand, and cob(III)alamin has two axial ligands [16]. As an ion in a solution, cobalamin may be surrounded by water ligands. Therefore, cob(I)alamin most likely has two water ligands, cob(II)alamin has one water ligand, and cob(III)alamin has no water ligand. The structures and coordination numbers of cobalamin oxidative states are summarized in Table 4.1.

Table 4.1 Cobalamin oxidative states structures and coordination numbers. Water ligands surrounding cobalamin ions are not illustrated. R = alkyls; N = nitrogen atom from DMB group; X = exogenous ligand (e.g. histidine from MS)

Oxidation state	Cob(I)alamin/Cbl(I)	Cob(II)alamin/Cbl(II)	Cob(III)alamin/Cbl(III)
Base-on structures	-		
Base-off structures		 	
Coordination numbers	4	5	6

The major dietary sources of cobalamin are meat, dairy, egg, fish and shellfish products [10]. While vegetarians are at risk of cobalamin intake deficiency, a number of plant-based foods such as dried purple laver (nori) is available as cobalamin sources for vegetarians [17], although nori only contains B12-analogues. The five forms of cobalamin mentioned in Figure 4.1B are readily available in food products, with AdoCbl and HOCbl present as the most dominant forms [2]. CNCbl presents in a small number of food extracts, indicating that CNCbl contributes the lowest for the intake of cobalamin from natural food sources [2]. In fact, most of CNCbl form is synthesized for the purpose of food fortification and supplementation [18]. HOCbl exists abundantly in food products inherently, but may also present as a result of photolysis of the light-sensitive cobalamins [2], [12]. While AdoCbl and MeCbl are the active coenzyme forms needed for essential enzymatic processes in cells [16], exogenous AdoCbl and MeCbl entering the cells are not readily active and need to undergo the same activation process as other exogenous cobalamins [12], [18].

MeCbl has an essential role as coenzyme for the methionine synthase (MS) enzyme in the cytosolic one-carbon cycle, the methionine cycle [3]. The methionine cycle is a crucial process in the cell, as failure in the cycle will impair the biosynthesis of DNA and other essential metabolites. AdoCbl is a coenzyme for methylmalonyl-CoA mutase (MCM) in the mitochondrial metabolism of amino acids and fatty acids [5]. The roles of cobalamin as coenzymes in human cells are discussed in detail in Section 4.4.

4.2. Cobalamin Uptake in Digestive System

The dietary recommendation of cobalamin for adults and elderly varies from 1.4 to 3.0 µg/day amongst European countries [19]. The cobalamin daily requirement has been determined based on the factorial approach in human studies, which measures the amount of daily cobalamin losses that need to be replaced by dietary intake [19]. Typically, daily losses of cobalamin are obtained using two different measurements: 1) radioactivity of administered labelled cobalamin, and 2) cobalamin excretion in bile. Cobalamin bioavailability is roughly assumed to be 40 – 50% in healthy adults [19], while biliary excretion of cobalamin is around up to 1.4 µg/day, which 50% of it is presumably reabsorbed in the intestines using intrinsic factor [19].

Reports on the bioavailability of cobalamin in food are diverse between different types of food [10], [19]. For example, cobalamin bioavailability in sheep, chicken, and fish meat are reported to be 56 – 89%, 61-66%, and 42% [10], respectively. However, most studies have limited number of samples and different measurement methods, such that the results are inconsistent amongst the reports. For example, one report showed that eggs have a relatively low cobalamin bioavailability, which is less than 9% [10], while others reported a higher bioavailability of eggs which is up to 36% [19].

The transport of cobalamin from food through digestive system to be activated in cells is a complex pathway involving a number of enzymes, protein transporters, and receptors. These factors are crucial to ensure optimum uptake of the vitamin. A simplified flowsheet of cobalamin transport from food to the cell is depicted in Figure 4.2.

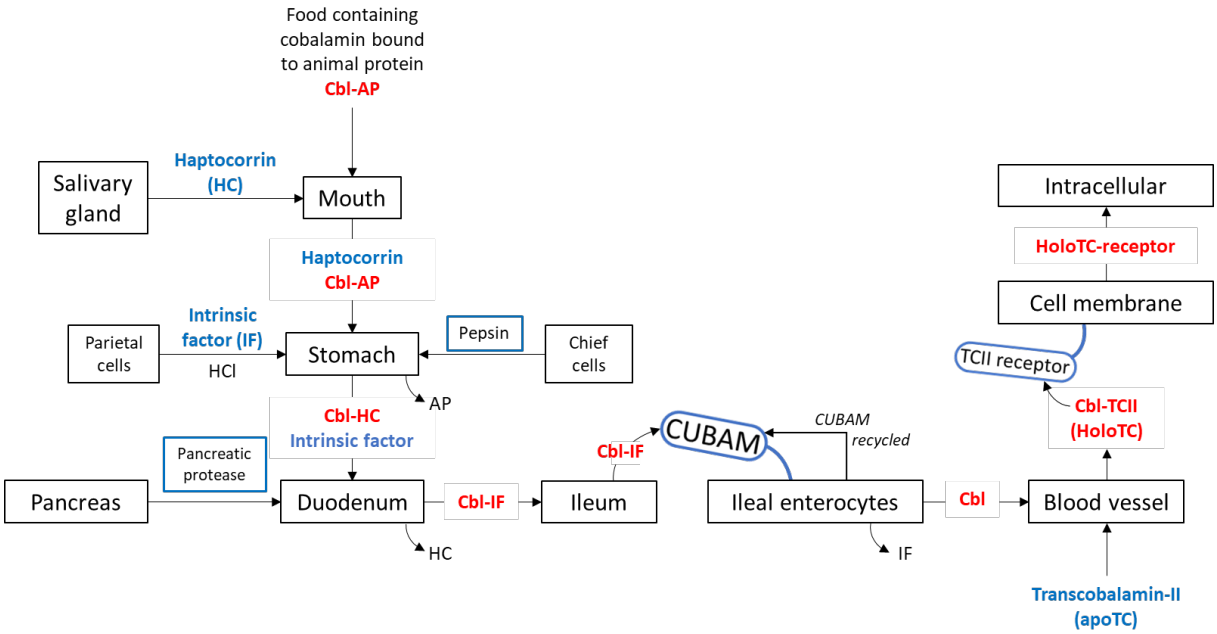


Figure 4.2 Cobalamin transport through digestive system to cell. Cobalamin and cobalamin complexes are in red bold text; Protein binders are in bold blue text; Receptors are in blue ellipses; Enzymes are in blue boxes.

Cobalamin in food is bound to animal protein (Cbl-AP). Cbl-AP enters the mouth and moves to the stomach together with a protein binder called haptocorrin (also known as R-protein or transcobalamin-I) that is produced by salivary gland. In the stomach, cobalamin is liberated from the animal protein by gastric acid (HCl) and enzyme pepsin. Free cobalamin binds immediately to haptocorrin (Cbl-HC complex), which protects cobalamin from hydrolysis due to the acidic environment in the stomach [20]. Parietal cells in the stomach produce intrinsic factor, an important protein chaperone which assists the absorption of cobalamin in ileum. Cbl-HC and intrinsic factor move to the duodenum, where the enzyme pancreatic protease is readily available to liberate cobalamin from the complex by proteolysis. Once freed, cobalamin binds to intrinsic factor (Cbl-IF complex) and moves throughout the small intestine. At the end of the small intestine, cubilin-amnionless (CUBAM) receptors are available on the enterocytes of the ileum, for the purpose of receptor-mediated endocytosis of Cbl-IF [21], [22]. The complex Cbl-IF-CUBAM enters the ileum enterocytes and is taken by endosome, where the CUBAM receptor is recycled back to the surface of enterocytes [21]. The remaining complex Cbl-IF enters the lysosome, where the intrinsic factor protein is proteolyzed and the cobalamin is freed in the cell. Next, free cobalamin exits the enterocytes through MRP1 transporter to the blood [21], where it binds to transcobalamin-II (apoTC), forming holoTC. The holoTC in the blood is transported to all cells in the body to perform as coenzymes, as well as other organs such as liver and kidney, for storage and reabsorption [21].

The three protein binders, haptocorrin, intrinsic factor, and transcobalamin-II, are polypeptides which bind cobalamin at their two domains (i.e. α and β domain) [23], [24]. Intrinsic factor and haptocorrin are highly glycosylated, while TCII is non-glycosylated [25]. These proteins bind base-on cobalamin in 1:1 molecular ratio [26]. However, there is limited report on the amount of each protein binder available in the body. Intrinsic factor is reported to limit the intestinal uptake of cobalamin to around 1.5-2.0 µg per meal [27]. Therefore, the bioavailability of cobalamin is highly dependent on intrinsic factor. However, some reports showed a higher cobalamin absorption amount with a higher cobalamin

intake [19]. Hence, one could hypothesize that there might be a positive feedback mechanism of intrinsic factor production, or other unreported mechanism in digestive system allowing a higher absorption of cobalamin. In the blood plasma, transcobalamin-II carries around 20-25% of the total plasma cobalamin, while the rest of cobalamin is bound to plasma haptocorrin [28], [29]. However, all cells in the body take up only holoTC through endocytosis by the TC-II receptor which is encoded by gene *CD320* [30], [31].

4.2.1. Cobalamin Uptake Problems

In general, the underlying cause of cobalamin deficiency is first examined with respect to the digestion pathway. Doctors usually simply pinpoint on the evident actors of cobalamin digestion and uptake, such as protein binders, receptors, and enzymes. The following factors are the (classical) causes of cobalamin deficiency which are centralized in the digestive system:

- A lower production of gastric acid

Gastric acid is particularly important for the liberation of dietary cobalamin from animal protein. It is common for elderly to experience a lower production of gastric acid which over time can result in a decrease of cobalamin absorption [32]. People with achlorhydria or hypochlorhydria also suffer from the lack of gastric acid production. Suppression of gastric acid production can also root from medications using histamine H_2-receptor antagonist, such as cimetidine, famotidine, nizatidine, and ranitidine, as well as proton pump inhibitors such as omeprazole, lansoprazole, rabeprazole, etc. [32]. These medications decrease gastric acid secretion by parietal cells.

- Pancreatic insufficiency

Exocrine pancreatic insufficiency is a condition where pancreas does not produce sufficient enzymes that aid digestion. Pancreatic protease is an enzyme produced by pancreas to liberate cobalamin from haptocorrin in duodenum. A lack of pancreatic protease will cause the failure of cobalamin uptake by the subsequent protein binder, which is intrinsic factor.

- Intrinsic factor deficiency

As briefly mentioned in Section 1.2, an autoimmune disorder can cause the antibody to attack the parietal cells [33], which then manifests in B12 deficiency. This disorder is known as pernicious anaemia. In this case, the production of intrinsic factor, the limiting factor of cobalamin uptake, from the parietal cells is disrupted.

- Gastrointestinal tract surgery

The production and the binding of intrinsic factor with cobalamin can also be interrupted due to surgery procedures, such as gastrectomy and gastric bypass. Gastrectomy is a procedure of partial or total removal of the stomach, while gastric bypass surgery is a procedure in which a part of the stomach is bypassed to a distal segment of the small intestine. Both procedures cause at least the following issues [34]:

  - the reduction in gastric acid,
  - the failure in the synthesis of intrinsic factor, and
  - the failure of cobalamin complexation with intrinsic factor in duodenum.

- Haptocorrin and transcobalamin-II disorders

Rare family hereditary disorders are observed to cause haptocorrin [35] and transcobalamin-II [36] (functional) deficiencies. Apart from genetic disorders and external procedures, one can expect cobalamin binder defects due to lack of protein building blocks as a result of DNA and protein damages.

On a side note, all hereditary/genetic issues related to cobalamin deficiency is out of scope of this project.

A vicious cycle between cobalamin deficiency and binders deficiency could be established as DNA and protein synthesis are two processes that are supported by cobalamin metabolism (i.e. through the methylation process).

- Cobalamin-protein complex receptors damages

Patients may also have rare disorders of impaired cobalamin complex receptors. For example, the intrinsic factor-cobalamin receptor (IFCR) may be poorly expressed at the apical brush border, resulting in a decreased IFCR activity at the designated cell surface [30]. Cubilin is found as an IFCR located at ileum [22]. Cubilin, which is also found in the kidney, serves a diverse function for the absorption of protein-vitamin complexes such as cobalamin-IF and vitamin D-binding protein, and proteins such as albumin and transferrin [22]. To function properly, the peripheral cubilin is anchored to the ileal brush border and the kidney proximal tubule by the membrane proteins amnionless and megalin, respectively [37]. The Imerslund-Gräsbeck syndrome is known as an autosomal recessive disorder which can be caused by mutation in genes encoding for either cubilin and/or amnionless genes [37]. In the kidney, cubilin is anchored by megalin, a low-density lipoprotein (LDL)-receptor [38], and forms a complex receptor to facilitate proteins and cobalamin reabsorption in the kidney [22]. Therefore, a defect in cubilin can also cause proteinuria.

- Other gastrointestinal disorders

The uptake of cobalamin in the digestive tract can be inhibited by GI tract diseases. For example, celiac disease is an immune disease which causes damage to the small intestine if the patient eats gluten. With celiac disease progression, the small intestine will be severely damaged and the uptake of cobalamin in the small intestine will be disrupted. Other GI disorders which cause cobalamin deficiency are Crohn's disease/inflammatory bowel disorder (IBD; autoimmune disease), small intestinal bacterial overgrowth, and tapeworms (parasites).

4.3. Cobalamin Cellular Activation

All forms of exogenous (i.e. ingested or injected) cobalamin are inactive before arriving in cells and therefore need to go through the cellular activation process to function as coenzymes [12]. Cobalamin activation mechanisms were commonly studied using genetically defected fibroblast cells to provide proof of the function of the relevant enzymes/proteins. Based on these, the mechanistic schemes were proposed.

The HoloTC-receptor complex enters the cell by receptor-mediated endocytosis, where it is directed to the endosome and lysosomes to free the cobalamin, while the TCII receptor is recycled to the outer membrane of the cell and the TCII protein is proteolyzed. Free cobalamin is then transported from the lysosome to the cytosol by proteins LMBD1 and ABCD4, which belong to *cbl* complementation group[2] F (*cbl*F) and J (*cbl*J), respectively [29]. Once arrived in the cytosol, cobalamin is taken up by the enzyme methylmalonic aciduria type C and homocystinuria (MMACHC), which belongs to complementation group *cbl*C. Different forms of cobalamin have different processing schemes in MMACHC: dealkylation for alkylcobalamin (e.g. MeCbl and AdoCbl) [39], decyanation for CNCbl [40], and reduction for HOCbl [41]. Defects in *MMACHC* gene may occur to affect *cbl*C patients with combined homocystinuria (excess level of homocysteine) and methylmalonic aciduria (excess level of methylmalonic acid) due to

[2] Complementation group = a collection of mutant alleles that fails to complement and restore the wild type when tested in all pair-wise combinations.

the early blocking in the synthesis of the essential cobalamin coenzymes [42]. A simplified flowsheet of cobalamin cellular activation is presented in Figure 4.3.

MMACHC is a polypeptide comprising of 282 amino acids and 2 modules, named the N-terminal and C-terminal [43]. MMACHC is the most diverse NADPH-dependent flavin reductase, and the structural study of MMACHC reveals that MMACHC forms a reductase fold when it binds cobalamin [43]. The reductase is then used to reduce cobalamin. Cobalamin configuration is switched from base-on to base-off after it binds to MMACHC [44]. The conversion from base-on to base-off configuration is driven by the slow protonation of DMB ligand with pKa of ~0.1 [45]. MMACHC does not provide a lower ligand for the bound cobalamin, but instead secures cobalamin at the cavity wall by polar interactions between cobalamin side chains and the core and cap of MMACHC [43]. The base-off configuration of cobalamin when it binds to MMACHC favours the reduction of cobalamin.

Figure 4.4 illustrates the activation schemes of different forms of cobalamin collected from the literature. On a side note, these schemes were drawn according to stand-alone experiments with no *in-vivo* proof. Therefore, the schemes may not accurately depict the real activation mechanism of cobalamins. Alkylcobalamin, e.g. methylcobalamin, undergoes nucleophilic displacement for dealkylation which involves glutathione (GSH) to yield cob(I)alamin [39], shown in Figure 4.4A. As cob(I)alamin has an unfavourable redox potential [46], it is oxidized to cob(II)alamin [39]. The latter form of cob(II)alamin most likely has a water ligand attached to the upper face of cobalamin, therefore depicted as OH_2-Cbl(II). Decyanation of cyanocobalamin involves a different reaction pathway (Figure 4.4B). Unlike dealkylation which is flavin-independent [43], decyanation by enzyme MMACHC requires an electron donor from NADPH, a diflavin oxidoreductase (methionine synthase reductase (MSR)), and reduced flavins from MMACHC to lose the cyanide ligand [40]. The reduction of cyanocobalamin produces OH_2-Cbl(II). Free hydroxocobalamin is known to react rapidly with reduced GSH to form glutathionylcobalamin (GSCbl) [41]. With a typically high concentration of cellular GSH of 1 – 2 mM [47], HOCbl entering the cytosol is expected to oxidize GSH and converts into GSCbl, then possibly binds to MMACHC (Figure 4.4C). Upon binding with MMACHC, it is suggested that the glutathionyl ligand is eliminated by another equivalent of reduced GSH, producing cob(II)alamin-MMACHC complex and glutathione disulfide (GSSG) [48], [49]. In summary, one could expect that cobalamin is delivered to MS and MCM enzymes in the form of OH_2-Cbl(II).

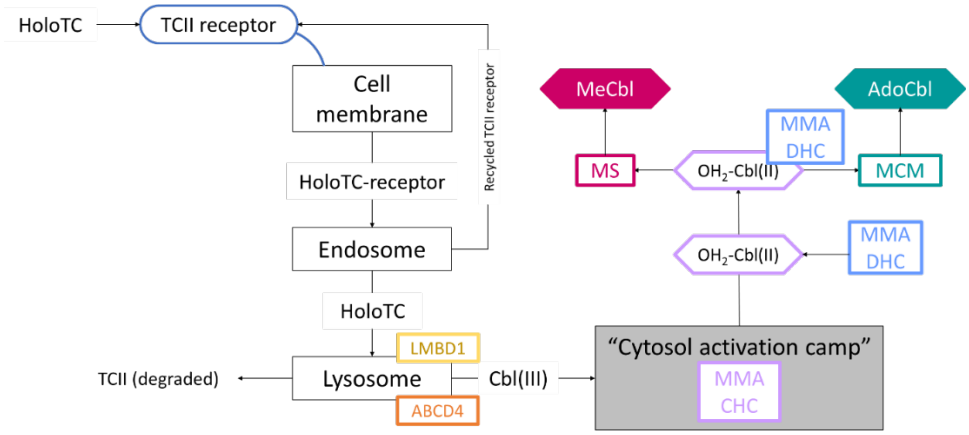


Figure 4.3 Cobalamin cellular activation scheme. Colored boxes with colored text depict enzymes; colored hexagons depict cobalamin binds to enzymes. An extended scheme of "cytosol activation camp" is presented in Figure 4.4.

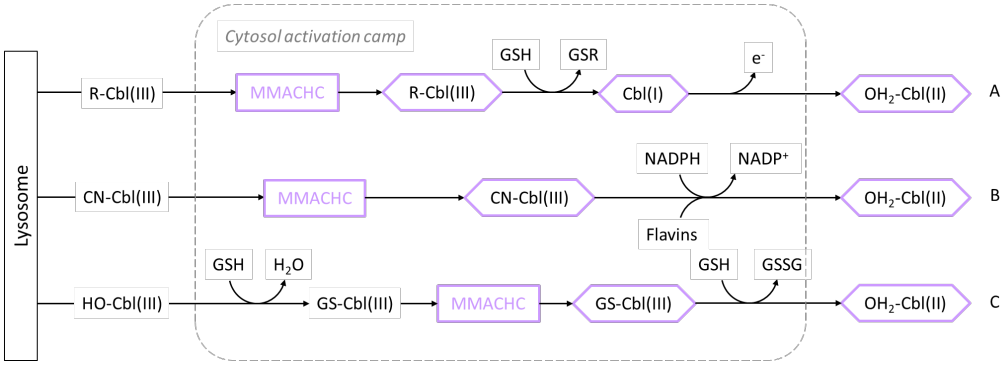


Figure 4.4 Proposed activation scenarios of cobalamin different forms. Notice that all cobalamin forms enter MMACHC as cob(III)alamin and exit the enzyme as cob(II)alamin with a water ligand. A) dealkylation by GSH. R = alkyls (e.g. methyl-, adenosyl-); B) decyanation by NADPH and reduced flavins; C) reaction of HOCbl and GSH, followed by reduction by GSH.

Currently there is lack of mechanistic proof to explain the pathway of cob(II)alamin trafficking from MMACHC to MS and MCM in cytosol and mitochondria, respectively. It has been strongly suggested that MMACHC interacts with MS to regulate the cellular processing of cobalamin coenzyme in cytosol [50]. Meanwhile, enzyme methylmalonic aciduria type D and homocystinuria (MMADHC), which belongs to complementation group *cbl*D, is suggested to assist the trafficking process [51], [52]. MMADHC does not bind to cobalamin to form another cobalamin-protein complex, but instead binds to cobalamin-MMACHC complex as an adaptor to support the trafficking process in a protein-protein complex [53]. There are two variants of MMADHC, i.e. *cblD*[3] variant 1 and *cblD* variant 2. A defect in *cblD* variant 1 causes deficiency in MS activity (i.e. homocysteinuria), while a defect in *cblD* variant 2 causes deficiency in MCM activity (i.e. methylmalonic aciduria) [54]. The apparent variants of MMADHC suggested that the protein is responsible for branching the cobalamin transport to the cytosolic and mitochondrial compartments [55]. The role of MMADHC in the downstream trafficking of cobalamin to the functional enzymes is strengthen by the fact that MMACHC is only cytoplasmic, while MMADHC is both cytoplasmic and mitochondrial [52].

To summarize, the cellular uptake and activation of cobalamin requires several exquisite enzymatic processes to traffic extracellular cobalamin to cytosolic MS and mitochondria MCM enzymes. Different forms of Cbl(III) from food and/or injection will be converted to OH_2-Cbl(II) by enzyme MMACHC before being delivered to MS and MCM. Glutathione is a key metabolite for the MMACHC reduction of the most dominant form of cobalamin in food, i.e. hydroxocobalamin HOCbl(III). Without a doubt, a defect in MMACHC will cause a major issue in the cellular function of cobalamin. Section 5.1: Hypothesis 3 will describe one hypothesis on the cause of MMACHC defect.

4.4. Cobalamin Cellular Functions as Coenzymes

The complicated pathway of cobalamin transport, uptake, and activation ensures successful delivery of cobalamin to cytoplasmic methionine synthase and mitochondrial methylmalonyl-CoA mutase. In MS, cobalamin is activated as coenzyme methylcobalamin which assists the one-carbon cycle [3], while in MCM, cobalamin is activated as coenzyme adenosylcobalamin which assists the synthesis of Succinyl-CoA for the tricarboxylic acid (TCA) cycle [56]. The ratio of MeCbl:AdoCbl synthesis is uncertain, but a study using bovine aortic endothelial cells confirmed that any form of cobalamin converts to MeCbl and AdoCbl in cells, with more forms are converted to AdoCbl [57]. For example, the ratios of cellular AdoCbl:MeCbl formed from exogeneous HOCbl, CNCbl, and AdoCbl are 12.9, 4.5, and 6.6, respectively [57]. This fact supports the notion that all exogeneous cobalamin is inactive and the activation of cobalamin happens inside cells.

[3] By the time both variants were found, MMADHC was still referred to as gene *cblD*.

4.4.1. Methylcobalamin-dependent Methionine Synthase

Methionine synthase is a cobalamin-dependent enzyme, which facilitates the regeneration of methionine from homocysteine in the one-carbon cycle. MS is important as it is involved in the central methylation pathway. MS is a single 136 kDa polypeptide consists of linearly arranged inter domain linkers, consisting of four modules [16] (Figure 4.7B):

1. Methyl donor module: binds 5-MTHF and connects with the cobalamin and transfers the methyl to cob(I)alamin yielding methylcob(III)alamin
2. Methyl acceptor module: binds a homocysteine molecule and moves to cob(III)alamin to obtain the methyl group to form methionine
3. Cobalamin-binding module: binds cobalamin at the α-ligand, also contains a four helix bundle referred to as "cap" to shield the face of β-ligand
4. Adenosylmethionine module: binds SAM which is required for reductive activation of the protein

Cobalamin in the form of OH_2-Cbl(II) coming from MMACMC binds to MS in the base-off configuration, in which the lower ligand can be replaced by histidine 759 from the enzyme [58]. Histidine 759 (his759) is linked to aspartate 757 (asp757) by a hydrogen bond, while asp is linked to serine 810 (ser810) by another hydrogen bond. His-Asp-Ser is known as the ligand triad (Figure 4.5). Thus, this particular cobalamin configuration is called "base-off, his-on". His-on and his-off configuration is interchangeable throughout the enzymatic conversion [16]. In human, the reduction of cob(II)alamin to cob(I)alamin takes place while bound to MS, utilizing an electron provided by MSR, an electron donor enzyme partner of MS [16]. The redox potentially unfavourable reduction to cob(I)alamin is facilitated by an increase in redox potential due to the base-off configuration [14].

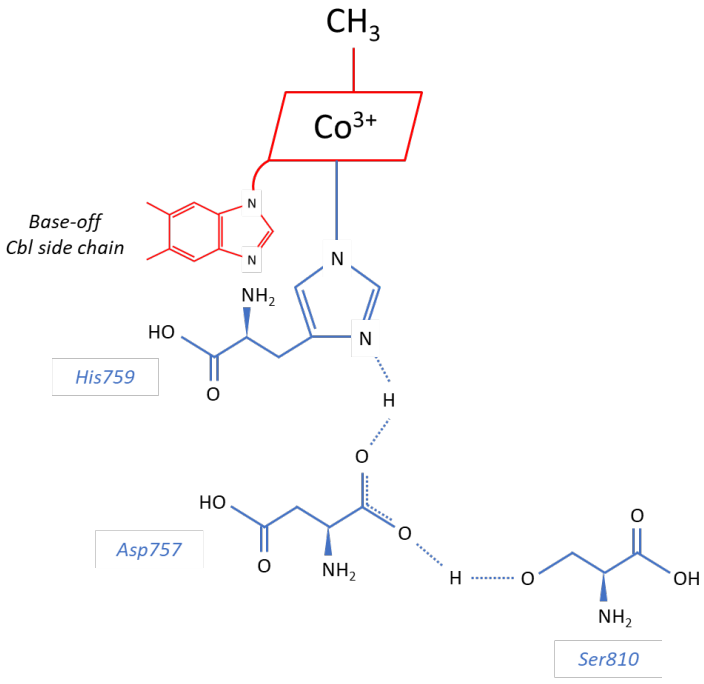


Figure 4.5 Methionine synthase ligand triad binding with methylcobalamin in base-off, his-on configuration. Red structure depicts cobalamin; blue structures depict the amino acids of MS. Adapted from [16].

- One-carbon cycle

Methionine synthase action in one-carbon cycle depends on methylcobalamin. The one-carbon cycle consists of two overlapping cycles, namely methionine and folate cycle. This cellular metabolic

pathway includes the addition, transfer, or removal of a one-carbon unit to and from relevant metabolites [3]. The importance of the one-carbon cycle is stemming from both overlapping cycles:

1. methionine cycle: the central methylation pathway, where S-adenosylmethionine (SAM) transfers its methyl unit to requiring agents, such as DNA, protein, etc;
2. folate cycle: for the synthesis of DNA building blocks such as deoxythymidine monophosphate.

One-carbon cycle is depicted in Figure 4.6 (and Figure A.1 in Appendix). The compartmentalization of folate pool involved in the cycle is depicted in Figure 4.9. The major carbon donor in one-carbon cycle is serine [59], which converts to glycine upon transferring its methyl group to 5,6,7,8-tetrahydrofolate (THF) [60]. The reversible transfer of this methyl group is catalysed by enzyme serine hydroxymethyltransferase (SHMT), producing methylenetetrahydrofolate (5,10-MTHF). Methylenetetrahydrofolate can be reduced to methyl-tetrahydrofolate (5-MTHF) by the enzyme methylenetetrahydrofolate reductase (MTHFR) and NADPH. In nucleus, 5,10-MTHF is converted to dihydrofolate (DHF) by enzyme thymidylate synthase (TS) and deoxyuridine monophosphate (dUMP) [61]. The latter reduction pathway is important as it synthesizes deoxythymidine monophosphate (dTMP/thymidine), which is a monomer for DNA synthesis (Figure A.2 in Appendix). Meanwhile, 5-MTHF is the active form of folate that actively transfers the methyl group to homocysteine (Hcy). This particular methyl transfer step is assisted by cobalamin-dependent MS. Upon the methyl group transfer, Hcy converts to methionine, which by the enzyme methionine adenosyl transferase (MAT) and adenosyl triphosphate (ATP) is converted to SAM. SAM is mainly synthesized in the liver, where around 50% of dietary methionine is metabolized [62]. SAM provides the methyl group acquired from methionine to the expecting metabolites, and converts into S-adenosylhomocysteine (SAH). Using enzyme SAH hydrolase (SAHh), SAH converts reversibly to Hcy, completing the methionine cycle.

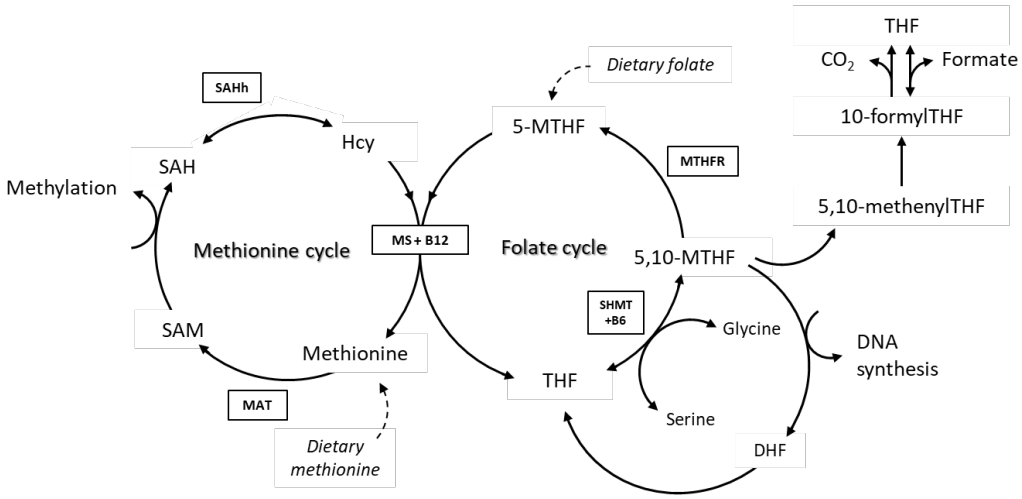


Figure 4.6 The One-carbon cycle includes the methionine cycle and the folate cycle. Enzymes are in boxes. Hcy = homocysteine; SAM = S-adenosylmethionine; SAH = S-adenosylhomocysteine; THF = tetrahydrofolate; 5-MTHF = methyl-tetrahydrofolate; 5,10-MTHF = methylenetetrahydrofolate; DHF = dihydrofolate; MS = methionine synthase; MAT = methionine adenosyl transferase; SAHh = SAH hydrolase; SHMT = serine hydroxymethyltransferase; MTHFR = methylenetetrahydrofolate reductase.

One of the most common manifestation of cobalamin deficiency is the elevation of homocysteine (hyperhomocysteinemia), although the issue is not specific for cobalamin deficiency as the elevation of homocysteine can also be cause by vitamin B6 and folate deficiency. Hyperhomocysteinemia has been associated with the risk of atherosclerosis [63]. However, there is lack of evidence on the reduction of cardiovascular risk by homocysteine-lowering interventions [64], such that the role of homocysteine as a risk factor of cardiovascular disease could still be debated. The toxicity of homocysteine was proposed to be due to its chemically reactive derivative, i.e. homocysteine-

thiolactone [65]. The excess levels of Hcy-thiolactone could promote *N*-homocysteinylation of proteins, and subsequently induce autoimmune activation and cellular toxicity [65].

- The methyl group transfer facilitated by MS

A scheme of the methyl group transfer to and from cobalamin is presented in Figure 4.7. In this turnover process, cobalamin bound to MS is interchangeable between the three oxidation states and his-on/his-off configuration. The primary turnover initiates folate binding to cob(I)alamin domain, activating the transfer of methyl from 5-MTHF to cob(I)alamin to form MeCbl(III) and releaseTHF. Subsequently, homocysteine (bound to the Hcy domain in MS) moves towards the MeCbl, thus the transfer of methyl from MeCbl to Hcy is activated. Cob(III)alamin converts back to cob(I)alamin, and Hcy is converted to methionine. Once every around 2000 turnovers, cob(I)alamin can be oxidized into cob(II)alamin, thus inactivates the enzyme [16], [66]. It could well be that is the latter cob(II)alamin form is similar to the fresh OH_2-Cbl(II) from MMACHC that is inserted in apo-MS. Thus, an electron from MSR is required to reduce cob(II)alamin back to cob(I)alamin [67], which is next remethylated into MeCbl by SAM [16]. To summarize, MSR is indicated as a chaperon of MS which has at least 2 functions:

  - to reduce cob(II)alamin coming from MMACHC to cob(I)alamin in MS, and
  - to reduce the deactivated cob(II)alamin during turnover in MS.

It is also suggested that MSR protects or stabilizes apo-MS and support the formation of holo-MS [68]. The kinetic of MS activity is also shown to be dependent on the concentration of MSR, with MS:MSR ratio of 1:1 found to be optimum [68]. Furthermore, a model of protein-protein interaction between MS, MSR, MMACHC and MMADHC, crucial for the intracellular cobalamin pathway was proposed [69], suggesting that defect in any one of the protein will impede the other, thus leads to failure of the functional cellular activity of cobalamin.

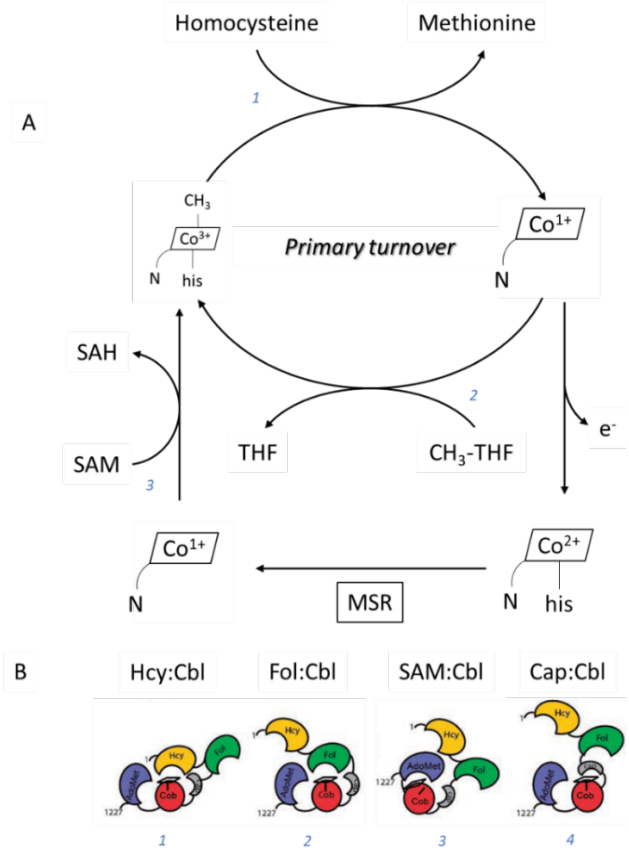


Figure 4.7 A) Transfer of methyl group to and from cobalamin; B) Illustration of MS-Hcy, MS- Folate, MS-SAM and MS-cap domains movement to bind with MS-cobalamin domain [16].

The cobalt ion of cob(I)alamin can also be oxidized by oxidizing agents. A commonly known example of an external cobalamin oxidizer is nitrous oxide (N_2O), which is commonly known as laughing gas. An abuse of N_2O by inhalation to create a feeling of euphoria leads to the irreversible inhibition of MS. The replacement of inactivated Cbl is slow since it requires recycling of the MS enzyme including the inactivated Cbl. It is not clear if thus inactivated MS can be restored in the cell, or it needs to be excreted.

Methionine synthase activity may also be disrupted due to DNA damage, caused by at least:

- the failure of DNA methylation in the one-carbon cycle,
- Errors in the transcription and translation of DNA to mRNA and proteins due to an excessive amount of formaldehyde (further discussed in Section 5.1: Hypothesis 12).

Any disruption in MS activity will further exacerbate cobalamin inactivity, as the MS domains may fail to protect cellular cobalamin. Examples of issues related to MS defects are further described in Section 5.1: Hypothesis 13.

- Methionine cycle alternative pathway

There is an MS-independent pathway of homocysteine methylation, in which the methyl group is provided by betaine (trimethylglycine), a product of choline metabolism [3]. Choline is also a precursor for the production of PC, bypassing the methylation by SAM. Betaine converts into dimethylglycine (DMG) after losing 1 methyl group (Figure 4.8). This particular pathway is folate-independent and catalyzed by enzyme betaine-homocysteine methyltransferase (BHMT), which is zinc-dependent [70]. A study of ethanol-fed rats showed that while MS activity was inhibited by 50%, BHMT activity was increased by more than 50% [71]. In addition, although MS activity was halted, SAM level remained controlled until betaine was exhausted. This study indicated that betaine could be a temporary fix to regulate SAM level while MS activity is slowed down. However, it is important to note that BHMT is absent in the brain [71], thus such alternative pathway is also absent in the brain. An elevated level of DMG was observed in patients with folate deficiency, but not in patients with cobalamin deficiency [72], [73]. However, an elevated level of plasma Hcy and DMG was also observed in patients with chronic renal failure [74], such that renal failure must be ruled out to avoid misdiagnosis.

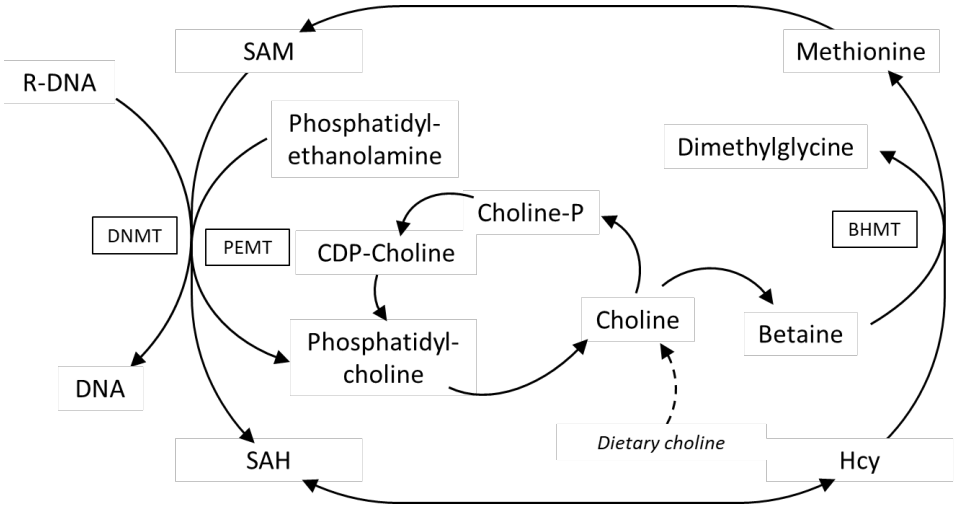


Figure 4.8 Alternative pathway of homocysteine methylation by betaine. BHMT = betaine-homocysteine methyltransferase; PEMT = phosphatidylethanolamine N-methyltransferase.

- Folate pool compartmentalization

While the MS-dependent one-carbon cycle only takes place in the cytoplasm, the folate pool, thus the folate cycle, is distributed between cytoplasm, mitochondria, and nucleus, with different dominant

forms in each site [75]. The compartmentalized folate pool is interconnected between different sites, as depicted in Figure 4.9. The enzymes involved are also shared between different sites. Folate pool originates from dietary folate (or folic acid, as the synthetic form), which is reduced to 7,8-dihydrofolate (DHF), then to THF by dihydrofolate reductase (DHFR) [75]. SHMT needs two cofactors, THF and pyridoxal phosphate, the active form of vitamin B6 [60]. Therefore, the conversion of serine to glycine requires an equimolar amount of THF [60], [76]. Apart from being the upstream part of the methyl donor, the folate cycle is also essential in producing glycine, an important building block of GSH. In fact, 85% of *de novo* glycine is synthesized via the folate cycle [60].

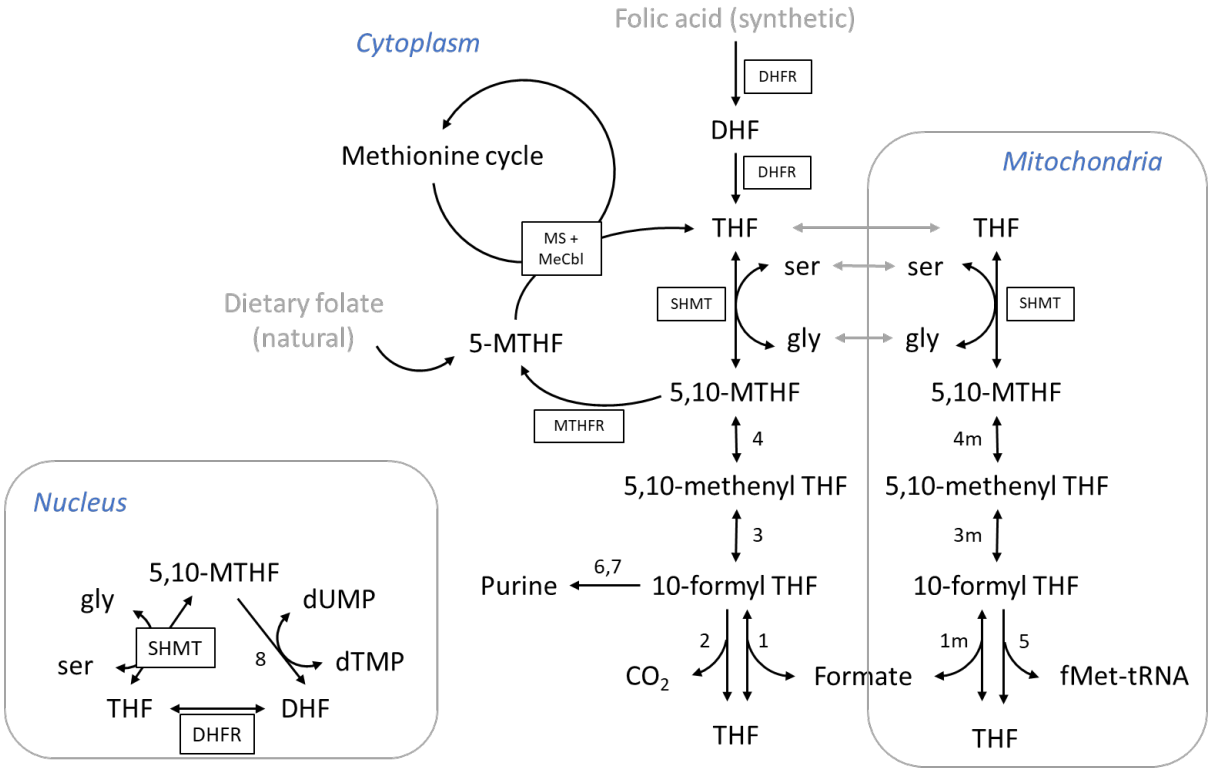


Figure 4.9 Compartmentalization of folate pools. Numbers represent enzymes; m represents mitochondrial enzymes. 1 = 10-formyl-THF synthetase; 2 = 10-formyl-THF dehydrogenase; 3 = 5,10-methenyl-THF cyclohydrolase; 4 = 5,10-methylene-THF dehydrogenase; 5 = Methionyl-tRNA formyltransferase; 6 = Phosphoribosylglycinamide formyltransferase; 7 = Phosphoribosylaminoimidazolecarboxamide Formyltransferase; 8 = Thymidylate synthase; DHFR = dihydrofolate reductase.

- Transsulphuration pathway

The one-carbon cycle is closely connected to the transsulphuration pathway, in which GSH is synthesized from Hcy, serine, glutamate, and glycine. GSH is a thiol tripeptide comprises of three amino acid (i.e. cysteine-glutamate-glycine), that is the major antioxidant in the body. Thus, GSH is essential for regulating redox cycle to prevent damage from elevated oxidative stress [47]. As described in Section 4.3, GSH is also essential for the activation of exogeneous cobalamin. In summary, the lack of GSH is a huge problem for B12 deficient patients, as they do not have enough substance to activate cellular cobalamin and have lack of defence against oxidative stress. The matter of oxidative stress is discussed further in Section 5.1: Hypothesis 6 and Hypothesis 7.

The synthesis pathway of GSH is illustrated in Figure 4.10. Serine (through Cysteine formation) and glycine, two building blocks of GSH, are also involved in the folate cycle. When the folate cycle is blocked, for example due to cobalamin inactivity, glycine synthesis will be deficient (discussed in Section 5.1: Hypothesis 1). This glycine deficiency issue is often overlooked, and can lead to several bigger problems, such as:

- B12 protein binders synthesis failure (discussed in Section 5.1: Hypothesis 1),

- B12 activation enzymes synthesis failure (discussed in Section 5.1: Hypothesis 3),
- Lower production of GSH (discussed in Section 5.1: Hypothesis 4), and
- Collagen deficiency (discussed in Section 5.1: Hypothesis 15).
- Mental issues, as glycine is a neurotransmitter acting on the NMDA receptor

4.4.2. Adenosylcobalamin-dependent Methylmalonyl-CoA Mutase

Cob(II)alamin is also escorted to mitochondria by MMACHC and MMADHC enzymes for activation into adenosylcobalamin, the coenzyme of methylmalonyl-CoA mutase. MCM has a role in the isomerization of (S)-methylmalonyl-CoA into succinyl-CoA [5], one of the important intermediates of tricarboxylic acid (TCA) cycle. The failure in the isomerization of (S)-methylmalonyl-CoA into succinyl CoA can be a result of at least two causes:

i) the deficiency/inactivity of adenosylcobalamin;
ii) the dysfunction of the MCM enzyme due to inborn errors (i.e. complementation group *mut*).

The accumulated (S)-methylmalonyl-CoA will by hydrolyzed into methylmalonic acid (MMA) and subsequently causing methylmalonic aciduria/acidemia (Figure 4.11). Patients with methylamonic aciduria/ acidemia often suffer from lethargy (excessive exhaustion), dehydration, muscular hypotonia, vomiting, acidosis, and mental retardation [77]. The elevated level of MMA is commonly used as a biomarker of cobalamin deficiency.

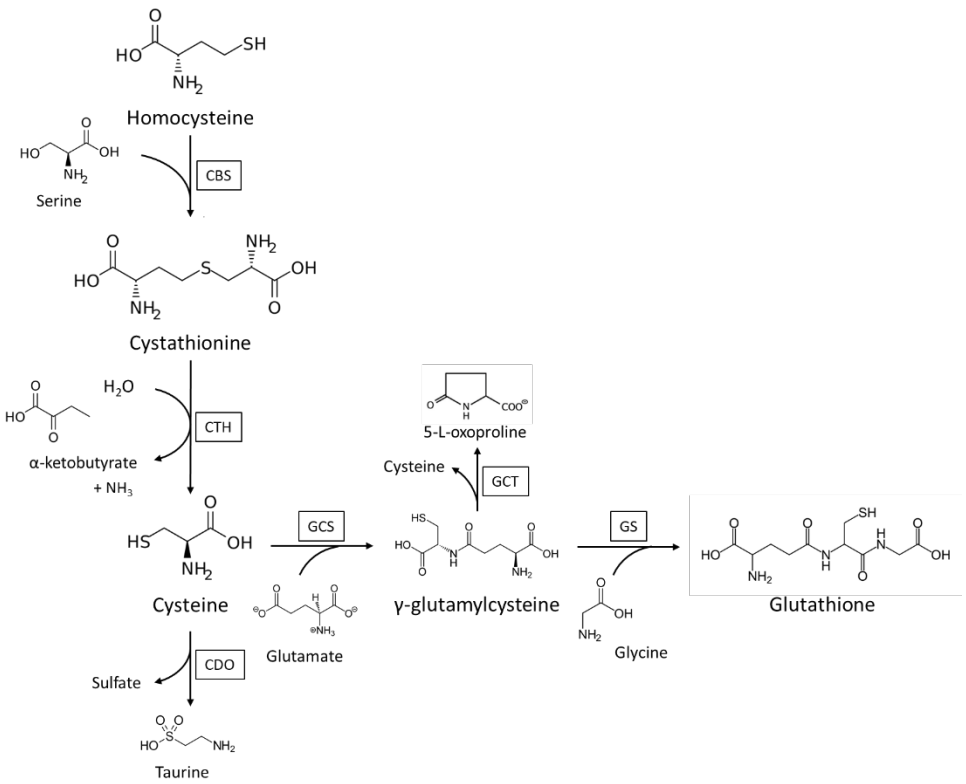


Figure 4.10 Transsulphuration pathway for Glutathione synthesis. CBS = cystathionine beta synthase; CTH = cystathionine γ-ligase; GCS = glutamylcysteine synthase; CDO = cysteine dioxygenase; GCT = γ-glutamyl cyclotransferase; GS = glutathione synthase.

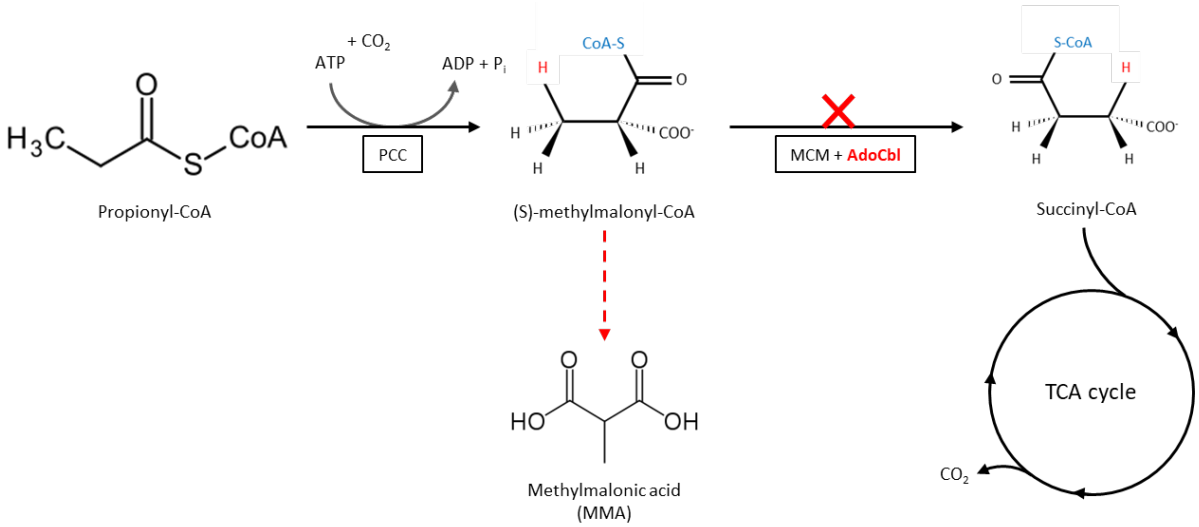


Figure 4.11 Adenosylcobalamin role in mitochondrial enzymatic conversion. Red dashed arrow indicates hydrolysis of (S)-methylmalonyl-CoA in case of Adenosylcobalamin inactivation (red cross).

The activation of inactive cob(II)alamin into AdoCbl requires enzyme adenosyltransferase (ATR) encoded by gene *cbl*B to catalyze the reductive adenosylation [78]. The activation process is in two steps:

1) the reduction of cob(II)alamin into cob(I)alamin, and then
2) the placement of adenosine alkyl from ATP to the upper ligand of cobalamin [53].

ATR binds cobalamin in the base-off configuration, thus the reduction of cob(II)alamin to cob(I)alamin can be thermodynamically favoured [14]. ATR has three identical units of polypeptide (homotrimer), for which 1 mole of ATR is observed to bind to 2 moles of cobalamin [79] and 2 moles of ATP [80]. When ATP is present, the water ligand of cob(II)alamin is eliminated [16]. However, what plays role as the reducing agent of cobalamin in mitochondrial ATR remains unclear. ATR is observed to serve a dual function of activating cobalamin into AdoCbl and delivering 1 unit of active AdoCbl to 1 unit of MCM by binding with another equivalent of ATP [80], [81]. ATP binds AdoCbl in five coordinate (base-off), while MCM binds AdoCbl in six coordinate (base-off, his-on) [53]. Figure 4.12 depicts the mechanism of AdoCbl tinkering and delivery to MCM.

Another protein that is expected to have a role in succinyl-CoA synthesis is methylmalonic aciduria type A protein (MMAA), which corresponds to *cbl*A complementation group [82]. The study of human MMAA is limited up to the observation of methylmalonic aciduria in *cbl*A patients, while the mechanistic explanation of its functional role is hardly elucidated. However, its bacterial protein homologue, MeaB, were understood to be a component of MCM [83], which is observed to protect MCM from oxidative inactivation, such that the conversion of AdoCbl to HOCbl is inhibited [84]. The inhibition in the isomerization of (S)-methylmalonyl-CoA to succinyl-CoA without lacking of AdoCbl is observed in bacteria with lacking of MeaB [83]. In contrast, human studies reveals that *cbl*A defected patients develop methylmalonic aciduria due to the lacking of AdoCbl [85]. Therefore, the human MMAA mechanistic function is of interest to be investigated further.

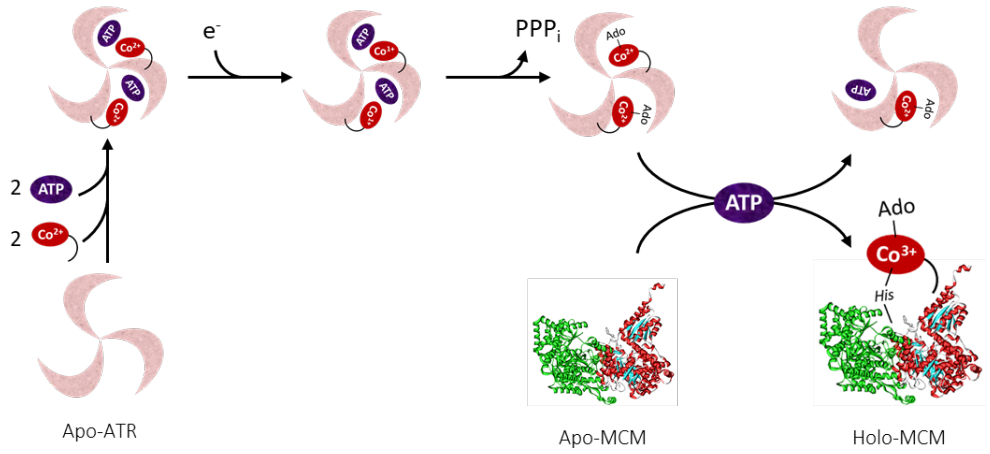


Figure 4.12 Adenosylcobalamin activation and delivery to methylmalonic-CoA mutase by adenosyltransferase. Redrawn from Banerjee et al. 2009 [53]

The MCM enzyme allows the enzymatic rearrangement of (S)-methylmalonic-CoA to succinyl-CoA [56]. The role of AdoCbl stems from the formation of a free adenosyl radical and the fluctuation between cob(III)alamin and cob(II)alamin [77]. With a relatively low dissociation energy of 75 – 125 kJ/mol [86], the ado-radical can easily dissociate from the cobalt ion. Furthermore, as the lower ligand is replaced by his610 from MCM domain, the cleavage rate of C-Co bond increases by ~10^{12} fold [87]. Simply, the isomerization rearrangement supported by adenosylcobalamin-dependent enzyme MCM is described in the following steps [77], [88]:

1. AdoCbl cleaves into ado-radical and Cbl(II),
2. The Ado-radical captures a hydrogen atom from (S)-methylmalonyl-CoA, which converts into methylmalonyl-radical,
3. The Methylmalonyl-radical undergoes rapid 1,2 rearrangement into succinyl-CoA radical (carbon skeleton rearrangement),
4. The Succinyl-CoA radical captures back the hydrogen atom from ado-CH_3, producing ado-radical, the Succinyl-CoA will enter the Krebs cycle which shifts the equilibrium
5. The Ado-radical binds back to Cbl(II), returning to adenosylcobalamin cofactor form.

The scheme of the catalytic isomerization by AdoCbl-dependent MCM is given in Figure 4.13.

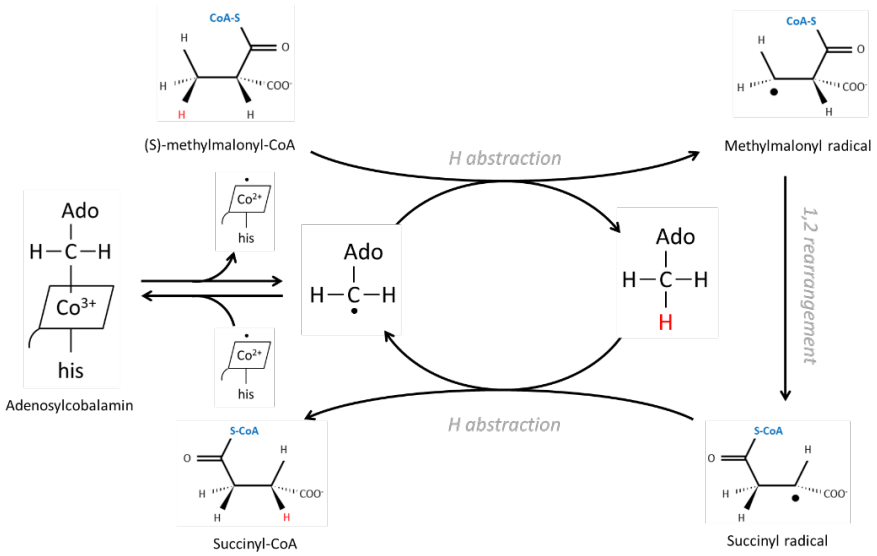


Figure 4.13 Reaction scheme of adenosylcobalamin-dependent isomerization by methylmalonyl-CoA mutase. AdoCbl is bound tp MCM in base-off, his on configuration.

4.5. Summary, Early Conclusions, and Unresolved Issues

This chapter has discussed the basic chemistry and mechanisms of cobalamin in human body, as well as the actors playing roles in each step. It is clear that the whole journey of cobalamin from food to the cell is very complex, such that the homeostasis of every actor involved in the process needs to be maintained in order to ensure successful delivery of active cobalamin in the site of action. Figure 4.14 summarizes the three phases of cobalamin metabolism: the digestion uptake, the cellular activation, and the cellular activity, that had been discussed in Chapter 4, in a simplified diagram.

Table 4.2 summarizes all proteins and enzymes that are discussed in this chapter, including the site of action, function, and class of genetic defects in patients. The mechanism of each protein action is still open to further elucidation as current proofs are mostly obtained by human studies of genetically defected fibroblast. Finally, amongst all knowledge that is on hand, there are still many missing links and unresolved issues that need to be addressed to put together a complete picture of cobalamin processing. Therefore, this chapter is closed with a series of preliminary conclusions and relevant questions of unresolved issues to be addressed in order to obtain a deeper understanding about the world of cobalamin, presented in Table 4.3. The biggest highlight is glutathione as a very important metabolite for controlling redox and activating cobalamins.

Following the extensive literature study that has been carried out in this chapter, Chapter 5 will carry on to extend the matters of cobalamin inactivity beyond the classical knowledge. Several brief introductions have been made earlier in a number of passage in Chapter 4, for example on the overlooked issues of glycine deficiency, an elevated oxidative stress, the accumulation of formaldehyde, and the failure of MS. Several strong hypotheses are discussed in Chapter 5 as stepping stones for further research in B12 deficiency.

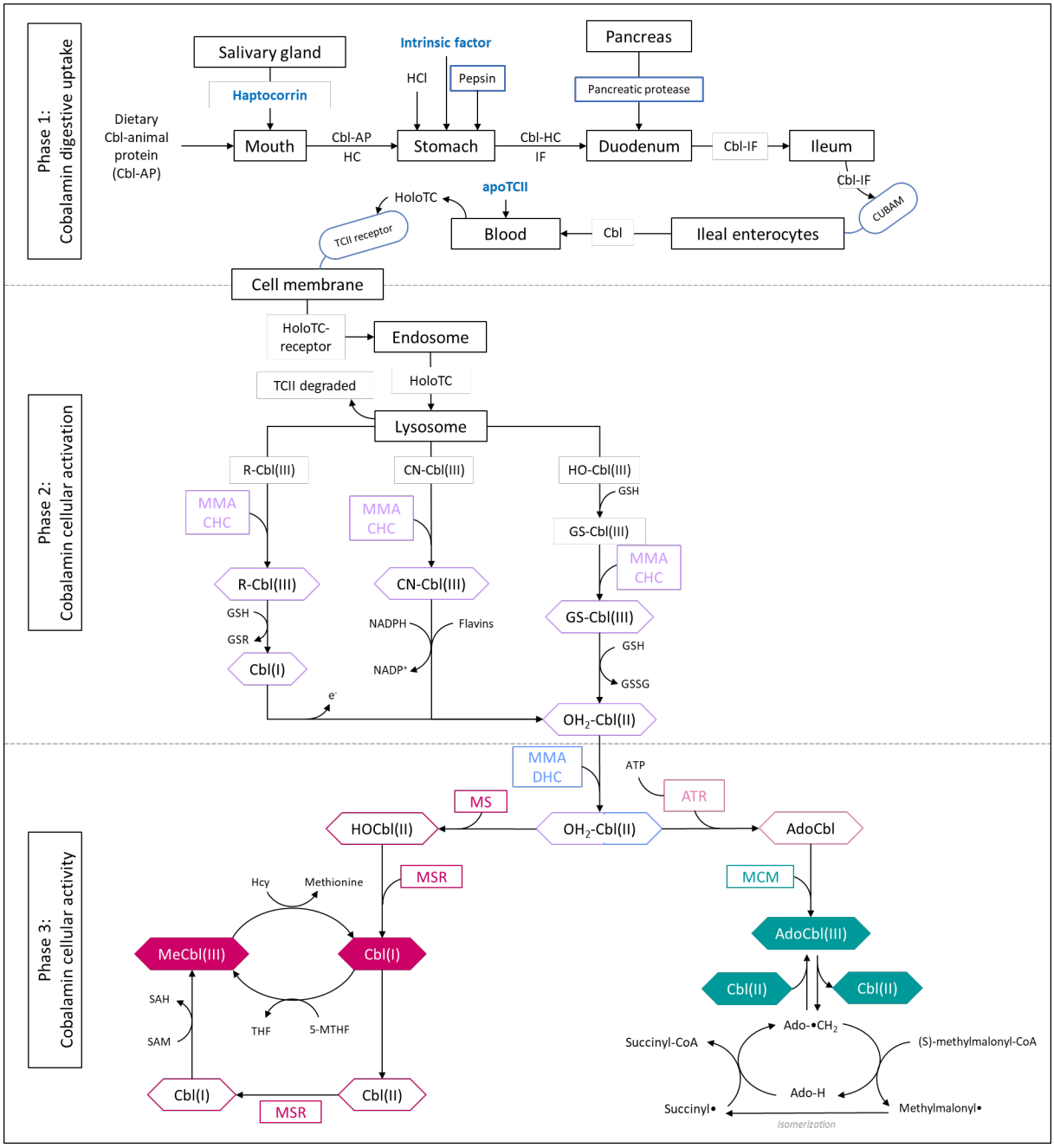


Figure 4.14 Summary of three phases of cobalamin metabolism.

Table 4.2 Summary of proteins and enzymes involved in the uptake, trafficking, and cellular activity of cobalamin.

Phase	***Actor***	***Site of action***	***Function***	***Class of disorders***
Digestion and blood	HCl and pepsin	Stomach	Liberates Cbl from animal protein	-
	Haptocorrin	Stomach	Protects Cbl from stomach acid	-
	Pancreatic protease	Duodenum	Liberates Cbl from haptocorrin	-
	Intrinsic factor	Duodenum	Forms Cbl-IF complex and binds with CUBAM for receptor-mediated endocytosis into ileal enterocytes	-
	CUBAM receptor	Ileum	The receptor for Cbl-IF complex to enter ileum enterocytes	-
	Transcobalamin-II	Blood	Forms holoTC and binds with *CD320* (TC-receptor) for receptor-mediated endocytosis into cells	-
	TC-receptor (*CD320*)	Cell membrane	The gate for Cbl to enter cells	-
Cellular activation	LMBD1	Lysosome	Transport Cbl from lysosome to cytosol	*cbl*F
	ABCD4	Lysosome	Transport Cbl from lysosome to cytosol	*cbl*J
	Methylmalonic aciduria type C and homocystinuria (MMACHC)	Cytosol	- Dealkylation, decyanation, reduction of Cbl - Transports Cbl to MS and MCM	*cbl*C
	Methylmalonic aciduria type D and homocystinuria (MMADHC)	Cytosol and mitochondria	Supports the trafficking of Cbl to MS and MCM	*cbl*D
Cellular activity	Methionine synthase (MS)	Cytosol	Converts homocysteine into methionine in one-carbon cycle	*cbl*G
	Methionine synthase reductase (MSR)	Cytosol	Reductive activation of inactive Cbl(II)	*cbl*E
	Adenosyltransferase (ATR)	Mitochondria	- Activation of AdoCbl - Transport AdoCbl to MCM	*cbl*B
	Methylmalonyl-CoA mutase (MCM)	Mitochondria	Enzymatic isomerization of (S)-methylmalonyl-CoA to succinyl-CoA	*mut*
	Methylmalonic aciduria type A (MMAA)	Mitochondria	Remains to be elucidated	*cbl*A

Table 4.3 Early conclusions/highlights and unresolved issues regarding cobalamin.

Section	**Highlights**	**Unresolved issues**
Digestion	Cbl binds to protein binders in equimolar ratio	How much are protein binders produced/exist (flux) in the body?
	Cbl uptake is most likely limited by intrinsic factor	Is there any positive feedback mechanism of intrinsic factor production due to more Cbl intake?
		Does it help to add IF to oral B12 supplements?
Cellular activation	All forms of exogenous Cbl(III) need further intracellular activation	
	The activation of Cbl is done by MMACHC enzyme, in base-off configuration	There is lack of *in-vivo* observation to provide direct proofs to these mechanisms
	Alkylcobalamin reduction needs GSH for nucleophilic displacement	Does reduction go through the Co(I) stage? Or is one electron stored somehow
	Cyanocobalamin reduction needs one electron from NADPH in a flavin-dependent mechanism	
	Hydroxocobalamin reacts rapidly with GSH to become GSCbl	How is GSCbl activated by MMACHC? Is GSH required for the activation of GSCbl in MMACHC?
		Could free HOCbl react with GSH which is available in blood already? Can GSCbl enter the cell through TC-II receptor?
	All cobalamin forms exit MMACHC as OH_2-Cbl(II) ion	
Methionine synthase	The reduction of OH_2-Cbl(II) happens in MS with the action of MSR which donates an electron	
	Cbl binds to MS in base-off, interchangeable between his-on and his-off throughout the enzymatic conversion	
	The active Cbl(I) can be oxidized to inactive Cbl(II); Cbl(II) needs reactivation by MSR and SAM	Is the oxidation from Cbl(I) to Cbl(II) elevated under elevated levels of oxidants?
		How many turnovers can 1 equivalent of Cbl be used in MS?
		How is the turnover of the existing Cbl with fresh new batch of Cbl?
Methionine cycle	Essential for central methylation processes	Can supplementing with methionine help to correct a deficiency?
	Utilizes MeCbl-dependent MS to convert Hcy to methionine	
		How much is PC synthesized from choline?
Folate cycle	Essential for biosynthesis (e.g. thymidine)	
	Essential for glycine synthesis, which leads to GSH synthesis	Is formaldehyde produced as an intermediate?
AdoCbl-dependent MCM	AdoCbl is activated and delivered to MCM by ATR	What is the reducing agent of Cbl(II) to Cbl(I) in ATR?
	Two moles ATP and two moles cobalamin are bound to 1 mole of ATR	
	The delivery of 1 mole of AdoCbl to MCM requires 1 mole of ATP	
	AdoCbl binds with MCM in base-off, his on configuration	

Section	**Highlights**	**Unresolved issues**
	The isomerization of (S)-methylmalonyl-CoA to succinyl-CoA requires adenosyl radical for hydrogen abstraction and MCM as carbon skeleton mutase	How important is the synthesis of succinyl-CoA by the isomerization? Is there any other source of succinyl-CoA?
	Enzyme MMAA is likely to be functional in MCM	What is the exact function of MMAA in MCM? How is the mechanism?

5 Collection of B12 Inactivity Issues Hypotheses

In this chapter, several issues related to cobalamin deficiency/inactivity are scrutinized and relevant potential biomarkers to support the diagnosis are explored. A hypotheses list relevant to the (non-classical) cause and consequences of cobalamin inactivity is generated. Subsequently, recommendations of analytical methods and corrective measures are provided.

5.1. The Non-Classical Cobalamin Inactivity Hypothesis

A disruption in any step of cobalamin metabolism can disturb the functional activity of cobalamin. A prolonged disturbance in cobalamin homeostasis is expected to cause cobalamin-dependent enzymes reduced activity, leading to neurological disorders and health issues. While the classical cobalamin deficiency is related to the lack of cobalamin uptake in the digestive system, the non-classical cobalamin deficiency proposition focuses on the later onset disorders which occur due to a prolonged inactivity of cobalamin-dependent enzymes. This section presents the list of hypotheses corresponding to more issues beyond cobalamin digestion, which have been described largely in Chapter 4. The hypotheses are described to widen the scope of cobalamin inactivity root causes (see Figure 5.1) and consequences (see Figure 5.2and Figure 5.3), such that the health issues of B12 deficient patients can be assessed and treated more properly. It is interesting to notice that several causes and consequences of B12 inactivity create vicious cycles.

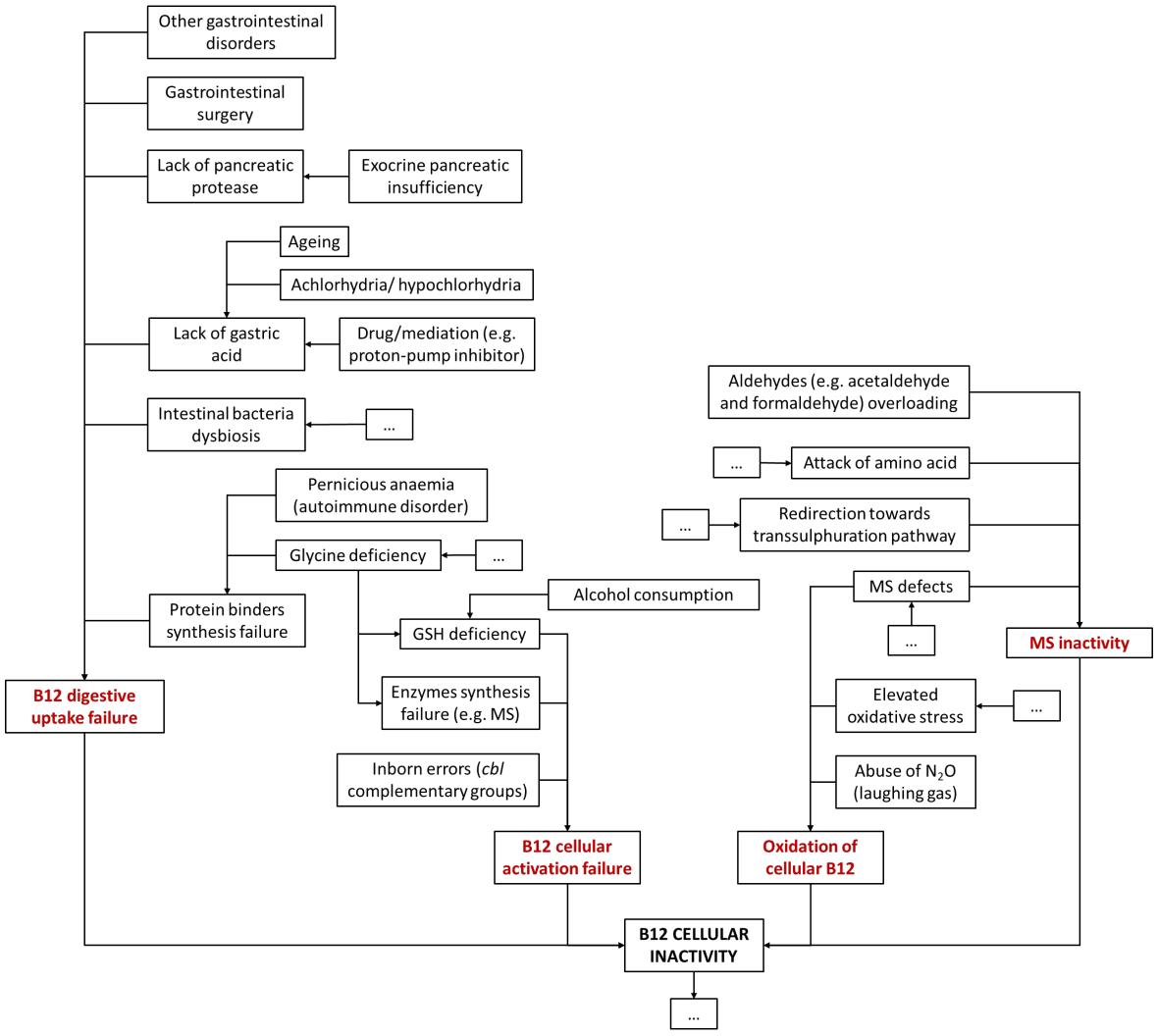


Figure 5.1 Causes of B12 cellular inactivity. Red texts indicate direct causes that are connected to consequences of B12 cellular inactivity (see Figure 5.2 and Figure 5.3).

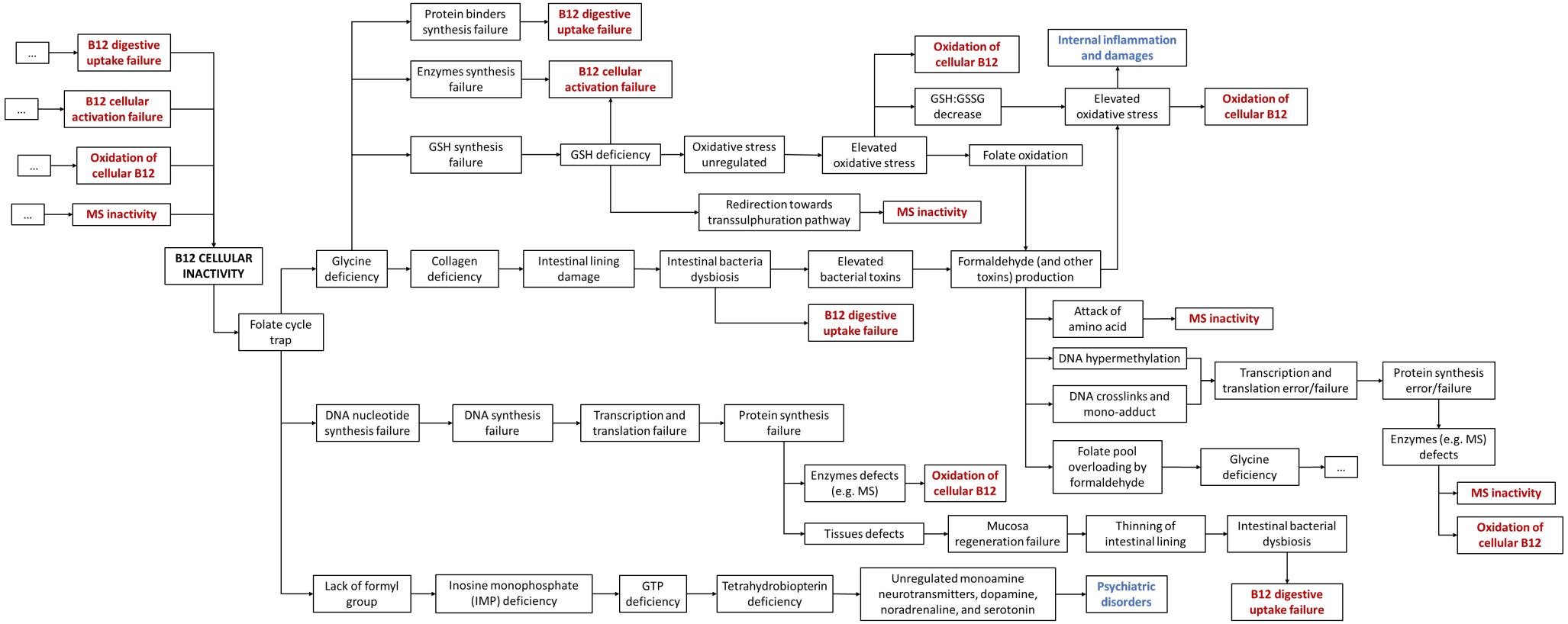


Figure 5.2 Consequences of B12 cellular inactivity (Part 1). Red texts indicate consequences that are connected to the direct causes of B12 cellular inactivity (Figure 5.1); Blue texts indicate the manifestation of the consequences.

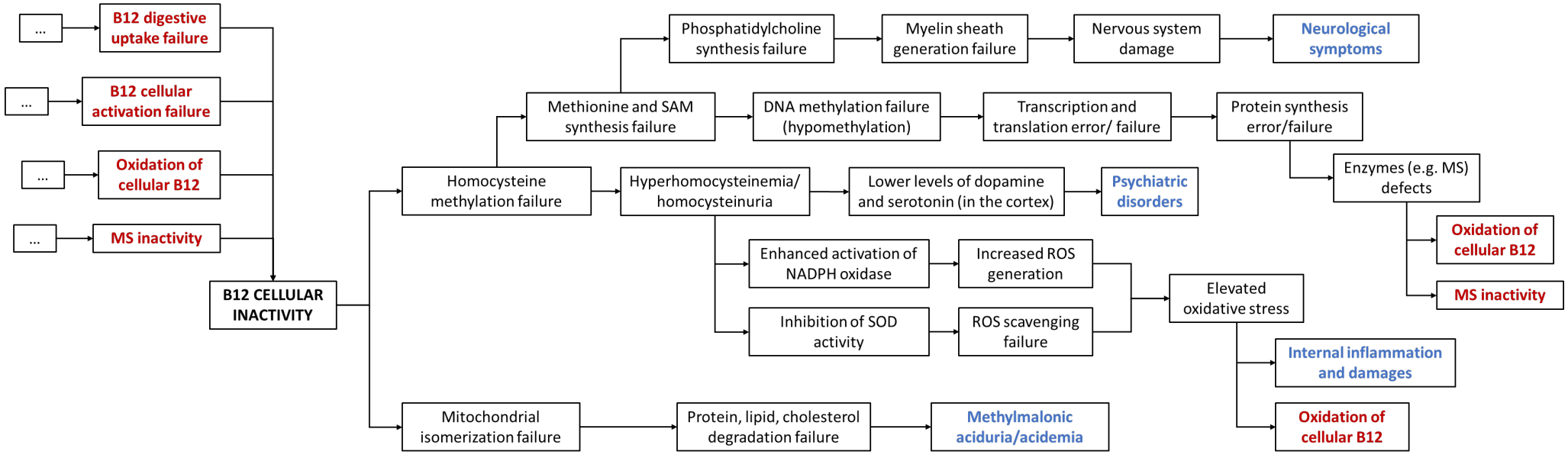


Figure 5.3 Consequences of B12 cellular inactivity (Part 2). Red texts indicate consequences that are connected to the direct causes of B12 cellular inactivity (Figure 5.1); Blue texts indicate the manifestation of the consequences.

Hypothesis 1 B12 cellular inactivity/deficiency causes glycine deficiency due to folate cycle block (see Figure 5.2)

Apart from being the central methylation site, the one-carbon cycle is also responsible for the *de novo* synthesis of glycine, the smallest amino acid, hence the smallest building block of every proteins in the body [60]. Besides being the building block of proteins, glycine also takes part in:

- the regulation of gene expression,
- protein configuration and activity
- the biosynthesis of several essential metabolites, such as glutathione, the major antioxidant in the human body, and
- acts as a neurotransmitter acting on the NMDA receptor [60].

As discussed in Section 4.4.1, around 85% of glycine synthesis *de novo* comes from the folate-dependent SHMT reaction of methyl transfer from serine in the folate cycle [60]. Initially, glycine is considered a non-essential amino acid, because the body can synthesize it. However, Melendez-Hevia et al. had suggested and predicted that the biosynthesis of glycine may fall significantly short of the amount required for the metabolic uses (e.g. a 70 kg human needs 10 grams of glycine per day for collagen synthesis)[89], hence glycine should be considered a semi-essential amino acid.

A prolonged inactivity of cobalamin-dependent MS directly blocks folate cycle, hence blocking glycine biosynthesis (Figure 5.4). In particular, the folate pool is trapped as 5-MTHF and the pool of THF may fall short. As the conversion of serine to glycine is THF-dependent, one can expect subsequent failure in glycine synthesis. Therefore, a prolonged MS inactivity may result in glycine deficiency if the patient does not get enough supply of dietary glycine. To obtain evidence on this hypothesis, one can simply measure the level of glycine in blood or urine.

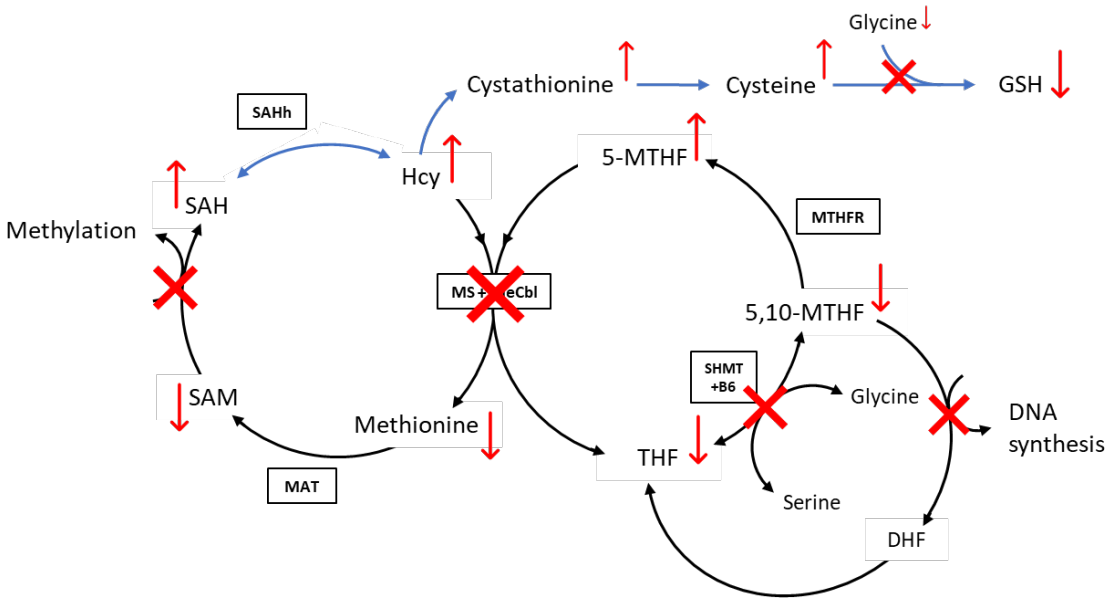


Figure 5.4 The aftermath of cobalamin-dependent methionine synthase blocking. Red cross indicate inhibition; up red arrows indicate accumulation of metabolites; down red cross indicate deficiency of metabolites; blue arrows indicate the redirection of Hcy conversion.

Hypothesis 2 Glycine deficiency causes B12 protein binders synthesis failure, thus decreases the B12 digestive uptake (see Figure 5.2)

As glycine is a major protein building block [60], severe glycine deficiency could result in the failure of protein binders production, e.g. intrinsic factor. Without protein binders, B12 digestive uptake is inhibited (Section 4.2).

Hypothesis 3 Glycine deficiency causes B12 activation enzymes synthesis failure, thus B12 cellular activation slows down (see Figure 5.2)

MMACHC, the major enzyme for the activation of cellular cobalamin (Section 4.3), may fail to activate cobalamin because of the enzyme defects due to the lack of glycine. An enzyme is generally made of a series of amino acid residues arranged in a protein fold. More specifically, glycine residue is suggested to provide flexibility for enzyme active sites [90].

Hypothesis 4 Glycine deficiency causes a lower production of GSH (see Figure 5.2)

Glycine is an essential building block for the synthesis of GSH via transsulphuration pathway [47]. A lack of glycine may cause GSH synthesis failure (Figure 5.4). When glycine is insufficient to maintain the synthesis of GSH, γ-glutamylcysteine consequently rises and may lead to the excretion of its metabolite, i.e. 5-L-oxoproline [91], as depicted in Figure 4.10. Therefore, apart from measuring glycine and GSH levels, one can use 5-L-oxoproline as an indirect biomarker for glycine and GSH insufficiency.

Hypothesis 5 The lack of GSH causes cobalamin cytosolic activation failure leading to cobalamin cellular inactivity (see Figure 5.2)

As discussed in Section 4.3, the activation of cellular cobalamin in MMACHC also requires reduced GSH for nucleophilic displacement of alkyls in alkylcobalamins and for reduction of hydroxocobalamin [39], [41], [48].

Hypothesis 6 The lack of GSH causes unregulated redox balance, thus an elevated oxidative stress (see Figure 5.2)

As GSH is the main antioxidant, the insufficiency of reduced GSH may disrupt the redox balance, hence elevates oxidative stress. Oxidative stress is a condition that occurs from the disruption in cellular redox homeostasis, leading to an elevated level of destructive ROS and toxins. Severe oxidative stress promotes inflammation and damages cells, proteins, and DNA, contributing to damages in tissues and organs. Oxidative stress develops in parallel to and may be implied in age-related diseases, such as neurodegenerative diseases, cardiovascular diseases, kidney diseases, etc. Some of the most notorious ROS are superoxide, H_2O_2, hydroxyl radicals (•OH), and singlet oxygen ($^1O_2$). In contrast to the toxic nature of ROS, the body can actually utilize ROS for signalling in cells [92], but probably at much lower concentration levels. However, the ROS signals should be quickly neutralized by the action of enzymes such as SOD, catalase, alcohol dehydrogenase (ADH), glutathione peroxidase (GPx), and others [93].

Apart from GSH, the body also requires intracellular antioxidant i.e. vitamin C (ascorbic acid) and vitamin E. Ascorbic acid and GSH are strong antioxidants which work synergistically to protect the cells from oxidative stress [94]. Human endothelial cells acquire ascorbic acid in two complementary forms, i.e. ascorbic acid (through sodium ascorbic acid transporter) and dehydroascorbic acid (DHA) (through the glucose transporters) [94]. Inside the cells, DHA is reduced to ascorbic acid by GSH. Thus, GSH level is essential in determining the level of reduced AA in the body.

Another oxidative toxin is formaldehyde, a notorious carcinogenic substance which is well-known to cause DNA damage. Formaldehyde detoxification to formic acid utilizes several enzymes and GSH in several pathways [95], as depicted in Figure 5.5. The urinary pH may be lowered when the body has an excessive amount of formic acid, which is a sign of acidosis. Thus, urinary pH and urinary excretion of formic acid can be measured as indirect biomarkers of formaldehyde, as it may be difficult to directly measure formaldehyde levels.

Formaldehyde may also decrease SOD activity [96], [97], although the mechanism is unknown. While oxidative stress is well-known as root cause in many diseases, formaldehyde is often overlooked as

another major cause of disorders. One source of formaldehyde we identifies is the food degradation by gut bacteria, further discussed in Hypothesis 17.

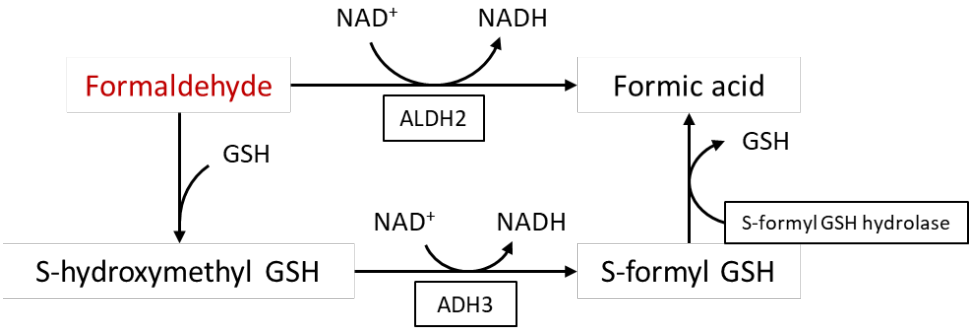


Figure 5.5 Formaldehyde detoxification pathways. ALDH2 = aldehyde dehydrogenase2; ADH3 = alcohol dehydrogenase3.

Hypothesis 7 Oxidative stress lowers the level of reduced GSH, and/or lowers the ratio of GSH:GSSG (see Figure 5.2)

An elevated level of oxidative stress will exacerbate the imbalance of reduced GSH and oxidized GSSG by overwhelming the pool of GSH. The normal molar ratio of GSH:GSSG in healthy adult is approximately 100:1, while the ratio is decreased significantly to down to 1:1 in case of oxidative stress [98]. While oxidative stress itself is a huge problem in the human body, a significant decrease of reduced GSH will impede the activation process of cobalamin (Hypothesis 5), causing a vicious cycle to exacerbate cobalamin deficiency.

Hypothesis 8 Oxidative stress redirects the homocysteine metabolism towards the transsulphuration pathway by inactivating the one-carbon cycle, with the aim to synthesize more cysteine and thus more GSH for counteracting the oxidative stress (see Figure 5.2).

When the redox balance is disrupted (i.e. elevated oxidative stress, lower GSH and/or lower GSH:GSSG ratio, etc.), the body has a metabolic consequence to counteract the redox imbalance. A hypothesis by Deth et al. suggests that *de novo* synthesis of GSH is critical during redox imbalance, such that the equilibrium will shift towards the synthesis cysteine, thus GSH, i.e. transsulphuration pathway [99]. As such, the activity of MS is automatically inhibited to redirect the conversion of homocysteine towards cysteine, subsequently GSH (Figure 5.6). In addition, the activity of CDO, the enzyme which facilitates cysteine conversion to taurine and sulfate, might also be inhibited to ensure all cysteine is converted to GSH [100]. However, glycine is required as the last building block to synthesize GSH and when it is deficit, the conversion of γ-glutamylcysteine to GSH is halted and its metabolite, 5-L-oxoproline, will be accumulated.

Hypothesis 9 Oxidative stress causes THF oxidative degradation to produce formaldehyde (see Figure 5.2)

One hypothesis of endogenous formaldehyde generation in cell is observed in the oxidative decomposition of folate backbone [59]. Folate derivatives that are susceptible to oxidation are THF, DHF, and 5,10-MTHF, while 5-MTHF and 5-formylTHF are relatively resistant to oxidation [59]. Folate is decomposed into pteridine and p-aminobenzoylglutamate (pABG), with formaldehyde produced from the methylene bridge (Figure 5.7). Apart from formaldehyde, the oxidation of 5-MTHF by H_2O_2 is found to produce a compound identified as 4-α-hydroxy-5-methyltetrahydrofolate (hmTHF) [101]. Therefore, it is expected that an excessive folic acid consumption without a proper flow of folate cycle may exacerbate oxidative stress by generating more formaldehyde. However, further investigation on the flux of formaldehyde from folate oxidation must be carried out to confirm the significance. In addition, it was suggested that the oxidative damage of the unstable folates can be enzymatically repaired in mammalian cells [102].

Hypothesis 10 A burst of formaldehyde from THF oxidation causes worsening of symptoms in patients with prolonged MS inactivity and 5-MTHF trap.

Following Hypothesis 9, the endogenous formaldehyde from folate oxidation might also explain the worsening of symptoms in patients at the early period of treatment. Patients with a prolonged MS inactivity may accumulate 5-MTHF. Once given a high dose of cobalamin, MS is activated and a burst of THF may occur. In the case of an elevated oxidative stress, the pool of THF may be oxidized into formaldehyde, which create more damages to the body.

We expect that it is a challenge to prove this hypothesis, as it will be difficult to simultaneously capture the moment of 5-MTHF trap release, THF oxidation, and formaldehyde production throughout the metabolism. Formaldehyde is a very reactive substance which will react rapidly with any reagents, such that it may not be visible using any analytical measurements. Instead, we can aim to use pABG and hmTHF as indirect biomarkers, which had been done in the literature [103]. Furthermore, we expect that there is other more significant source of formaldehyde, such as from bacterial toxins (Hypothesis 17).

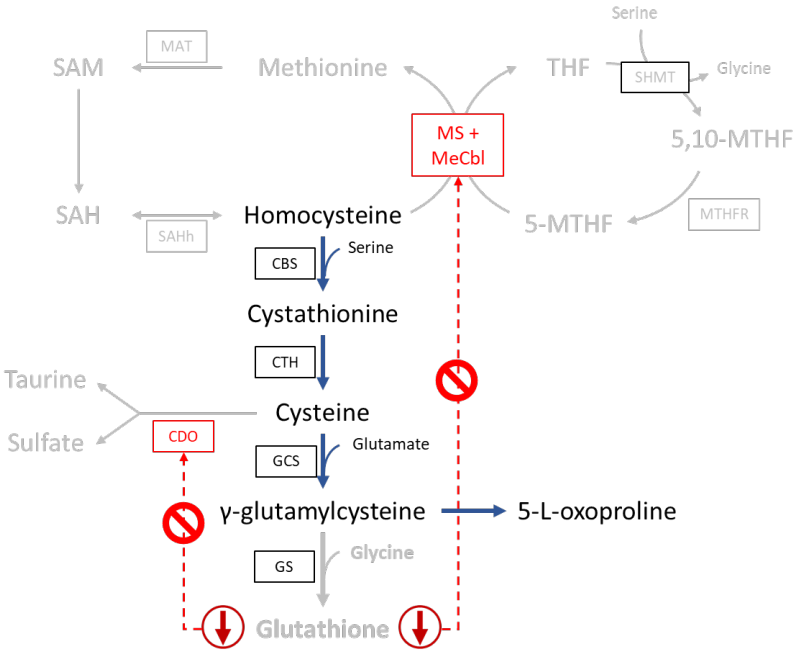


Figure 5.6 Proposed metabolic consequence of lowered glutathione level and redox imbalance. Red dashed lines indicate the inhibition of MS and CDO activities to redirect the equilibrium towards GSH synthesis.

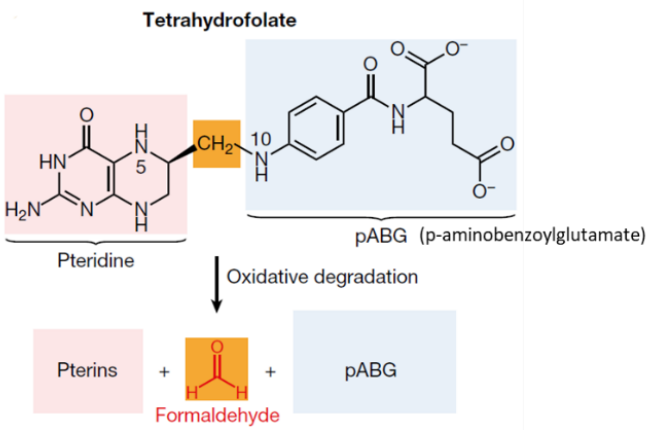


Figure 5.7 Oxidative degradation of tetrahydrofolate backbone to formaldehyde [59].

Hypothesis 11 Formaldehyde serves as a methyl donor to the THF pool, taking it away from glycine synthesis, causing glycine deficiency (see Figure 5.2)

Formaldehyde can react with tetrahydrofolate to produce 5,10-MTHF [104], thus overloading the one-carbon cycle as a methyl donor [105]. This postulate is supported by the notion that formaldehyde is generated as an intermediate in the THF-dependent conversion of serine to glycine [59], although in this case, it is highly unlikely that formaldehyde is freed from the enzyme [76] (Figure 5.8A). Nevertheless, if THF is converted to 5,10-MTHF by formaldehyde, serine conversion to glycine is halted as it is dependent on THF (Figure 5.8B), which brings us back to the issue of glycine deficiency. The methyl group from formaldehyde will be converted via the folate pool to either CO_2 or formate (Figure 5.8C). Thus, in addition to formate produced from formaldehyde enzymatic detoxification (Figure 5.5), more formate will be accumulated. Although formate will be taken back by the folate cycle via 10-formylTHF by 10-formylTHF synthase, we expect that an elevated level of formate (or formic acid) may cause acidosis. As discussed in Hypothesis 6, urinary pH and formic acid are potential indirect biomarkers for formaldehyde issues.

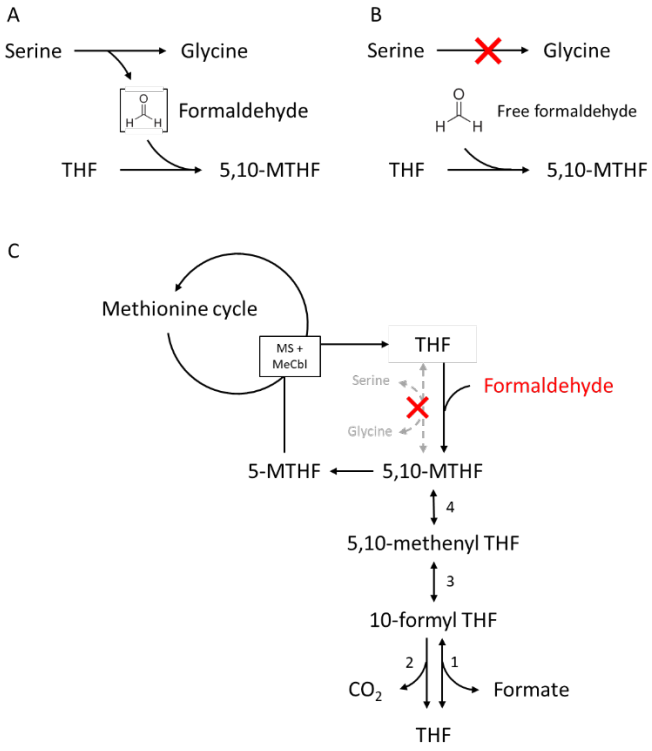


Figure 5.8 A) Formaldehyde as an intermediate of serine to glycine conversion by THF-dependent SHMT. Formaldehyde is not released from the enzyme; B) Proposed scheme of free formaldehyde reacts with THF, causing serine to glycine conversion fails to happen; C) 1-carbon from formaldehyde is converted to CO_2 and/or formate via the folate pools. 1 = 10-formyl-THF synthetase; 2 = 10-formyl-THF dehydrogenase; 3 = 5,10-methenyl-THF cyclohydrolase; 4 = 5,10-methylene-THF dehydrogenase.

Hypothesis 12 Formaldehyde causes errors/failure in the synthesis of proteins, including MS (see Figure 5.2)

An excessive amount of formaldehyde is well-known to cause damage to DNA and being carcinogenic. Particularly, formaldehyde induces the mono-adduct of *N*-hydroxymethyl to guanine, adenine, and cytosine, as well as the crosslinks of *N*-methylene between adjacent purine in DNA [106]. The crosslinks cause single-strand breaks, and formaldehyde exacerbates the damage by inhibiting of resealing to repair DNA [107].

In addition, SAM-dependent DNMT is found to catalyse reversible covalent addition of exogenous aldehydes, including formaldehyde, to DNA residues [108]. It was evident that the product of DNA-

cytosine (a methyl group acceptor) and formaldehyde is DNA-5-methyl-cytosine [109]. DNA hypermethylation can manifest in the failure of DNA to RNA transcription, especially if the hypermethylation happens at the promoter site (i.e. the site where the transcription process initiates). Hence, an excessive formaldehyde may result in errors of the transcription and translation of DNA to mRNA and proteins, including enzymes such as methionine synthase.

Hypothesis 13 MS defects cause the oxidation of cellular B12, thus cobalamin inactivity (see Figure 5.2)

An incorrect transcription of hypomethylated/hypermethylated and damaged DNA to mRNA and subsequently a wrong translation to MS may result in an improper function of MS. For example, an age-dependent decrease and an alternative splicing in MS mRNA was first observed in post-mortem human cortex [110], and later was observed in SH-SY5Y human neuroblastoma cells [67]. The alternative splicing of MS mRNA may cause deletion in a number of exons, which results in an impaired MS binding mechanism. For example, deletion in exon 16-18 (i.e. folate domain) causes the reduction in folate affinity, while deletion in exon 19 and 20 (i.e. cap domain) causes cobalamin to be susceptible to oxidation as it loses its cap protector [67].

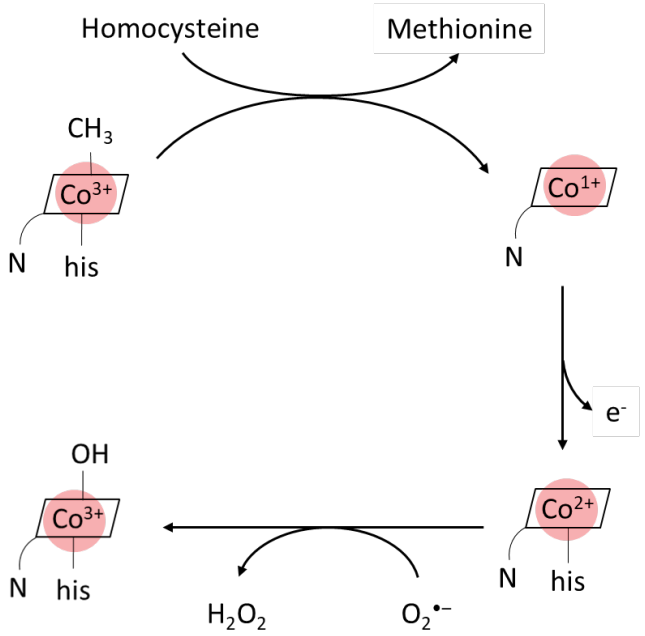


Figure 5.9 Further oxidation of cob(II)alamin to hydroxocob(III)alamin in MS due to cap domain absence.

The cap domain defect could most likely increase the oxidation rate of cob(I)alamin to inactive cob(II)alamin. Normally, cob(II)alamin will be reduced by MSR and methylated by SAM to reactivate into MeCbl (Figure 4.7A). However, with the lack of the upper face protector, cob(II)alamin can be oxidized further to HOCbl(III) by reactive oxygen species (ROS), such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), or by formaldehyde (Figure 5.9). In fact, a kinetic study shows that the oxidation rate of cob(II)alamin to HOCbl/H_2O^+Cbl by superoxide (K = 7 x 10^8 M/s) is relatively close to that of superoxide peroxide dismutase (SOD) (K = 2 x 10^9 M/s) [7], which is the primary enzyme for the defence against superoxide. Therefore, a reactivation scheme is required to convert HOCbl(III) back to MeCbl in MS.

Hypothesis 14 The reactivation of HOCbl(III) in MS happens *in situ* utilizing GSH, MSR and SAM.

Figure 5.10 presents the proposed mechanism of HOCbl(III) *in situ* reactivation in MS. The hypothesis is partially adapted the hypothesis of Waly et al. which includes GSH as an essential factor in the reduction process of HOCbl [67], [111]. In a stand-alone experiment, Waly et al. showed that the

absence of GSH is observed to inactivate HOCbl-based MS activity, and subsequent addition of GSH to the inactive HOCbl-MS pool reactivates the enzyme activity [67]. GSCbl is hypothesized to be an intermediate in this reactivation pathway and it requires SAM to convert to methylcobalamin [67]. However, this hypothesis lacks in the steps of GSCbl reduction and remethylation into MeCbl. Therefore, we add to the hypothesis that GSCbl requires another equivalent of GSH to eliminate the glutathionyl ligand and generate cob(II)alamin. This proposed scheme is similar to the scheme of GSCbl activation in MMACHC [112]. Cob(II)alamin could then be reduced by MSR back to cob(I)alamin, only by then SAM is required to remethylate cob(I)alamin to methylcobalamin.

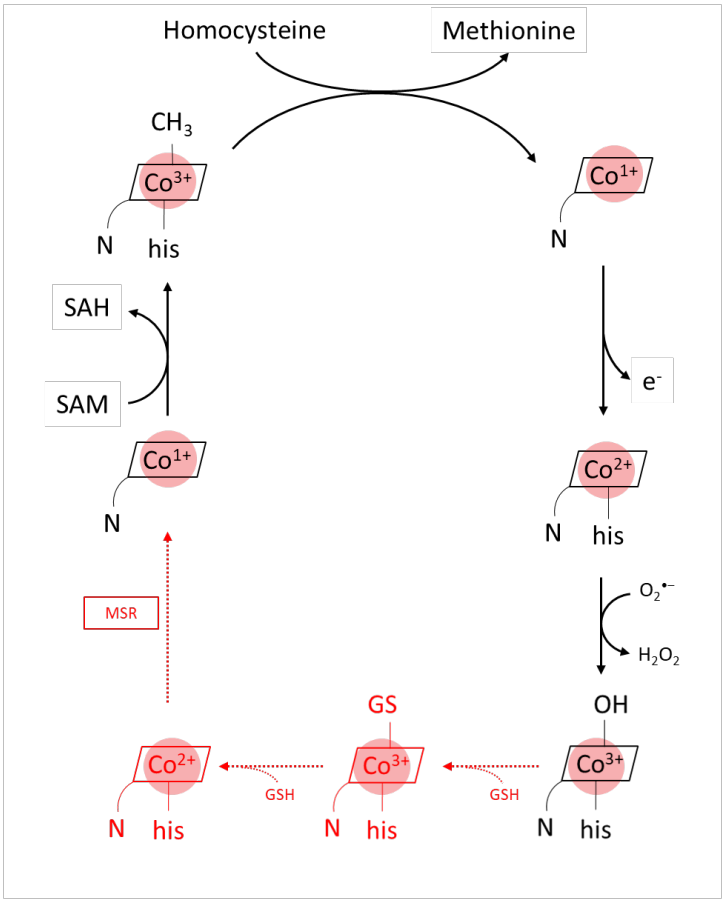


Figure 5.10 The proposed mechanism of HOCbl in situ *reactivation in MS by GSH, MSR, and SAM.*

Hypothesis 15 Glycine deficiency causes collagen deficiency (see Figure 5.2)

Collagen synthesis relies heavily on glycine. In fact, 33% of collagen is made of glycine [113]. According to Hypothesis 1, a severe cobalamin deficiency may lead to glycine deficiency, thus collagen synthesis failure. The lack of collagen might result in the deviation of the intestinal bacterial population (elaborated in Hypothesis 16).

Hypothesis 16 Collagen deficiency (thus glycine deficiency) elevates the risk of intestinal bacterial dysbiosis and elevates bacterial toxins (see Figure 5.2)

Collagen is a major component to build the extracellular matrix, and to maintain the structure and strength of all tissues [114]. When a tissue is injured, collagen is required for the rapid repair. In particular, the human intestinal smooth muscle is found to synthesize collagen that is required to repair the bowel wall [114]. According to Hypothesis 15, collagen synthesis might be inhibited when glycine is deficient. Without a sufficient supply of collagen, the risk of the intestinal lining injury increases, thus elevates the risk of intestinal bacterial homeostasis disruption. As a result, the balance of bacterial population may be disrupted, causing the intestinal bacterial dysbiosis. In addition, glycine

is also required to build digestive enzymes (Hypothesis 3), which have an important role of inhibiting bacterial overgrowth. Especially, glycocholic acid, a bile acid, depends directly on glycine availability. Therefore, cobalamin deficiency may indirectly generate intestinal disorders, causing elevated digestive issues and a disrupted bacterial homeostasis.

Several patients at the B12 Institute had experienced gastrointestinal disorders symptoms (e.g. diarrhoea, constipation). In most cases, the symptoms are relieved after receiving B12 injection. However, if left untreated and become severe, the condition can develop into a more recurring disorder such as small intestinal bacterial overgrowth (SIBO). SIBO is a condition where the colony of bacteria from the large intestine moves to the small intestine. The small intestine, which is generally sterile or has low level of bacteria (up to 10^3 bacteria/ml), is invaded by more than 10^5 bacteria/ml [115]. The invasion of bacteria (e.g. *bacteroides*) in the small intestine results in the undesired competition of cobalamin uptake between intrinsic factor and the bacteria in duodenum [116]. Cobalamin may also play a role in maintaining the balance of gut bacteria, as cobalamin may regulate gut microbial ecology through corrinoid-dependent gene regulation of gut microbiome [116], [117].

The apparent malabsorption of cobalamin in the small intestine is exacerbated by the production of toxins by the abnormal population of bacteria. The dislocated bacteria in the small intestine may degrade food blocks (e.g. protein, carbohydrate, lipid, pectin). Food blocks degradation by bacteria produces toxic products, such as methanol (CH_3OH), methylamine (CH_3NH_2), dimethylamine (C_2H_7N), and trimethylamine (C_3H_9N) [118], [119], and methylglyoxal [120], amongst others. These toxins contribute to the excessive production of formaldehyde and other advanced glycation end products, causing an elevated oxidative stress [119], [121]–[123]. For example, an overconsumption of pectin-containing foods can elevate formaldehyde production in the body [124]. Pectin-containing foods, e.g. tomatoes, have an enzyme called pectin methylesterase (PME) which is able to convert pectin into methanol [125]. In addition, PME is also found in pathogenic fungi and bacteria [126]. In case of bacterial dysbiosis or a more severe SIBO, methanogenesis (i.e. the formation of methane from methanol by bacteria) will fail due to the absence of methanogen bacteria in the large intestine, as illustrated in Figure 5.11. Methanogens are slow growers, so they may be washed out by diarrhoea form the colon. The use of antibiotics could also be a cause of methanogenic bacteria absence. Methanol is then converted to formaldehyde by cytosolic alcohol dehydrogenase 1 (ADH1), mostly in the liver [95].

It is interesting to use bacterial toxins as biomarkers of intestinal issues such as SIBO. For example, an elevated amount of trimethylamine in urine, also known as trimethylaminuria, can be measured. Methanol breath test had been used to proof an elevation in blood methanol after an intake of aspartame [127]. These indirect methods offer less invasive diagnostics to assess the health status of the intestines, compared to for example colonoscopy.

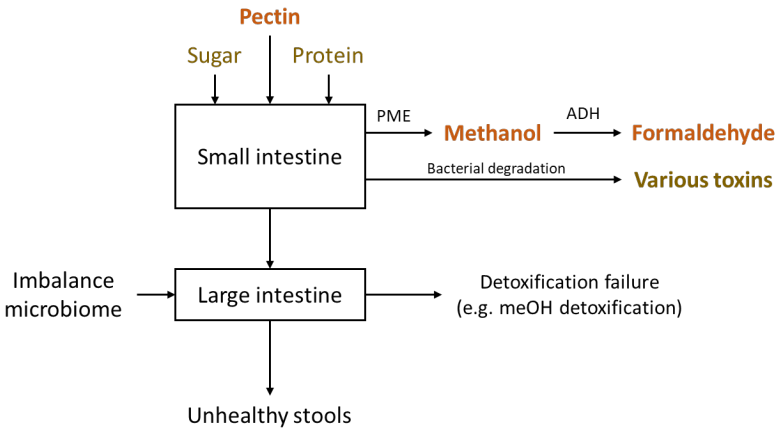


Figure 5.11 Proposed mechanism of oxidative stress and toxins generation in case of intestinal bacterial dysbiosis.

Hypothesis 17 Intestinal bacteria significantly elevate the production of formaldehyde (see Figure 5.2)

Formaldehyde is also produced from toxic products of bacteria (Figure 5.12). For example, the deamination of methylamine (i.e. the degraded product of protein) is catalysed by enzyme semicarbadize-sensitive amine oxidase (SSAO) and produces formaldehyde, H_2O_2, and ammonia [128]. Trimethylamine from bacterial choline degradation is converted by flavin monoxidase (FMO) to trimethylamine-N-oxide (C_3H_9NO), which can be further converted to dimethylamine and formaldehyde by trimethylamine oxide aldolase (TMAOase) [118].

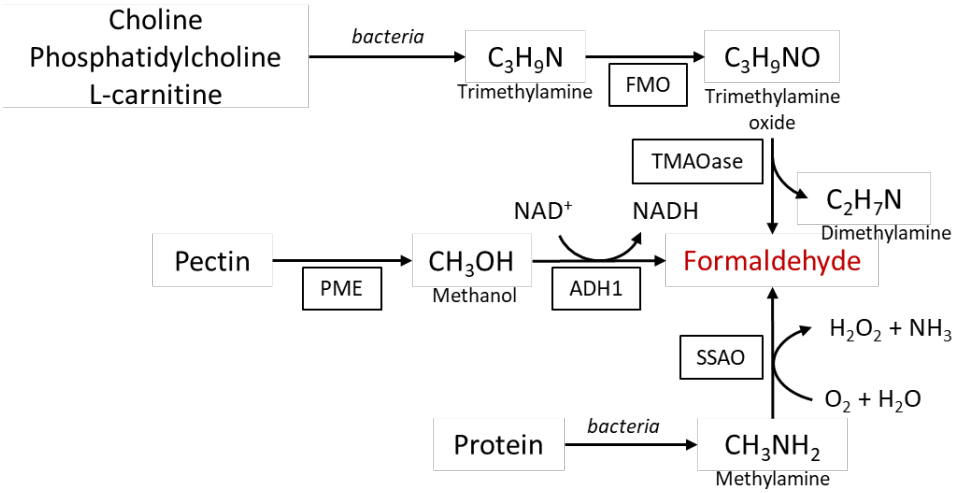


Figure 5.12 Formaldehyde generation from food degradation.

Hypothesis 18 Alcohol lowers GSH level, thus inhibiting MS activity (see Figure 5.1)

Alcohol, particularly ethanol, is found to inhibit MS activity [129]. Although the exact mechanism remains to be investigated, it has been suggested that acetaldehyde, the derivative product of ethanol, may have a direct effect on MS activity [130]. Ethanol is also found to lower the GSH level in the body [111], [131]. According to Hypothesis 8, a lower level of GSH may inhibit the activity of MS by redirecting homocysteine towards the synthesis of GSH (Figure 5.6).

Hypothesis 19 Aldehydes (i.e. formaldehyde and acetaldehyde) slow down MS activity by attacking amino acids (see Figure 5.1)

Formaldehyde is reported to react with amino acids, including cysteine, histidine, serine, etc. [132]. Cysteine is found to react the most effectively with formaldehyde producing a stable thiazolidine. Other aldehydes and ketones are also found to react with cysteine [132]. Therefore, amino acids in MS could be damaged under an elevated level of aldehyde. A defect in for example the ligand triad (his-asp-ser) of MS could be another cause of MS inactivity.

Hypothesis 20 Choline supplementation helps to restore phosphatidylcholine (and betaine), thus restores myelin sheath.

Phosphatidylcholine, the major constituent of myelin sheath, is biosynthesized via two pathways:

- The CDP-choline pathway (Kennedy pathway) using choline as an essential precursor [133]
- By phosphatidylethanolamine methylation by SAM [134]

During cobalamin deficiency/inactivity, the methylation of PE into PC is inhibited due to the lack of SAM. Therefore, while the methionine cycle is slowly repaired by cobalamin supplementation, choline may act in two ways to accelerate the repair of myelin sheaths:

- choline supplementation can help to accelerate the synthesis of PC via the Kennedy pathway,
- choline is also the precursor of betaine, the coenzyme of BHMT for the alternative methylation of homocysteine; thus choline could increase the synthesize SAM during MS slow running.

The scheme choline function is illustrated in Figure 4.8.

Hypothesis 21 Folate trap directly leads to psychiatric disorders (see Figure 5.2)

Under the inactivity/deficiency of B12, folate trap may indirectly interrupt the synthesis of the neurotransmitters (i.e. dopamine, noradrenaline, and serotonin). These substances are fundamental for the normal function of the brain, which strongly relates to important mental health disorders. Folate trap could disrupt the supply of formyl groups, which are necessary for *de novo* synthesis of inosine monophosphate (IMP) [135]. IMP is the precursor of ATP and GTP; the latter is an essential precursor of tetrahydrobiopterin (BH_4). BH_4 is an essential regulatory cofactor for the biosynthesis of the monoamine transmitters. As depicted in Figure 5.13, the 5-MTHF trap will lead to neurotransmitters synthesis disruption, which manifest in several symptoms of psychological complaints (see Appendix A).

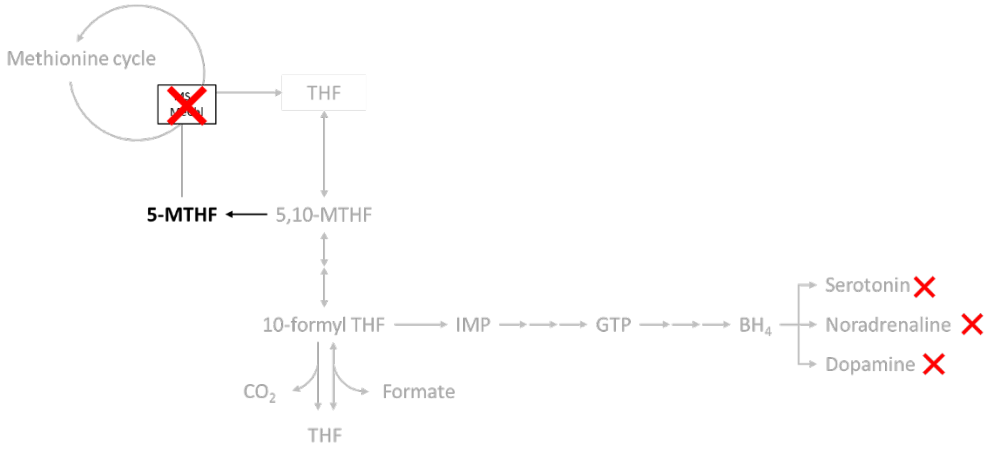


Figure 5.13 Neurotransmitters synthesis disruption due to folate trap. IMP = inosine monophosphate; GTP = Guanosine-5'-triphosphate; BH_4 = tetrahydrobiopterin.

5.2. Summary of Potential Biomarkers

The list of hypotheses in Section 5.1 has offered a number of overlooked issues related to B12 deficiency/inactivity. These hypotheses have led us to explore several direct and indirect biomarkers to assess the health of the patients. We identified several interesting biomarkers in Table 5.1, amongst others.

Table 5.1 List of potential biomarkers explored from the hypotheses list.

Biomarkers	Sample	To measure	References
Glycine	Blood	Glycine level	[89]
Glutathione	Blood	GSH level	
		Ascorbic acid levels	[94]
		Oxidative stress status	[98]
5-L-oxoproline	Blood/Urine	Glycine level	[91]
		GSH level	
Urinary pH	Urine	Acidosis	-
		Formaldehyde level	
Formic acid + MMA + propionic acid	Urine	Acidosis	-
		Formaldehyde level	
p-aminobenzoylglutamate	Blood	Folate oxidation	[103]

Biomarkers	Sample	To measure	References
4-α-hydroxy-5-methyltetrahydrofolate	Blood	Folate oxidation	[103]
Trimethylamine	Urine	Bacterial toxins	[118]
Trimethylamine-N-oxide	Blood	Bacterial toxins	[118]
Methanol	Breath	Bacterial toxins	[127]

5.3. Strategies of Diagnosis and Treatment

We have explored several overlooked issues related to B12 deficiency in Section 5.1. Next, we compose and discuss some strategies for the diagnosis and treatment for the issues. For this purpose, the hypotheses list is regrouped into 5 big groups:

- Group 1: Issued related to glycine deficiency
- Group 2: Issues related to oxidative stress and glutathione reduction
- Group 3: Issues related to intestinal bacterial dysbiosis
- Group 4: Issues related to formaldehyde damages
- Group 5: Issues related to the oxidative damage and inhibition of the one-carbon cycle

Table 5.2 presents the classification of each hypothesis in the groups.

Table 5.2 Classification of the hypotheses list.

Group 1	Group 2	Group 3	Group 4	Group 5
Hypothesis 1	Hypothesis 5	Hypothesis 16	Hypothesis 11	Hypothesis 9
Hypothesis 2	Hypothesis 6	Hypothesis 17	Hypothesis 12	Hypothesis 10
Hypothesis 3	Hypothesis 7			Hypothesis 13
Hypothesis 4	Hypothesis 8			Hypothesis 14
Hypothesis 15				Hypothesis 18
				Hypothesis 19
				Hypothesis 20
				Hypothesis 21

5.3.1. Hypotheses Group 1: Glycine deficiency

The importance of glycine is often dismissed as it is the smallest amino acid and is considered to be a non-essential amino acid. However, as it has been mentioned several times throughout this report, glycine deficiency is a hugely overlooked issue tightly related to B12 deficiency. The hypotheses group 1 collects and summarizes disorders branches to and from glycine deficiency, such glutathione synthesis failure, B12 activation failure, and intestinal damage, and the vicious cycle thereof (see Figure 5.14).

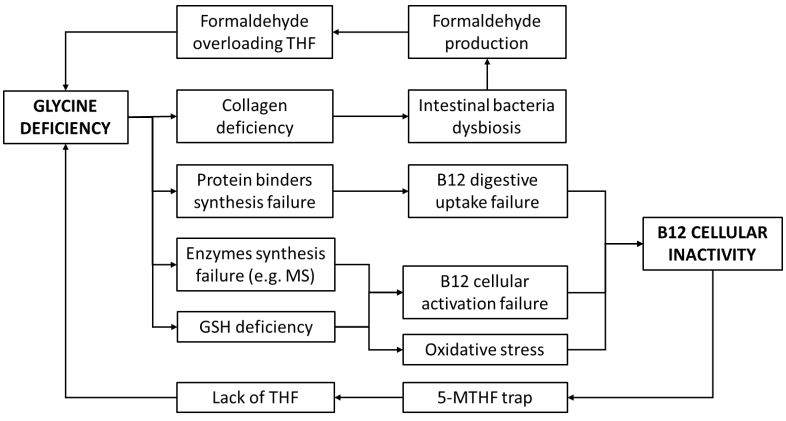


Figure 5.14 Proposed vicious cycle of glycine deficiency and B12 deficiency.

The diagnosis of glycine deficiency is quite straightforward, which can be done by a direct measurement of glycine blood or urinary level. In addition, as discussed in Hypothesis 4, 5-L-oxoproline is a valuable indirect biomarker of glycine (and glutathione) deficiency. However, the quantification of glycine deficiency effects to B12 protein binders and enzymes, as well as to collagen level remains a challenge.

The treatment of glycine deficiency can be done by simply supplementing with glycine. As discussed in Section 5.3.2.2, glycine is safe to be given even in a dose as high as 60 g/day. The dose of glycine to fulfil all processes requiring the amino acid (e.g. proteins and enzymes synthesis, GSH synthesis, etc.) remains to be investigated. We then recommend future research studies involving the metabolic fluxes of the glycine-dependent processes.

5.3.2. Hypotheses Group 2: Low GSH and High Oxidative Stress

The issues of low GSH and high oxidative stress form a vicious cycle which exacerbate each other, no matter which one comes first. We argue that both issues cannot be separated from the B12 deficiency disorder, even though they have always been underestimated. Therefore, we offer a strategy to cut the vicious cycle, which can hopefully improve the health of the patients.

5.3.2.1. Diagnosis of oxidative stress

Several biomarkers have been used to measure the oxidative stress status in the literature. For example, levels of exogenous antioxidants (e.g. vitamin C, vitamin E, selenium, etc.), endogenous antioxidants (e.g. GSH/GSSG, thiols, urate, GPx activity), and oxidative DNA damage (8-hydroxy-2'-deoxyguanosine) had been measured to identify oxidative stress in distinct diseases [136]. Malondialdehyde (MDA), the product of polyunsaturated fatty acids peroxidation in the cell, has been long used as a biomarker of oxidative stress [137]. In addition, C-reactive protein (CRP) test can also be considered to assess the level of inflammation in the body. In spite of the many choices of oxidative stress biomarkers, we strongly suggest to first measure the levels of the reduced and the oxidized glutathione, because the low ratio of GSH/GSSG is a major sign of oxidative stress.

5.3.2.2. Treatment of oxidative stress

The importance to restore the levels of GSH is obvious in case of oxidative stress. Several methods to administer GSH had been investigated in the literature, including orally, intravenously, sublingually, intranasally, via inhalation, and via transdermal [138]. It is unfortunate, however, that the efficacy of oral GSH, which is the most non-invasive way, has not been proven well due to some unresolved GSH digestion issues [138]. Therefore, alternative strategies to restore the levels of GSH may include the administration of other compounds to enhance the *de-novo* synthesis of GSH, i.e. glutamate, cysteine, and glycine. Below, we discuss the existing literature studies of each compound as well as the combinations.

- Glutamine

  Glutamine is a free amino acid which will be converted into glutamate through normal metabolic pathways. The reports on glutamine effects to GSH synthesis are inconclusive. A study on HIV+ patients showed that 20 g/day oral glutamine for 7 days increased the plasma GSH [139]. On the other hand, a dose of 0.3 g/kg/day oral glutamine for 10 days on healthy subjects decreased GSH level by 37% instead [140]. Glutamine is found to share a similar transporter as cystine [141], thus may compete with the other amino acid, and consequently reduces the availability of cysteine [142]. Another metabolic side effect of a long-term glutamine supplementation is increasing ammonia production, and impairing ammonia transport and detoxification [143]. The safe dose of glutamine is set at 14 g/day [144].

Considering the past literature studies, we do not recommend to give glutamine to patients without further knowledge of the other two GSH building blocks sufficiency. Glutamate and cysteine are required in the upstream reaction of GSH synthesis. If glycine at the downstream is insufficient, the product of cysteine and glutamate will be degraded into 5-L-oxoproline and excreted. Therefore, supplementation of glutamine/glutamate (and cysteine) should only be considered if glycine is for sure sufficiently supplied.

- N-acetylcysteine (NAC)

  NAC is given as the precursor of cysteine. While cysteine supplementation is normally oxidized into disulfide cystine, oral NAC supplementation is readily absorbed in the stomach and the intestines to be converted almost entirely in the liver and kidney into cysteine [138]. Moreover, supplementation with cysteine has risks of toxicity and mutagenicity [138].

  The effects of NAC supplementation on GSH elevation were observed in HIV+ patients. GSH was elevated with a dose of 8 g/day for 8 months [145], while a lower dose of 1 g/day achieved almost two folds of GSH increase [139]. NAC supplement is relatively safe, although there are some side effects observed, i.e. nausea and gastric distress. However, it is unclear whether the side effects are caused by solely NAC or by its excipients. We hypothesize that the side effects could be a result of acidosis due to an excessive 5-L-oxoproline production, which would happen when NAC is dosed under glycine deficiency.

- Glycine

  Although there is limited human study on the effect of a single glycine supplementation of GSH levels, studies indicated that supplementation with glycine may attenuate inflammation and oxidative stress. Glycine was reported to decrease the intracellular homocysteine *in-vitro* [146], although the mechanism is still open for investigation. Glycine was also reported to improve the symptoms and spirometric variables on cystic fibrosis patients, as it lowered serum TNF-α and sputum IL-6 and G-CSF [147]. Patients with metabolic syndrome who received a daily dose of 15 g glycine had their thiobarbituric acid reactive substances (TBARS) decreased by 25%, which indicated the reduction of oxidative stress and a more balanced redox reactions in their body [148]. In rats, the no-observed-adverse-effects-level (NOAEL) of glycine is at least 2000 mg/kg/day [149], and a study on schizophrenia patients indicated that a dose of 60 g/day glycine has not manifested in adverse effects [150].

- Glutamine + NAC

  An *in vitro* study by Wessner et al. [151] on myelomonocytic cells suggested that a combination of NAC and glutamine can improve the glutathione synthesis, compared to glutamine alone. The results of the study showed that while glutamine alone reached the optimum of GSH at 0.6 mmol/l (which reflects the normal human plasma glutamine), the addition of 0.5 mmol/l of NAC may boost the synthesis of GSH to the optimum level (Figure 5.15) [151]. It will be interesting to observe the effect of glycine addition to the combination on the GSH level, whether GSH synthesis can be boosted further, which had not yet carried out in the study.

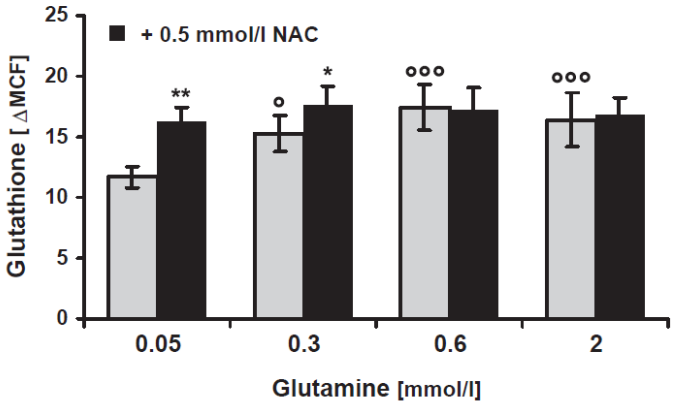


Figure 5.15 The effects of glutamine and NAC on glutathione levels [151]. *MCF = mean channel fluorescence.*

- Glutamine + glycine

The study by Wessner et al. [151] revealed that a combination of glycine and glutamine may decrease the GSH level. Interestingly, this peculiar effect was only observed significantly when the dose of glycine and glutamine are both high (Figure 5.16) [151]. The authors also observed that a high level of glycine decreased the level of intracellular glutamine (Figure 5.17). They suggested that the addition of glycine may lower the uptake of glutamine as these amino acids share the same transporter systems for their uptake into the cell [151]. It is not yet clear whether this effect will be apparent in human studies.

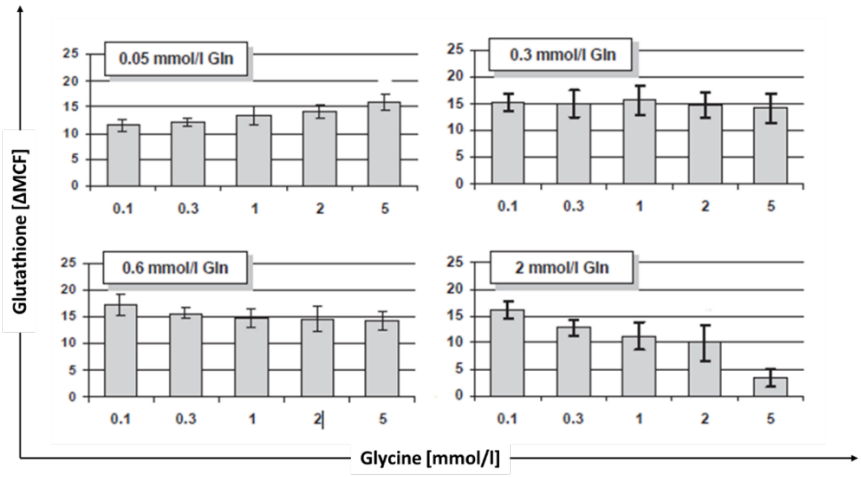


Figure 5.16 The effects of various concentrations of glutamine (GLN) and glycine on glutathione levels [151].

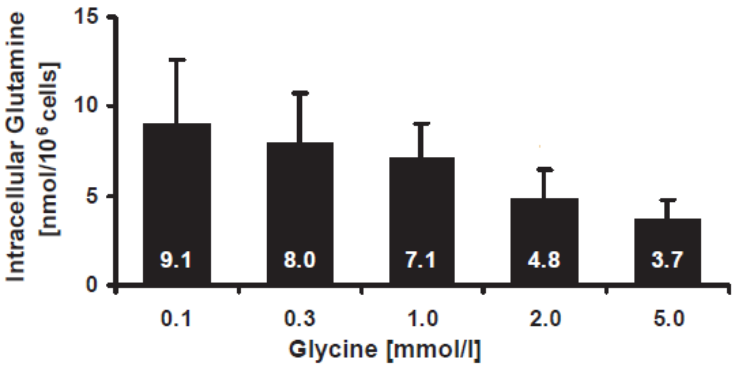


Figure 5.17 The effect of glycine on intracellular glutamine [151].

- NAC + glycine

  There is limited yet promising results on the study of the combination of NAC and glycine on the glutathione and oxidative stress status. An animal study on the cardiac function of mice revealed that NAC in combination with glycine (1.6 g/kg each) for 7 weeks may improve the cardiac function, such as improving the diastolic function, increasing the peak early filling velocity, and reducing relaxation time, left atrial volume, and left ventricle end diastolic pressure [152]. Interestingly, NAC alone did not demonstrate the similar effect on cardiac function [152].

  Sekhar et al. studied the effect of the supplements on elderly, whose GSH and oxidative stress status were noticeably abnormal compared to young subjects [153]. Relatively high doses of 100 mg/kg/day of oral NAC and 100 mg/kg/day of oral glycine were given to the elderly for 14 days, which resulted in a 94.6% increase in red blood cells GSH [153]. There was no change in RBC GSSG, therefore the ratio of GSH:GSSG was increased. The oxidative stress state of the post-supplemental elderly was comparable to the young subjects, as the oxidant markers (i.e. plasma F_2-isoprostanes and lipid peroxides) were decreased [153].

- Glutamine + NAC + glycine

  There is little to no studies on the effect of combined glutamine, NAC, and glycine on GSH levels.

We conclude that it may be the safest and the most effective to give glycine and NAC to patients to increase *de-novo* synthesis of GSH. The apparent side effects of NAC supplementation may be due to acidosis, due to an excessive production of 5-L-oxoproline, which is derived from γ-glutamylcysteine under a deficit glycine. Therefore, it would be safe to give both compounds together, to avoid the undesired side reaction and to ensure GSH can be synthesized with enough building blocks. However, the appropriate doses remain to be determined, as these amino acids are also required to make up for other processes when they are deficient. This needs to be estimated based on metabolic fluxes.

The natural synthesis of cysteine needed for GSH in the body is via the transsulphuration pathway with homocysteine as the upstream metabolite. Homocysteine conversion to cystathionine in the transsulphuration pathway requires vitamin B6-dependent cystathionine beta synthase enzyme. Thus, the supply of vitamin B6 should be sufficient to support this reaction. Vitamin B6 is included as a part of vitamin B group. Vitamin B6 includes 6 vitamers, i.e. pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and their phosphorylated forms (i.e. PN-phosphate, PL-phosphate, and PM-phosphate) [154]. Upon digestion from diets and/or supplements, all forms of B6 vitamers are converted into the active form of vitamin B6, which is pyridoxal-5-phosphate (PLP) [154]. The threshold of an adequate plasma PLP is 20 nmol/L [155]. Even though it seems more beneficial to supplement B6 in the form of PLP, the bioavailability of oral PLP is limited by the fact that PLP will be hydrolysed into the non-phosphorylated form in the small intestine by the enzyme alkaline phosphatase [156]. Therefore, vitamin B6 is commonly supplemented in the inactive form of pyridoxine.

Two enzymes in the one-carbon cycle and transsulphuration pathway are dependent on vitamin B6, i.e. CBS [157] and SHMT [158]. Therefore, a supplementation with vitamin B6 is expected to enhance the conversion of homocysteine by the enzyme CBS. However, the anticipated effects are not conclusive in several human studies. The study in healthy elderly showed the reduction of total plasma homocysteine at the dose of 1.6 mg/day for 12 weeks [159]. It is important to note that in this study, the elderly are repleted with folic acid and riboflavin as well [159]. On the other hand, supplementation with 10 – 25 mg/day of vitamin B6 did not affect the levels of homocysteine in hyperhomocysteinemia [160] and healthy subjects [161]. A study on patients with coronary artery disease showed that a dose of up to 50 mg/day of vitamin B6 failed to reduce the fasting plasma homocysteine after 12 weeks of supplementation [162]. In this study, a slight increase of homocysteine and a slight decrease of serum folate were observed during 12 weeks of 10 and 50 mg vitamin B6 supplementation, although the

mechanism of folate reduction by vitamin B6 supplementation remains unclear [162]. Thus, the use of vitamin B6 supplementation to reduce homocysteine levels remains controversial, considering the number of studies disproving the positive effects of vitamin B6 on homocysteine levels.

An excess of vitamin B6 is well-known as toxic and can lead to sensory neuropathy [163]. The manifestation of sensory neuropathy is commonly reported at doses of pyridoxine above 1000 mg/day, although some cases may occur at doses below 500 mg/day [163]. There is lack of consensus on the safe dose of vitamin B6 supplementation. The European Food Safety Authority (EFSA) sets a safe dose of 18 mg/day [164], while an older safe dose was set at 100 mg/day by the Institute of Medicine in 1998 [165].

In summary, a sufficient amount of vitamin B6 is essential for the transsulphuration pathway. However, considering the side effects reported in the literature, we strongly suggest to first measure the level of plasma B6 before supplementing with extra vitamin B6. In case of deficiency, we suggest 18 mg/day of vitamin B6 as safeguard for the transsulphuration pathway. Finally, the side effects of higher dose of vitamin B6 is still open for investigation.

Apart from restoring GSH, a boost of other antioxidants will also be required to suppress the elevated oxidative stress. We studied antioxidants such as vitamin C, vitamin E, lipoic acid, and selenium, amongst others.

- Vitamin C

  Vitamin C, also known as ascorbic acid, is a potent antioxidant which functions by scavenging ROS and protecting proteins from alkylation by electrophilic lipid peroxidation products [166]. Vitamin C is also dependent on vitamin E to neutralize lipid hydroperoxyl radicals [166]. In addition, the activity of vitamin C depends on the level of glutathione which is required for the recycling or the reactivation of its oxidized form, i.e dehydroascorbic acid [167].

  A number of human studies had been carried out to investigate the role of vitamin C as an antioxidant. The effects of vitamin C in several diseases had been reviewed early in the literature [168]. While *in vitro* experiments showed superior actions of vitamin C as antioxidant, there is lack of evidence on the significance of vitamin C treatment on oxidative stress biomarkers or in clinical outcomes [168]. The radioprotective effect of vitamin C was also studied in hyperthyroidism patients undergoing radioactive treatment [169]. By taking 1000 mg/day of vitamin C throughout 1 week starting from 1 day before the treatment, the total oxidant status, malondialdehyde, and 8-Oxo-2'-deoxyguanosine (i.e. the product of DNA oxidation) were reduced, while the total antioxidant status was increased slightly [169]. Healthy women who did a 30-minute moderate-intensity cycling had their levels of MDA decreased by 1000 mg of vitamin C, compared to the placebo group [170]. However, a daily dose of 250 mg of vitamin C for 2 months had failed to normalize the plasma levels of carbonyls, CRP, albumin, and GSH levels in hemodialysis patients [171].

  As a water soluble vitamin, the plasma concentration of vitamin C is tightly controlled and the excess of vitamin C is excreted. An intake of 1 gram of oral vitamin C resulted in 70 – 80 µM of plasma concentration, with a transient peak at 120 µM, while a parenteral administration could give 10 times of the plasma concentration [168]. A Recommended Dietary Allowance (RDA) for vitamin C is set at 90 mg/day for adult men and 75 mg/day for adult women [172]. These doses are approved to maintain near-maximal neutrophil concentration with minimal urinary excretion [172]. The tolerable upper intake level (UL) for adults is 2000 mg/day, where a dose above this level is possibly unsafe with adverse effects including osmotic diarrhoea and gastrointestinal disturbances [173]. A risk of renal calcium oxalate stones elevation upon ingesting a high dose of vitamin C had been expressed early in the literature [174]. Healthy subjects may have an increase

in urinary oxalate excretion upon administration of high doses of vitamin C [175], although it may not be a major concern [174]. The risk is higher in calcium stone-forming patients, as they may have the risk of calcium oxalate crystallization by taking 1 to 2 g/day of vitamin C [175], [176].

- Vitamin E

  Vitamin E is a fat-soluble group of vitamin, including 8 forms, i.e. α-, β-, γ-, and δ-classes of tocopherol and tocotrienol [177]. The active form of vitamin E is α-tocopherol, which is the only form that fulfils the human requirement [178]. For its purpose, vitamin E depends on other antioxidant (e.g. vitamin C, or other reductants serving as hydrogen donors) to maintain α-tocopherol in its reduced form [179].

  As vitamin E is a fat-soluble vitamin, the bioavailability of vitamin E is dependent on the fat content of the meal. The absorption of vitamin E is similar t the absorption of other dietary lipids: solubilization by bile, further absorption by the small intestine, and finally transported to the blood via lymphatics. Therefore, vitamin E is found to be relatively poorly absorbed without food [180]. The amount of vitamin E is measured as International Unit (IU), which is commonly used to measure fat-soluble vitamins. The conversion of an IU to milligrams differs amongst vitamins. One IU of vitamin E is equivalent to 0.45 mg and 0.67 mg for synthetic and natural forms, respectively [181].

  The antioxidant function of vitamin E is based on the prevention of oxidative damage by lipid peroxidation [182]. All vitamin E forms act as lipid-soluble chain-breaking antioxidants which could prevent further autooxidation of lipids [182]. The effects of vitamin E on the reduction lipid peroxidation and its products was well-observed in healthy young and elderly volunteers [183]. In patients undergoing knee-osteoarthritis surgery, 400 IU/day for 2 months before the surgery helped to reduce oxidative stress and improve the clinical symptoms [184]. On the other hand, a randomized, double-blinded, placebo-controlled study on healthy subjects failed to obtain evidence of either a protective or deleterious effect on DNA damage, resistance of DNA to oxidant challenge, or lipid peroxidation by 400 IU/day of vitamin E [185]. Furthermore, the study did not find evidence on the synergistic interaction between vitamin C and E [185].

  The tolerable UL of vitamin E is 1000 mg/day [178]. The side effects of vitamin E is limited, even in high doses [154]. Vitamin E is commonly used for disease prevention rather than for resolving chronic diseases [154].

- Lipoic acid

  Lipoic acid is one of the biological reductors [186], [187]. As its reduced form of dihydrolipoic acid (DHLA), lipoic acid serves the function to reduce and regenerate other antioxidants from their oxidized or inactive forms [187]. Lipoic acid is involved as co-factor in the glycine cleavage system, a reversible reaction between glycine and 5,10-MTHF [188]. In addition, lipoic acid can reduce cystine to cysteine, such that it can bypass the transport system of cystine and enable cysteine to be utilized for GSH synthesis [189]. Lipoic acid had been used in the treatment of several diseases related to oxidative stress, which is well-summarized in the literature [186]. Amongst the results, the effects of lipoic acid to oxidative stress are rather inconsistent. Lipoic acid demonstrated superior function as an antioxidant in diabetic patients [190]–[192] and in symptomatic peripheral diabetic neuropathy [193]. On the other hand, lipoic acid did not to normalize MDA and total oxidant status in healthy elderly [194] and renal failure patients [195].

  The redox potential of lipoate/dihydrolipoate system is low, with E_0 = -0.29V [187]. As such, the reduction of lipoic acid to dihydrolipoic acid is rather easy. Furthermore, lipoic acid can be easily absorbed in the stomach [187]. However, studies suggested that R-lipoic acid is better used than

S-lipoic acid, as the former has better pharmacokinetic parameters and better bioavailability [196]. The no-observed-adverse-effect level (NOAEL) is considered to be 61.9 mg/kg/day [197]. However, possible hypoglycemia can be a risk for diabetic patients as high doses of lipoic acid may improve glucose utilization [198].

- Selenium

  Selenium is anticipated to increase the expression of glutathione peroxidase (GPx), an enzyme which reduces hydrogen peroxide to water and oxygen, and reduces peroxide radicals to alcohol and water. GPx is a selenoprotein, which means that it includes a selenocysteine amino acid residue. The use of selenium as a treatment had been studied in coronary artery disease case [199]. Doses of 200 and 500 µg/day of selenium for 12 weeks increased the activity of GPx 1 by more than 10%, although there was no relevant change in biomarkers of oxidative stress and inflammation [199]. The platelet, serum, and whole blood seleno-glutathione peroxidase were increased in women taking 200 µg/day of sodium selenium for 4 weeks [200]. The use of selenium supplementation in radiotherapy had been well-summarized in a review, which concluded that selenium could improve the life quality of the patients and reduce the side effects of radiotherapy without reducing the effectiveness of the radiotherapy [201]. Other positive outcomes of selenium on GPx activities were observed in chronic kidney disease patients [202], early stage chronic renal failure patients [203], patients undergoing hemodialysis [204]. However, other studies suggested that selenium has no effect in healthy individuals [205]–[207] and in end-stage chronic renal failure [203].

  The RDA and the tolerable UL of selenium is 55 µg/day and 400 µg/day, respectively. Selenium can be toxic in very high concentration. A report on several cases of selenium toxicity suggested that blood selenium concentration above 1400 µg/l is associated with acute poisoning [208].

In conclusion, we suggest to supplement with vitamin C as a boost of antioxidant action only when GSH is sufficiently supplied. Under GSH deficiency, the antioxidant function of vitamin C may not be in its fullest capacity. Lipoic acid should also be considered because it is required in the glycine cleavage system, while still functioning to restore other essential antioxidants.

5.3.3. Hypotheses Group 3: Intestinal Bacterial Dysbiosis

Patients with prolonged B12 deficiency have high risk of suffering from an imbalance of intestinal bacteria. The prolonged deficit of B12 may cause damages to DNA, subsequently to tissues constructing the intestinal lining, such that the homeostasis of bacterial population in the small and the large intestines is disrupted. In addition, as glycine is one of the major constituent of collagen, the intestinal lining damage is exacerbated by the lack of collagen. Therefore, B12 deficient patients often suffer from intestinal issues with common symptoms, such as diarrhoea, constipation, etc. If the intestinal condition is kept unresolved, the patients may develop SIBO, which is a worse condition that is hard to treat.

It would be nice to have a method to identify, or even quantify, the intestinal health of the patients apart from assessing the symptoms. The assessment of bacterial dysbiosis is often done by stool microbiome analysis. However, this method cannot differentiate bacteria residing the small or the large intestine. As a result, the imbalance between the two sites cannot be identified. As we have learned that the bacterial dysbiosis may produce several toxins, we recommend to utilize the toxins as biomarkers of the intestinal health. The toxins, as described in Hypothesis 16 and Hypothesis 17, are methanol, trimethylamine, trimethylamine-N-oxide, amongst others. Methanol can be quantified in blood, but a more rapid methanol test had been carried out using breath test with a promising result [127]. Trimethylamine and trimethylamine-N-oxide can be measured in plasma and urine using ultra-high pressure liquid chromatography (UHPLC) system [209]. In addition, urinary pH and formic acid can

also be measured to assess the excess of formaldehyde that is not completely oxidized to CO_2 and leaves the body as formic acid (formate).

The B12 Institute had successfully resolved intestinal issues in numerous patients by the B12 injection in the past. However, if left prolonged, the intestinal issues might be difficult to reverse. We thus recommend an extended treatment by including diet plan and, if necessary, antibiotic. The diet of the patient may affect the health more than expected, as food is the substrate of bacteria to produce toxins. As depicted in Figure 5.12, proteins, pectin, and other food component can be broken down by bacteria into toxins, with the end result of excessive formaldehyde. Therefore, the treatment of the patients should be accompanied by a nutritionist who can create a personalised diet plan for each patient. For example, the consumption of protein could be reduced to starve the bacteria, and pectin-containing food should be avoided. A more aggressive strategy is by using antibiotic. Antibiotics could be used when the patients have very bad and chronic symptoms, which could be a sign of SIBO. The patients might also have excessive bad bacteria in gut (e.g. *Clostridium perfringens*, *Staphylococcus*, and *Escherichia coli*, etc.), which may be identified from for example the stool microbiome analysis. An example of the antibiotic is Rifaximin, an antibiotic medication used to treat travellers' diarrhoea, irritable bowel syndrome, and hepatic encephalopathy, which can flush out all bacteria in the gut. Subsequently, more good bacteria (e.g. methanogens) might be restored by taking probiotics, and also by following a tight long-term diet plan.

5.3.4. Hypotheses Group 4: Formaldehyde Damages

Following hypotheses group 3, formaldehyde that is produced excessively by the gut bacteria will continue to exacerbate the condition of B12 deficiency. Formaldehyde does not only damage DNA, but also overwhelms the folate pool, such that glycine synthesis is inhibited and formic acid is accumulated. This condition will end up to result in acidosis, which will worsen the condition of the patients. Therefore, although currently overlooked, the existence of formaldehyde in the body should be notified and treated urgently.

Formaldehyde is very reactive such that it is a challenge to trace, identify, and quantify the level of formaldehyde in the body. We suggest indirect biomarkers such as CRP (to identify inflammation), urinary pH (to identify acidosis), and urinary formic acid (to identify the end product of formaldehyde enzymatic detoxification).

The treatment can be focused on the effort to reduce formaldehyde sources and to reverse the damages. We believe that the source of formaldehyde varies vastly, although we strongly argue that one of the major source is the bacterial degradation of food (discussed in Hypothesis 17). Therefore, to reduce the production of formaldehyde, we need to eradicate the intestinal bacterial dysbiosis. The strategy to this particular issue has been discussed in Section 5.3.3.

Another simple strategy we suggest is to supplement with glycine, due to several reasons:

- Under formaldehyde excess, glycine synthesis via the folate cycle will be halted (Hypothesis 11), therefore an exogenous source of glycine is urgently required;
- The condition of the intestines will be worsened if the lack of collagen, thus glycine, is not resolved;
- Glycine is the building block of GSH, which is needed for the enzymatic detoxification of formaldehyde.

In addition, acidosis could be treated by getting rid of formate through the kidneys by control of blood and urine pH, e.g. by administering potassium bicarbonate ($KHCO_3$) or sodium bicarbonate ($NaHCO_3$).

Reversing other damages caused by formaldehyde, such as DNA damages, is challenging and will take time. Therefore, while continue treating with vitamin B12, we can do extra efforts such as changing

the diet of the patients and supplementing with safe supplements such as glycine to accelerate the recovery of the patients.

5.3.5. Hypotheses Group 5: Oxidative Damages and Inhibition of One-Carbon Cycle

Hypotheses group 5 includes the matter of oxidative stress damages on the one-carbon cycle, such as the oxidation of folate pool and MS, as well as the inhibition of MS by alcohols and aldehydes. This group of hypotheses is rather difficult to diagnose, as the main issue is located on a cellular level. We may be able to identify the abnormal levels of folate pool and its oxidative degradation products, e.g. p-aminobenzoylglutamate and 4-α-hydroxy-5-methyltetrahydrofolate [103], to identify the oxidation of folate pool. We may not be able to directly assess the activity of MS, but we can analyse the profile of the one-carbon cycle metabolites (e.g. SAM, SAH, folate derivatives, etc.) to see any abnormalities. However, this strategy may not be able to differentiate between the inhibition of MS due to

1. B12 deficiency itself or due to
2. damages by alcohol, aldehydes, and
3. other sources of oxidation.

In summary, we have not found the best strategy to approach this particular group of hypotheses, but good enough to identify the abnormalities.

While MS is inhibited, we may be able to make use of the alternative methylation pathway (Figure 4.8) to keep the balance of homocysteine to methionine flux. Supplementation with betaine or choline could be considered.

- Betaine

  Betaine is an alternative methyl group donor for homocysteine in one-carbon cycle. The reaction between betaine and homocysteine is catalysed by BHMT and therefore is independent from MS and cobalamin. Thus, a sufficient betaine intake is expected to lower homocysteine during MS and B12 inactivity.

  A meta-analysis of five randomized controlled trials suggested that betaine could lower plasma homocysteine concentration in healthy adults [210]. The studies showed that 4 to 6 g/day of betaine supplementation lowered plasma homocysteine by 11.8% of baseline values [210]. A high dose of betaine, i.e. 6 g/day or higher, had been used as homocysteine-lowering therapy for hyperhomocysteinemia patients with inborn errors [211]. Along with folic acid supplementation, betaine may be a useful treatment for patients with the MTHFR 677C→T genetic mutation [210]. Another study had shown that betaine supplementation could inhibit the increase of homocysteine thiolactone in trained men [212].

  On the other hand, betaine could adversely affect serum lipid concentrations [211], such as increasing the levels of total and low-density lipoprotein (LDL) cholesterol. Therefore, betaine supplementation should be cautiously used in patients with the risk of high cholesterol. Other minor side effects include nausea, stomach upset, and diarrhoea. Betaine is considered safe at a dietary intake of 9 to 12 g/day [213].

- Choline

  Choline is the precursor of betaine. Therefore, it is expected to produce similar homocysteine-lowering effects to betaine. Choline is also the precursor of phosphatidylcholine, the major constituent of the myelin sheath. The proposed benefits of choline have been described in Hypothesis 20. One could expect the advantage of taking sufficient dietary choline for supporting myelin sheath regeneration. A dose of 2.6 g/day of choline (as phosphatidylcholine) had lowered

fasting and postmethionine-loading plasma total homocysteine concentrations in health men [211]. However, there is lack of study on the effects of choline in hyperhomocysteinemia patients.

In spite of the expected effects of choline or phosphatidylcholine, an excessive amount of phosphatidylcholine in the intestines could be degraded by the gut bacteria into trimethylamine. An accumulation of trimethylamine will cause trimethylaminuria, or better known as the fishy odour syndrome. Cases of trimethylaminuria had been reported in the treatment of Huntington's chorea and Alzheimer's diseases after high administration of 8 – 20 g/day of choline [214]. Therefore, the use of choline as a supplementation or treatment should not exceed the safe intake levels. The daily upper intake levels of choline are:

- 1 gram daily for children 1-8 years,
- 2 grams for children 9-13 years,
- 3 grams for children 14-18 years, and
- 3.5 grams for adults over 18 years of age.

Other side effects of high administration of choline includes gastrointestinal distress, diarrhoea, and vomiting.

Finally, we suggest the supplementation with betaine or choline for a short period of time to rapidly suppress high homocysteine. While the blocking of methionine cycle can be temporarily fixed by betaine or choline, the folate cycle remains inhibited if MS and B12 functions are not restored. A prolonged blocking of folate cycle will be harmful because the capacity of the folate pool to reduce formaldehyde levels (by oxidizing them to formic acid and CO_2) and synthesize glycine will be significantly lowered.

5.4. Conclusion

This chapter have explored and discussed a list of hypotheses regarding disorders and deficiencies related to B12 deficiency/inactivity. It could be preliminary concluded that the issues and symptoms experienced by the B12 deficient patients do not only stem from the vitamin B12 deficiency itself, but are also the manifestation of other essential metabolites, amino acids, and vitamins deficiencies. We have shown in Figure 5.1, Figure 5.2, and Figure 5.3 that the issues around B12 deficiency create several vicious cycles, where one issue exacerbates the others and vice versa. Therefore, it is important for the future B12 deficiency research direction to identify as many related issues as possible to create better strategies for the treatment of the patients. A simplified diagram of the vicious cycles is presented in Figure 5.18.

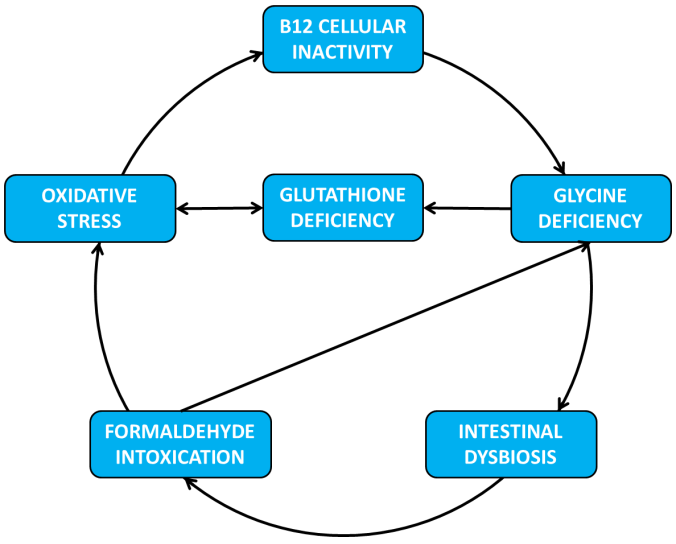


Figure 5.18 The vicious cycle of B12 deficiency.

We proposed that B12 deficiency/inactivity leads to severe additional deficiencies that are difficult to reverse in short time. Essential amino acids and metabolites, such as glycine, glutathione, and collagen, are often overlooked as key actors to determine the health status of the patients. The lack of glycine is caused by folate cycle inhibition, a manifestation of B12 cellular inactivity. Glycine deficiency leads to the failure of glutathione synthesis, the oxidation of cellular B12, B12 digestive and cellular uptakes, and finally the inactivity of methionine synthase (Figure 5.14). Glycine deficiency is also tightly related to the elevation of oxidative stress, the source of internal inflammation and damages.

Besides, when glycine is deficient, the synthesis of collagen may fail and the regeneration of the intestinal lining may be disrupted, such that the intestinal health is compromised. The intestinal issues lead to the production of toxins from bacterial degradation of food in the small intestines. These toxins include methanol, trimethylamine, and formaldehyde, amongst others.

The excessive production of formaldehyde is extremely harmful for the human body, as this notorious substance causes inflammation, DNA damages, autoimmune responses, and further elevation of oxidative stress. In this case, treating the patients with high dose of B12 injections will take a long period of time, because we need to reverse many disorders in the body.

To improve the health of B12 deficient patients more effectively, we recommend several supportive supplementations which may accelerate the recovery of the patients. We summarize the hypotheses groups, biomarkers, and treatment suggestions in Table 5.3. Although we found the safe dosages of the supplements from literatures (see Section 5.3), we have yet to determine the appropriate dosage for the targeted function of each suggested supplement. Therefore, a study on the metabolic flux and material balance of the related compounds is interesting to be pursued in the future research studies.

Table 5.3 Summary of hypotheses groups, biomarkers, and treatment suggestions.

GROUP	PROPOSED ISSUES	BIOMARKERS	SAMPLES	TREATMENTS
1	Glycine deficiency	Glycine	Blood, urine	Glycine
		5-L-oxoproline	Urine	
2	Oxidative stress and low GSH	GSH:GSSG	Blood	NAC + glycine
		CRP	Blood	Vitamin C
		MDA	Blood	Lipoic acid
3	Intestinal Bacterial dysbiosis	Methanol	Breath	Diet plan
		Trimethylamine	Plasma, urine	Antibiotics
		Trimethylamine-oxide	Plasma, urine	Probiotics
		Formate	Urine	
		Urinary pH	Urine	
4	Formaldehyde damages	CRP	Blood	Glycine + NAC
		Formate	Urine	$KHCO_3$
		Urinary pH	Urine	$NaHCO_3$
5	One-carbon cycle disruption	SAM:SAH	Blood	Betaine
		Folate derivatives	Blood	Choline

6 Conclusions and Recommendation

We have found several strong hypotheses which may explain the unresolved bad condition of the B12 deficient patients, which are included in various vicious cycles leading to a locked-in state of quite some metabolic pathways.

- Glutathione is a key metabolite to activate cellular B12

Without glutathione, the reduction by MMACHC will not happen and the exogenous B12 cannot be utilized in cells. Low glutathione levels need to be corrected to activate B12.

- B12 deficiency leads to folate cycle block, which causes glycine deficiency

A prolonged inactivity of cellular B12 and/or the overloading of the folate cycle by external formaldehyde oxidative stress leads to the inhibition of glycine synthesis in the folate cycle. This issue is very serious and it is often overlooked. Glycine deficiency may cause:

  - Glutathione synthesis failure, leading to oxidative stress and B12 cellular activation failure;
  - Collagen synthesis failure, leading to intestinal issues;
  - Possibly proteins and enzymes synthesis failure (e.g. intrinsic factor and MMACHC), leading to B12 digestive uptake and cellular activation failure;
  - Glycocholic synthesis deficiency, leading to malabsorption of fats in the intestines, and to bacterial overgrowth (bile is bactericidal).

The level of glycine can be measured from urine or blood. Glycine deficiency is relatively easy to correct by supplementation with glycine. Glycine is safe to consume, with a safe dose at least 60 g/day reported in the literature [150]. However, as glycine is required for the synthesis of many proteins and metabolites, the appropriate dosage for the treatment should be studied further.

- Glutathione deficiency must be corrected to eliminate oxidative stress

Glutathione deficiency leads to the elevation of oxidative stress. The impacts of oxidative stress are at least two folds:

1) the oxidative damaging effects of the oxidant to cells, tissues, and organs, and
2) the overwhelming of glutathione as the major antioxidant in the body (a vicious cycle).

Thus, the levels of glutathione (i.e. the reduced and oxidized glutathione) is a good biomarker for indicating oxidative stress. Supporting the treatment with extra NAC and glycine, as well as antioxidants such as vitamin C may help to accelerate the recovery of the patients. NAC should not be given alone, because under glycine deficiency, NAC supplementation could cause an elevation in 5-L-oxoproline, which could lead to acidosis and manifest in side effects.

- Intestinal bacterial dysbiosis produces toxins which contribute to oxidative stress

The patients with a prolonged cobalamin deficiency may also suffer from an intestinal bacterial dysbiosis due to a damaged intestinal lining. The intestinal lining may be damaged due to two major issues:

1) DNA and tissue damage due to B12 deficiency, and
2) collagen deficit due to glycine deficiency.

Bacteria in the small intestine can intervene with the uptake of cobalamin, as well as degrade food into toxins and ROS such as ammonia, H_2O_2, and formaldehyde, contributing to the elevate oxidative stress. We propose that the production of toxins and ROS to be used as biomarkers of the intestinal health.

The dysbiosis of the gut bacteria is a relatively difficult issue to reverse, as we need to ensure the repair of the intestines. Therefore, we suggest to seek for personalized diet plans for the patients to help reducing the toxin production in the guts, for example reducing the consumption of protein and pectin-containing foods. By this strategy, we expect to starve and subsequently kill the bad bacteria colonies and restore the homeostasis. Antibiotics and probiotics can also be considered for more severe cases.

- Formaldehyde is the most dangerous toxin produced by intestinal bacterial dysbiosis

The most dangerous consequence of the bacterial dysbiosis is the excessive production of formaldehyde. Apart from being extremely damaging to cells and tissues, formaldehyde gives direct impacts to cobalamin metabolism.

  - Formaldehyde can methylate the pool of tetrahydrofolate, thus blocking the synthesis of glycine.
  - Formaldehyde in the body is detoxified into formic acid by glutathione and dehydrogenase enzymes linked to the folate cycle. Hence, it is important to keep a sufficient level of reduced glutathione to decrease the level of oxidative stress by formaldehyde.
  - The of formic acid from the folate mediated oxidation of formaldehyde may cause acidosis. Therefore, the production of (endogenous) formaldehyde is not desired under any circumstances.

The excess of formaldehyde and/or acidosis can be well measured by urinary pH and urinary formate analysis. In case of acidosis, the administration of $KHCO_3$ or $NaHCO_3$ could help to get rid of high formic acid in kidneys.

- Oxidative stress and toxins inhibit the activity of methionine synthase

Lastly, we hypothesize that there is an inhibition in the activity of methionine synthase by oxidative stress and other toxic chemicals such as alcohols (forms aldehydes) and aldehydes (due to consumption and/or production by the gut bacteria). We have not yet found a good strategy to identify this issue other than by measuring the levels of the one-carbon cycle metabolites (e.g. SAM:SAH and folate pools) as the indicators of MS activity. If the homocysteine level is very high, indicating the inactivity of B12 and/or MS, we can restore the methylation function by giving combinations of betaine and choline. These compounds can be a temporary fix to methylation failure through the pathway using betaine-homocysteine methyltransferase. However, the main issues with B12 and MS inactivity and the external formaldehyde overload should be treated first to resolve the folate cycle inhibition.

Recommendations

As the follow up of this project, we recommend:

1. Research studies to obtain the proof-of-concept of the hypotheses groups are recommended, especially for the strong hypotheses such as glycine and glutathione deficiency, as well as oxidative stress.
2. Further study of the relations between the proposed hypotheses to the B12 deficiency common symptoms (see Appendix A). For example, one could argue that the patients headache and/or migraine could be partially caused by toxins production, i.e. methanol, from the intestinal issues. It would be a challenge to disclose the roots of the symptoms, especially the general ones (e.g. fatigue, headache, infections), but we expect it to be extremely helpful for the diagnosis and treatment.
3. A study on the metabolic fluxes and the requirements of essential metabolites (e.g. NAC, glycine) for better treatment strategies.

At last, we offer a design of a mini study for new biomarkers in the next additional chapter. Towards the end of this project, B12 Institute has started the study with some selected patients. Within the limited time left, we present the methodology and expect some preliminary results from the mini study.

7 Design of a Mini Study for New Biomarkers

This additional chapter describes a design of a mini study to investigate the feasibility of new biomarkers to assess the B12 cellular activity. The new biomarkers are selected to focus on the assessment of oxidative stress and glutathione issues, as well as on the monitoring of B12 cellular function. The mini study is divided into 2 phases: 1) the diagnostic study phase, and 2) the comparison study phase. We chose the most feasible biomarkers which are readily available in the Netherlands.

7.1. Biomarkers and Phases of the Mini Study

As discussed in Chapter 5, B12 deficiency leads to other issues which deteriorate the health of the patients. Some highlighted issues are oxidative stress, glutathione deficiency, and glycine deficiency, which have huge impacts on the activity of cellular B12. Therefore, this section offers the strategy to use new biomarkers to identify and diagnose the issues.

To measure the activity of cellular B12, the methylation metabolites can be a useful group of biomarkers. As proposed in Section 5.3.5, the profile of the metabolites can inform us about the status of the one-carbon cycle, thus the activity of the cellular B12. World Health Laboratories (WHL), a private analytical laboratory situated in Bunnik, The Netherlands, offers an analytical package to measure the complete methylation metabolites, namely the Methylation Panel Plus. The sample report of the Methylation Panel Plus, which includes the list of metabolites and the reference ranges, is presented in Figure 7.1. The Methylation Panel Plus also includes the measurement of reduced and oxidized glutathione, the indicators of antioxidant levels. Theoretically, the ratio of reduced/oxidized GSH would be lowered under a high oxidative stress. In addition, the level of reduced GSH may indicate the activity of cellular B12, as the activation of B12 requires reduced GSH.

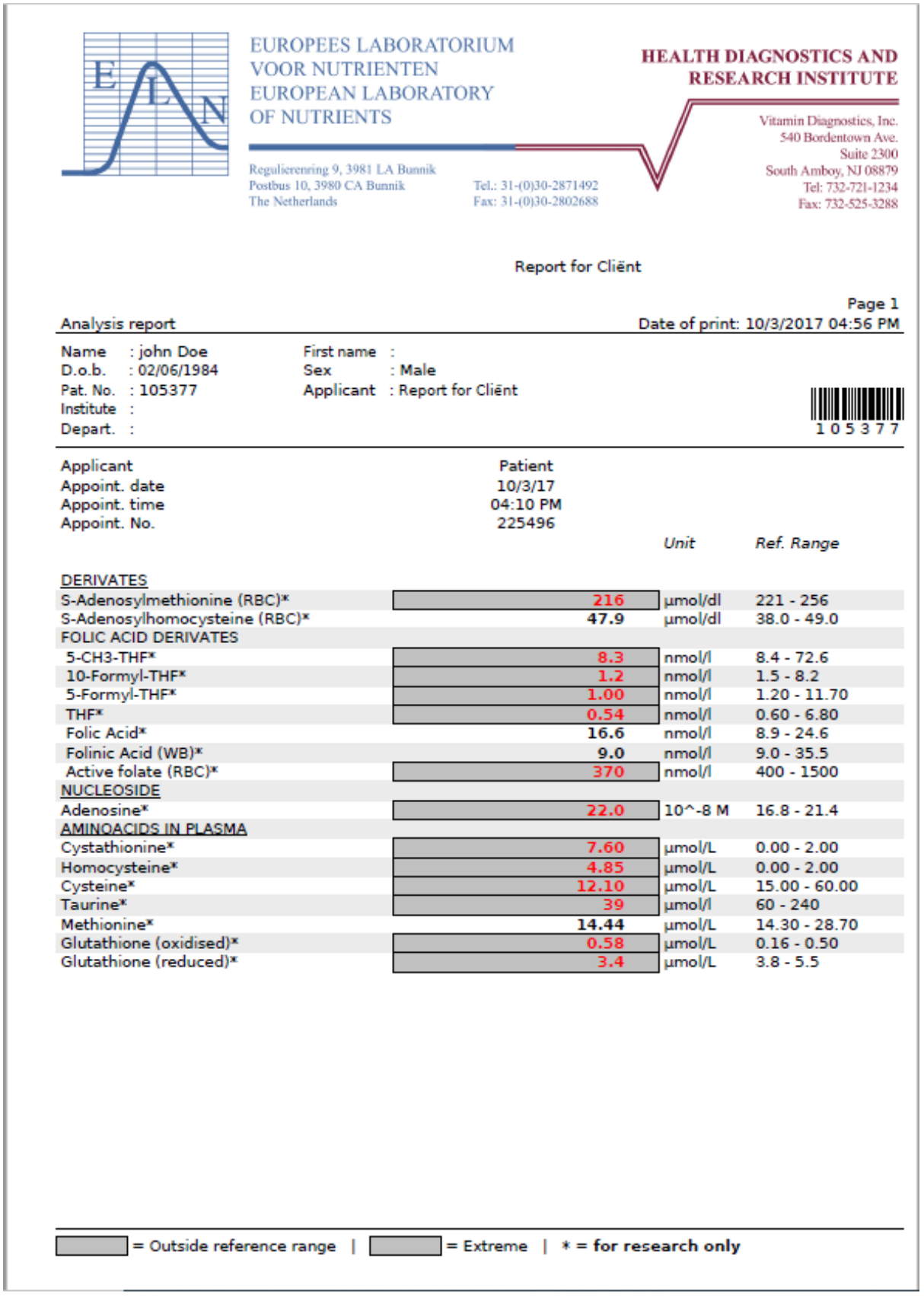
To assess the oxidative stress status, it would be valuable to measure CRP and MDA levels. CRP is a protein produced by the liver which is sent to the bloodstream in response to inflammation. Thus, CRP test is used to measure the level of inflammation in the body, which would be elevated in case of formaldehyde accumulation. MDA is the product of polyunsaturated fatty acids peroxidation in the cell, thus is a commonly used biomarker of oxidative stress [137]. In addition, urinary pH and urinary formate can also indicate acidosis, one of the manifestation of excessive formaldehyde. Urinary pH can be measured by a simple acid-base titration method, or simply using commercial pH test strips. However, we have not yet found any laboratory which could measure the urinary formate.

To measure the glycine level, we can refer to the complete amino acids measurement package offered by WHL. In addition, the level of urinary 5-L-oxoproline may be useful to indicate the lack of glycine (as discussed in Hypothesis 4), although currently we are not sure which laboratory in the Netherlands offers 5-L-oxoproline measurement.

The summarized list of biomarkers are provided in Table 7.1.

Table 7.1 Recommended new biomarkers of B12 deficiency.

BIOMARKERS	LOCATION	SAMPLE	PRICE (EURO)
METHYLATION PANEL PLUS	World Health Lab	Blood	400
C-REACTIVE PROTEIN (CRP)	Erasmus MC, Rotterdam	Blood	
MALONDIALDEHYDE (MDA)	World Health Lab	Urine	20
URINARY PH	N/A	Urine	N/A
FORMATE	N/A	Urine	N/A
AMINO ACIDS	World Health Lab	Urine	150
5-L-OXOPROLINE	N/A	Urine	N/A



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Report for Cliënt

Page 1

Analysis report — Date of print: 10/3/2017 04:56 PM

Name : john Doe
D.o.b. : 02/06/1984
Pat. No. : 105377
Institute :
Depart. :
First name :
Sex : Male
Applicant : Report for Cliënt

105377

	Patient		
Applicant			
Appoint. date	10/3/17		
Appoint. time	04:10 PM		
Appoint. No.	225496		
		Unit	*Ref. Range*
DERIVATES			
S-Adenosylmethionine (RBC)*	216	µmol/dl	221 - 256
S-Adenosylhomocysteine (RBC)*	47.9	µmol/dl	38.0 - 49.0
FOLIC ACID DERIVATES			
5-CH3-THF*	8.3	nmol/l	8.4 - 72.6
10-Formyl-THF*	1.2	nmol/l	1.5 - 8.2
5-Formyl-THF*	1.00	nmol/l	1.20 - 11.70
THF*	0.54	nmol/l	0.60 - 6.80
Folic Acid*	16.6	nmol/l	8.9 - 24.6
Folinic Acid (WB)*	9.0	nmol/l	9.0 - 35.5
Active folate (RBC)*	370	nmol/l	400 - 1500
NUCLEOSIDE			
Adenosine*	22.0	10^-8 M	16.8 - 21.4
AMINOACIDS IN PLASMA			
Cystathionine*	7.60	µmol/L	0.00 - 2.00
Homocysteine*	4.85	µmol/L	0.00 - 2.00
Cysteine*	12.10	µmol/L	15.00 - 60.00
Taurine*	39	µmol/l	60 - 240
Methionine*	14.44	µmol/L	14.30 - 28.70
Glutathione (oxidised)*	0.58	µmol/L	0.16 - 0.50
Glutathione (reduced)*	3.4	µmol/L	3.8 - 5.5

= Outside reference range | = Extreme | * = for research only

Figure 7.1 Sample report of Methylation Panel Plus by World Health Laboratories.

We aim to execute the mini study towards the end of this project. Due to the limitation of time and budget, we chose the study using the Methylation Panel Plus offered by WHL. We chose this group of biomarkers because at this stage, we expect it to provide a sufficient additional information about the B12 cellular status in various groups of patients.

7.1.1. Phase I: Diagnostic Study

Phase I is aimed to find evidence to the hypothesis that patients who suffer from severe and unresolved symptoms even after B12 treatment are suffering from an elevated oxidative stress and a low glutathione, thus an inactive B12 cellular function. Thus, phase I only targets a small group of patients (i.e. up to 5 patients) with severe symptoms, with the objective to diagnose their condition using the new biomarkers. The inclusion criteria for further classification of patients is as follows:

- Patients with severe symptoms who have not received any treatment yet;
- Patients with severe symptoms who received/are receiving oral treatment;
- Patients with severe symptoms who are receiving a maintenance dose.

We aim to focus on the patients with undetermined root causes of B12 deficiency/inactivity, therefore patients with genetic disorders (e.g. MTHFR polymorphism, CBS mutation, *cbl* complementation groups, etc.) are supposedly excluded in this mini study. However, the B12 Institute does not have a routine of gene sequencing to identify such genetic disorders. Therefore, the early diagnostic study may unknowingly include patients with genetic disorders. The process scheme of phase I is presented in Figure 7.2.

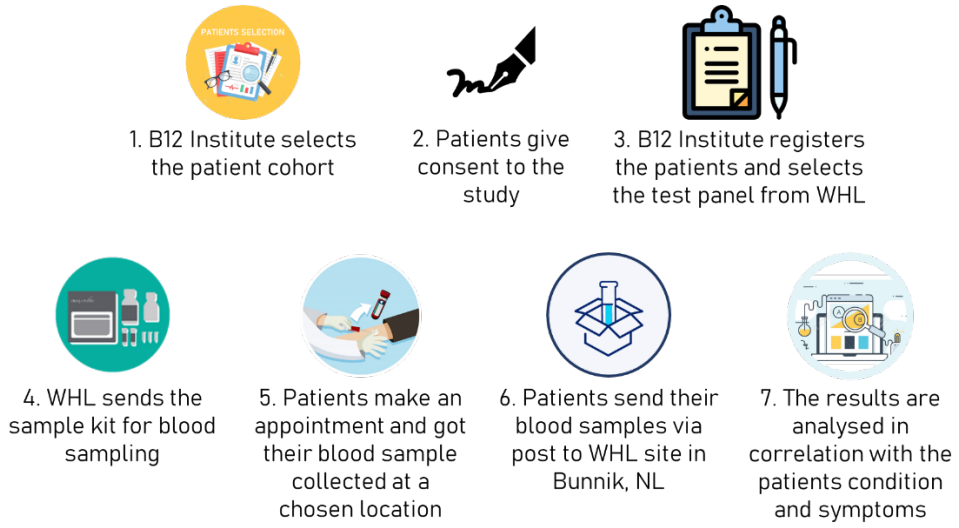

Figure 7.2 Phase I: Diagnostic Study process scheme.

7.1.2. Phase II: Comparison Study

Phase II is aimed for a larger group of patients with distinctive conditions. In this phase, the new biomarkers are expected to distinguish and quantify different conditions of patients with and without severe symptoms. As the study involves subjects with mild conditions (relieved symptoms), the study of phase II would need a review and an approval from an accredited Medical Research Ethics Committees (MRECs) or the Central Committee on Research Involving Human Subjects (CCMO). The patients are divided into four groups:

- Patients with relieved symptoms who are receiving maintenance dose;
- Patients with severe symptoms who have not received any treatment yet;
- Patients with severe symptoms who received/are receiving oral treatment;
- Patients with severe symptoms who are receiving maintenance dose.

The process scheme of phase II is presented in Figure 7.3.

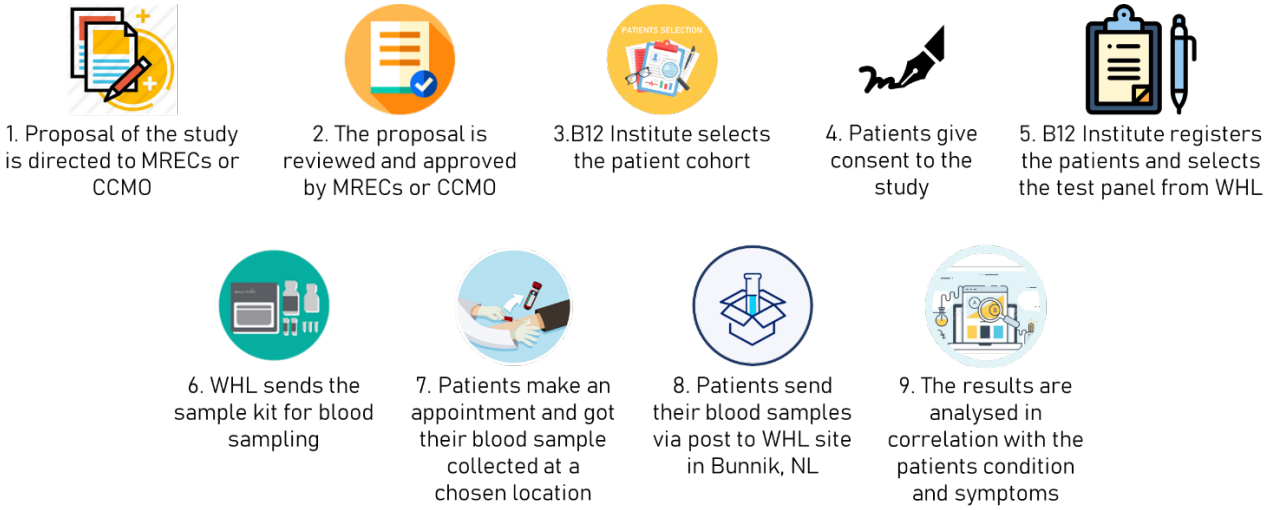

Figure 7.3 Phase II: Comparison Study process scheme.

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Appendix A

List of B12 Deficiency Symptoms

General

1 Fatigue (without cause, when waking up, no energy, exhausted)
2 Flu symptoms, feeling hot or feverish
3 Susceptible to infections (cavity infections (sinusitis), cystitis etc.)
4 Headache and/or migraine
5 Hair loss
6 Blurred/impaired vision/eye problems

Thinking and learning ability, intellectual capacity

7 Blurry in the head/"brain-fog".
8 Memory problems, forgetful,
9 Dementia-like complaints (Moca test)
10 Concentration problems
11 Aphasia (difficulty in coming up with words)

Neurological complaints

12 Tingling in the hands and/or feet,
13 Abnormal reflexes
14 Insensitive spots on the skin
15 Sleeping, numbness in the arms/legs
16 Walking uncertainly/uncoordinated, clumsy (ataxia)
17 Sleeping problems
18 Decreased smell and/or taste
19 Hearing problems (ringing in the ears (tinnitus), deaf)

Psychological complaints

20 Depressed (mild to severe), suicidal
21 Rapidly irritated, emotionally unstable,
22 Anxiety or panic attacks
23 Manic behaviour, psychosis
24 Confusion

Personality changes

25 Mood swings, tantrums
26 Autistic traits (somewhat withdrawn, may have few stimuli)
27 Apathetic, flat response
28 Alienating feeling (e.g. “is this my hand?”)

Muscle complaints

29 Signs of a stroke and/or paralysis symptoms (legs, arms, face)
30 Muscle weakness and loss of strength (from weakness/acidification to lowering through the legs)
31 Muscle pain and/or muscle cramps
32 Stiff and/or rigid movement pattern
33 Tremor and/or tics
34 Restless legs (unable to keep legs still)

Heart and lung complaints

35 Palpitations, irregular heartbeat (arrhythmias)
36 Shortness of breath, especially with exertion
37 Cold hands/feet

Urogenital system (urinary and reproductive system)

38 Menstrual problems (irregular and/or heavy blood loss and/or painful)
39 Problems with fertility (then or now)
40 Miscarriages and/or preterm births
41 Frequent urination (day/night) and/or incontinent

Digestive tract

42 Irregular bowel movements and/or diarrhoea and/or constipation
43 Poor appetite, nauseous
44 Abdominal pain/bloating
45 Ulcers, rapid bleeding gums
46 Sore tongue and/or mouth,

Skin complaints

47 Vitiligo (whitish patches on the skin)
48 Painful surface skin
49 Constitutional eczema

Appendix B

One Carbon Cycle and Transsulphuration Pathway

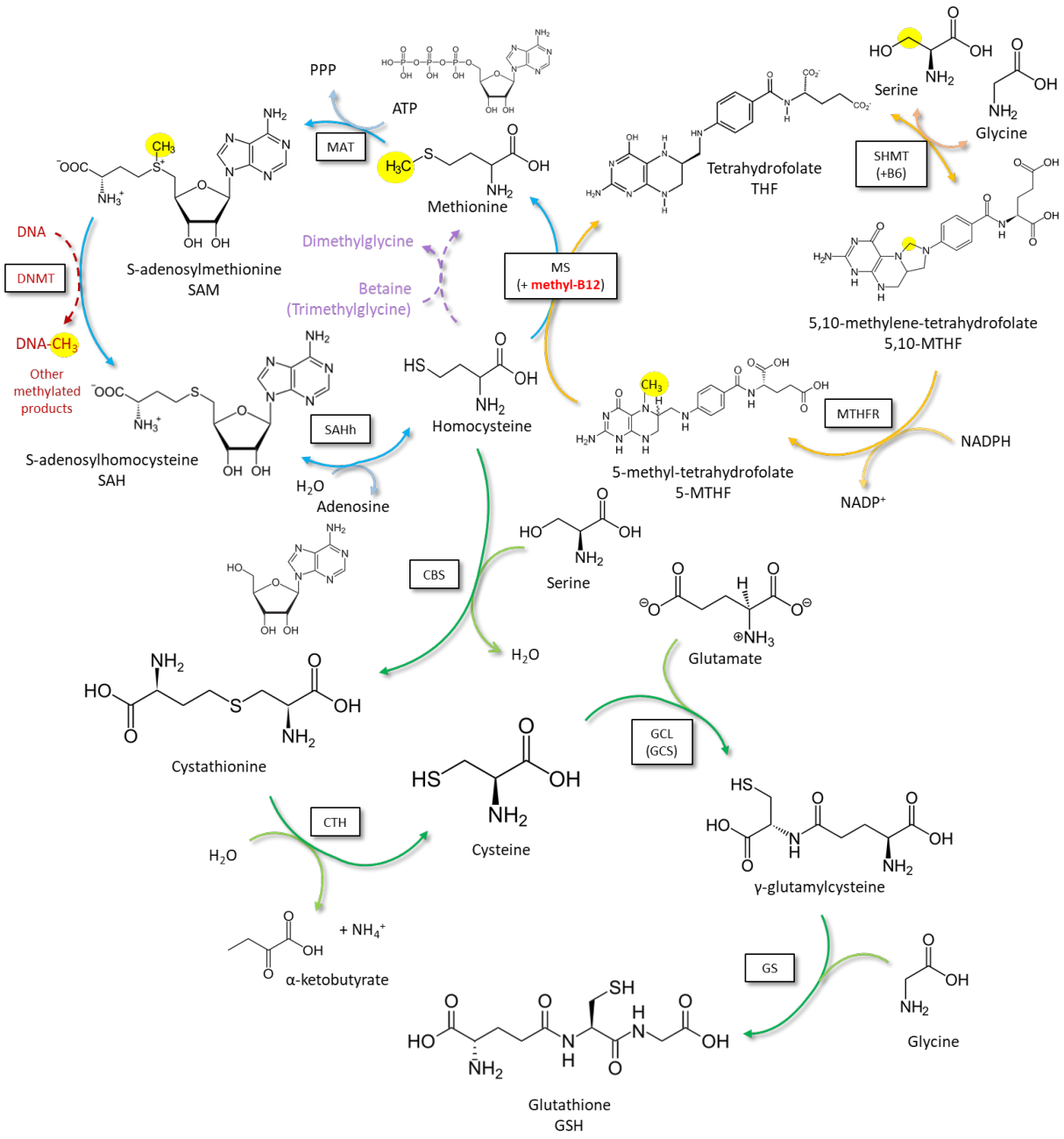

Figure A.1 One-carbon cycle and transsulphuration pathway with chemical structures of metabolites. Blue arrow is methionine cycle; Orange arrow is folate cycle; Green arrow is transsulphuration pathway; Purple dashed arrow is alternative pathway of homocysteine methylation; Red dashed arrow is methylation. Yellow circles highlight the transfer of methyl group. Enzymes are in boxes: MS = methionine synthase; MAT = methionine adenosyl transferase; DNMT = DNA methyltransferase; SAHh = SAH hydrolase; MTHFR = methylenetetrahydrofolate reductase; SHMT = serine hydroxymethyltranferase; CBS = cystathionine beta synthase; CTH = cystathionine γ-lyase; GCL(GCS) = glutamate-cysteine ligase (γ-glutamylcysteine synthetase); GS = glutathione synthase.

DNA Monomer Synthesis from Folate

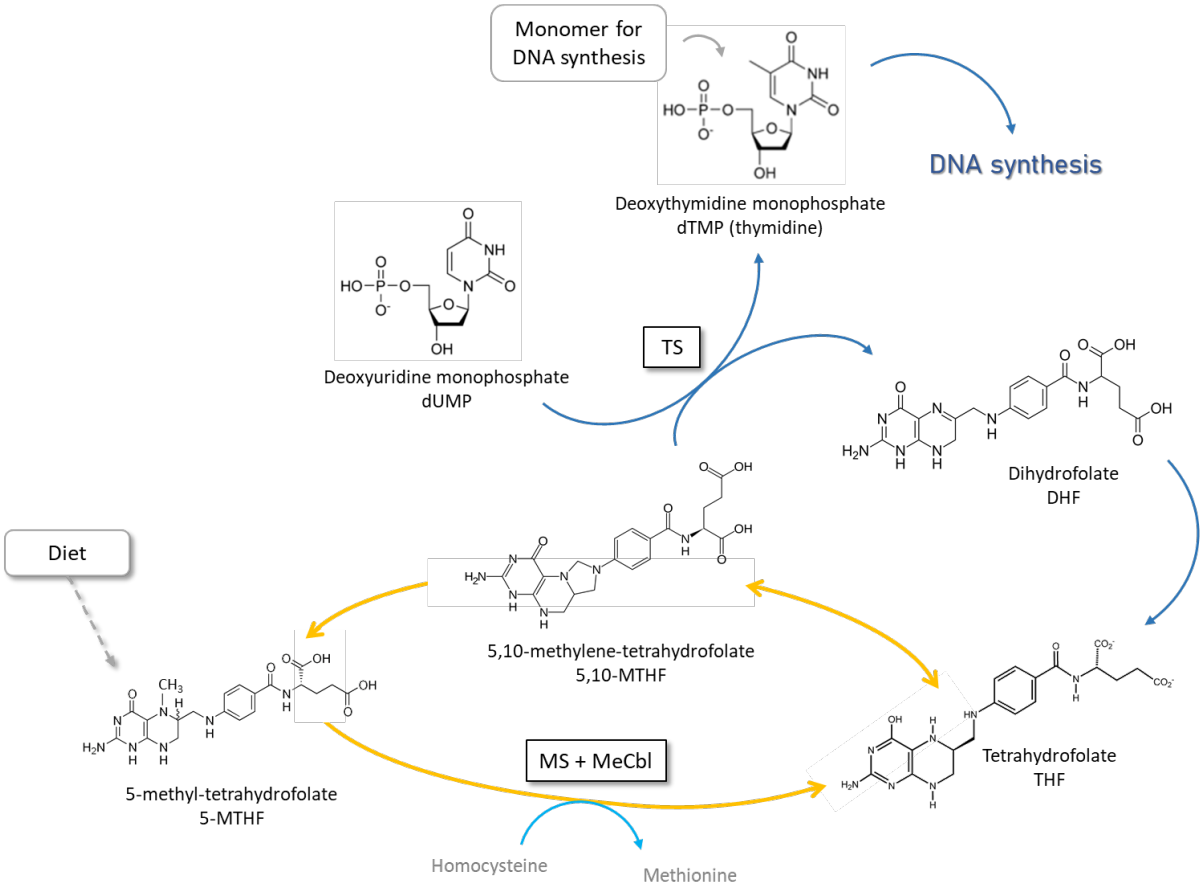

Figure A.2 Folate cycle mediates biosynthesis of thymidine, a monomer for DNA synthesis. TS = thymidylate synthase.

Appendix C

Biomarkers Selection Using Selection Tables

Earlier in the project, we tried to select feasible and valuable biomarkers using selection tables. However, we have not yet found the proper use of the tables and have not drawn a conclusive conclusion from the selection. Therefore, we put the tables in the appendix for reference, if needed.

The biomarkers selection is carried out using a Pugh matrix. For that purpose, a series of criteria and scores is generated. The selection criteria are classified into three categories: usefulness, accessibility, and easiness. The criteria for biomarkers selection are described as follows:

- Specificity to B12 issue : Is the biomarker specific only for B12 lowered activity issue?
- Sensitivity to B12 issue : Does the biomarker involve directly, indirectly, or separately from B12 metabolism?
- Supplementation : Can the deviation be corrected by supplementation?
- Analytical technique : Is the analytical technique to quantify the biomarker conventional or advanced?
- Commercial clinical test : Is there clinical test available for the biomarker?
- Commercial test kit : Is there commercial test kit available for purchase?
- Sample type : Sample preference: Breath > Urine > Blood > Cell
- Standard level reference : Is there standard for the concentration level of the biomarker?
- Stability of sample : Is the biomarker stable during extraction, preparation, and quantification?

Each categories carries different weights to fulfil the purpose of marking. Usefulness carries the highest weight as it is the most important category, followed by accessibility and easiness. Table C.1 presents the criteria and the corresponding scores. The Pugh matrices to select biomarkers for cobalamin metabolism and oxidative stress are given in Table C.2 and Table C.3, respectively.

Table C.1 Marking of criteria for biomarker selection.

Usefulness (weight = 5.0)					
	Specific to B12 issue		*Sensitive to B12 issue*		*Supplementation*
+1	Yes	**+2**	Metabolite	**+1**	Correctable
0	No	**+1**	By-product	**0**	Not correctable
		0	Not related		
Accessibility (weight = 3.5)					
	Techniques availability		*Commercial clinical test*		*Commercial test kits*
+1	Conventional	**+2**	Hospital labs	**+1**	Exist
0	Advanced	**+1**	Private labs	**0**	None
		0	None		
Easiness (weight = 1.5)					
	Sample Type		*Standard level*		*Stability of sample*
+3	Breathe	**+1**	Exist	**0**	Stable
+2	Urine	**0**	None	**-1**	Degrade easy
+1	Serum/plasma				
0	Cell/tissue				

Table C.2 Cobalamin metabolism biomarker selection using Pugh matrix. Average score = 7.33.

Criteria	Weight	5-MTHF	THF	5,10-MTHF	MS	SAM	SAH	Met	PC
Specificity	1.7	0	0	0	1	0	0	0	0
Sensitivity	1.7	2	2	2	2	2	2	2	1
Supplementation	1.7	0	0	0	0	0	0	1	0
Techniques	1.2	1	1	1	0	1	1	1	1
Clinical test	1.2	1	1	0	0	2	2	1	0
Test kit	1.1	1	1	0	0	1	1	1	1
Sample type	0.5	1	1	1	0	2	2	1	1
Standard level	0.5	0	0	0	0	1	1	1	0
Stability	0.4	-1	-1	-1	0	0	0	0	-1
Total		**7.0**	**7.0**	**4.7**	**5.1**	**9.6**	**9.6**	**9.6**	**4.1**

Criteria	Weight	$^{13}CO_2$	Gly	Ser	GSH	GSSG	Cys	Glu
Specificity	1.7	1	0	0	0	0	0	0
Sensitivity	1.7	2	1	1	2	1	1	1
Supplementation	1.7	0	1	1	1	0	1	0
Techniques	1.2	1	1	1	1	1	1	1
Clinical test	1.2	0	1	1	1	1	1	1
Test kit	1.1	0	1	1	1	1	1	1
Sample type	0.5	3	2	2	1	1	2	2
Standard level	0.5	1	1	1	1	0	0	0
Stability	0.4	0	0	0	-1	0	0	-1
Total		**8.3**	**8.4**	**7.9**	**9.2**	**5.7**	**7.9**	**5.8**

Table C.3 Oxidative stress biomarkers selection using Pugh matrix. Average score = 3.71.

Criteria	Weight	TMA	TMAO	DMA	CH_3NH_2	NH_3	pABG	TOS
Specificity	1.7	0	0	0	0	0	0	0
Sensitivity	1.7	0	0	0	0	0	1	0
Supplementation	1.7	0	0	0	0	0	0	1
Techniques	1.2	1	1	1	1	1	1	1
Clinical test	1.2	0	1	0	0	2	1	0
Test kit	1.1	0	0	0	0	1	0	1
Sample type	0.5	2	2	2	2	2	2	1
Standard level	0.5	0	0	0	0	1	0	0
Stability	0.4	-1	0	0	0	0	0	0
Total		**1.8**	**3.4**	**2.2**	**2.2**	**5.1**	**5.1**	**4.5**

Criteria	Weight	HCOH	MeOH	EtOH	MeCHO	$HCOO^-$	CRP	AA
Specificity	1.7	0	0	0	0	0	0	0
Sensitivity	1.7	1	0	0	0	**0**	0	0
Supplementation	1.7	0	0	0	0	0	0	1
Techniques	1.2	1	1	1	1	1	1	1
Clinical test	1.2	0	0	0	0	0	2	2
Test kit	1.1	0	0	0	0	0	1	1
Sample type	0.5	2	3	3	3	2	1	1
Standard level	0.5	0	0	0	0	0	1	1
Stability	0.4	-1	0	0	0	0	0	-1
Total		**4.6**	**2.7**	**2.7**	**2.7**	**2.2**	**5.7**	**7.0**