Running title: Vitamin B_{12}

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Abbreviations

AD, Alzheimer's disease

AdoCbl, adenosylcobalamin

ApoE, apolipoprotein E

B_{12}, cobalamin

CDR, clinical dementia rating

DMB, dimethylbenzimidazole

DTI, diffusion tensor imaging

HC, haptocorrin

HoloHC, holohaptorcorrin (HC-cobalamin)

HoloTC, holotranscobalamin (TC-cobalamin)

IF, intrinsic factor

MCI, Mild Cognitive Impairment

MMA, methylmalonic acid

MMSE, mini-mental state examination

RDA, recommended daily allowance

SAH, S-adenosylhomocysteine

SAM, S-adenosylmethionine

SPECT, single-photon computed tomography

TC, transcobalamin

tHcy, plasma total homocysteine

Abstract

The biosynthesis of B_{12}, involving up to 30 different enzyme-mediated steps, only occurs in bacteria. Thus, most eukaryotes require an external source of B_{12}, and yet the vitamin appears to have only two functions in eukaryotes: as a cofactor for the enzymes methionine synthase and methylmalonylCoA mutase. These two functions are crucial for normal health in humans, and in particular, the formation of methionine is essential for providing methyl groups for over one hundred methylation processes. Interference with the methionine synthase reaction not only depletes the body of methyl groups but also leads to the accumulation of homocysteine, a risk factor for many diseases. The syndrome pernicious anemia, characterised by lack of intrinsic factor, leads to a severe, sometimes fatal form of B_{12} deficiency. However, there is no sharp cut-off for B_{12} deficiency; rather, there is a continuous inverse relationship between serum B_{12} and a variety of undesirable outcomes, including neural tube defects, stroke and dementia. The brain is particularly vulnerable; in children, inadequate B_{12} stunts brain and intellectual development. Suboptimal B_{12} status (serum B_{12} < 300 pmol/L) is very common, occurring in 30-60% of the population, in particular in pregnant women and in less-developed countries. Thus, many tens of millions of people in the world may suffer harm from having a poor B_{12} status. Public health steps are urgently needed to correct this inadequacy.

1. INTRODUCTION

This Chapter will review a selection of topics of current interest in the biochemistry, function and clinical relevance of vitamin B_{12} (B_{12}). We have chosen topics that the authors are familiar with and regret that a more extensive survey was not possible for space reasons. Nevertheless, we hope that the reader will find material of interest and will be provoked by our recommendations for clinical practice. We believe that B_{12} is a neglected area of public health and that more attention needs to be placed upon this water-soluble vitamin.

2. BIOCHEMISTRY OF VITAMIN B_{12}

2.1 Chemistry

A brief history of the discovery of B_{12} helps to explain the nomenclature associated with this remarkable molecule. The compound was first identified as a nutrient, or extrinsic factor, through the research efforts of Minot, Murphy and Whipple in the 1920's, who demonstrated that the symptoms of pernicious anemia could be overcome through the addition of liver to the diet (Minot & Murphy, 1926; Whipple & Robscheit-Robbins, 1925). The nutrient was eventually isolated from liver samples after addition of cyanide and extraction into organic solvent, from which it was crystallized to reveal the presence of a deep red pigment (Rickes et al., 1948; Smith, 1948). The structure of the compound was solved through the pioneering work of Dorothy Hodgkin by the application of X-ray crystallography, revealing that the vitamin was a cyanolated, cobalt-containing, amidated tetrapyrrole (Hodgkin et al., 1956). Consequently, it was called cyanocobalamin and, therefore, technically, B_{12} specifically refers to cyanocobalamin.

[Figure 1 near here]

The structure of B_{12} is shown in Figure 1. The cobalt is located in the centre of a ring-contracted modified tetrapyrrole macrocycle, coordinated via the four pyrrole nitrogen atoms. This core of the molecule is known as a corrin ring and is similar to, though quite distinct from, the tetrapyrrole-derived ring systems found in hemes and chlorophylls. Attached to the corrin ring, through a side chain linked to ring D of the macrocycle, is a lower nucleotide loop that contains an unusual base called dimethylbenzimidazole (DMB), which also coordinates to the cobalt ion. In B_{12}, therefore, the cobalt ion is ligated not only through the four pyrrole ring nitrogens of the corrin ring, but also through upper (beta) and lower (alpha) ligands. The upper ligand in B_{12} is the cyano group, whereas the lower ligand is the nitrogen from the DMB. In

biological systems, the upper cyano ligand is replaced by either an adenosyl group, to give (AdoCbl), a methyl group, to give methylcobalamin, or water to give hydroxo or aquacobalamin. Similarly, some biological systems utilise a different lower base to which the cobalt is attached (Gruber et al., 2011). In these systems the DMB is replaced with bases such as adenine, substituted benzimidazoles that include hydroxy or methoxybenzimidazole, or phenolic compounds encompassing phenol or cresol (Hazra et al., 2013).

These variations in the structure of the molecule give rise to a great deal of confusion in terms of nomenclature ("Nomeclature of corrinoids ", 1976). Technically, B_{12} refers only to cyanocobalamin, but the term B_{12} is used ubiquitously to refer to the different forms of cobalamin, such as methyl or AdoCbl and they are also referred to as complete corrinoids. The variants that have a different lower ligand are referred to as cobalamin analogues, or are called by trivial names. This review relates largely to cobalamin (the complete corrinoids) and hence throughout the text we will use the term B_{12} loosely to refer to cobalamin.

2.2 B_{12} as a coenzyme

As a nutrient, B_{12} acts as either a cofactor or coenzyme in a number of essential biochemical processes (Banerjee & Ragsdale, 2003; Gruber et al., 2011; Krautler, 2005). The biochemistry of B_{12} revolves around the chemistry that is mediated via the central cobalt ion, and especially the ability of the cobalt ion to form direct cobalt-carbon bonds. The chemistry reflects cobalt's ability to switch between different oxidation states, Co(I), Co(II) and Co(III), although as the oxidation state of cobalt changes, so the coordination of the metal changes from 4 to 6, respectively. Of these oxidation states, Co(I) is the most unstable and acts as a super-nucleophile. The corrin ring appears to be optimally constructed to help stabilise this form (Widner et al., 2016). Cobalamin-dependent enzymes fall into four broad classes that involve methylation, isomerisation, reductive dehalogenation and radical S-adenosylmethionine (SAM) processes (Bridwell-Rabb & Drennan, 2017; Vey & Drennan, 2011). All these B_{12}-mediated reactions are facilitated by the ability of the cobalt ion to change its state of oxidation.

Cobalamin-dependent methylation reactions. These reactions involve the transfer of methyl group from methylcobalamin to an acceptor molecule. This is best exemplified with methionine synthase, where methylcobalamin methylates homocysteine to give methionine. The cobalamin is then recharged through methylation from methyltetrahydrofolate, which results in the formation of tetrahydrofolate (Drennan et al., 1994). In this respect, methylcobalamin is a cofactor that needs to be reactivated. Mechanistically, the process

depends on the ability of the central cobalt ion to form a Co(I) species, which acts as a powerful nucleophile to form the methylcobalamin. The close relationship with the requirement for folate explains why deficiencies in folate and cobalamin share some similar symptoms. It is also interesting to note that nature has evolved a cobalamin-independent methionine synthase, which is found in a range of different organisms, including plants and yeasts, but that the cobalamin-independent forms of the enzyme are less efficient in terms of their turnover number. This means that there is a strong selection pressure for biological systems to retain the cobalamin-dependent methionine synthase.

Other known cobalamin-dependent methyltransferases are deployed by specific bacteria and archaea, especially those involved in methanogenesis, anaerobic methane oxidation and acetogenesis. Here, cobalamin plays key roles in the formation of methane as well as in the fixation of C1 substrates such as methane or carbon dioxide. These processes are reviewed elsewhere (Ragsdale, 2008).

B_{12}-dependent isomerases. Adenosylcobalamin acts as a coenzyme in enzymes that are able to catalyse complex rearrangement reactions of their substrates. Most of these reactions are involved in bacterial fermentation processes, although enzymes such as methylmalonylCoA mutase have application in more general biochemical pathways (Roth wt al., 1996). In the example of methylmalonylCoA mutase, the enzyme is able to interconvert methylmalonylCoA and succinylCoA; it is the only isomerase present in animals. The underpinning process that allows this to happen is the ability of AdoCbl to undergo a homolytic cleavage of the cobalt-carbon bond, which results in the formation of a Co(II) species as well as an adenosyl radical (Dowling et al., 2012). The radical promotes hydrogen abstraction from the substrate and initiates the rearrangement chemistry. In bacteria, there are many other AdoCbl enzymes associated with a broad range of isomerisation reactions, and all are dependent upon the ability of AdoCbl to undergo homolytic cleavage to generate the adenosyl radical.

Reductive dehalogenation. It is only recently that molecular insight has been gained on the role of B_{12} in the process of reductive dehalogenation, which takes place in certain bacteria (Bridwell-Rabb & Drennan, 2017; Fincker & Spormann, 2017; Payne et al., 2015). Reductive dehalogenases are able to remove a halogen component from a substrate with the simultaneous addition of electrons. In this case, the enzyme appears to function through the ability to form a

direct cobalt-halogen bond. These processes have the potential to be used for the bioremediation of contaminated land and water.

Radical SAM enzymes. The final class of B_{12}-dependent enzymes outlined above are the radical SAM enzymes (Bridwell-Rabb & Drennan, 2017). They are an extremely broad class of enzymes that house at least one Fe-S centre and employ the use of SAM to generate an adenosyl radical (Sofia et al., 2001). However, within this broad class of radical SAM enzymes, the largest group also have a requirement for B_{12}. Again, we are only just starting to understand how cobalamin chemistry is being coupled with radical SAM chemistry to perform these complex reactions. These enzymes are largely restricted to prokaryotes.

Non-enzymatic functions. B_{12} is also involved in a number of non-enzymatic processes outside the animal kingdom. B_{12} is able to bind to specific nucleic acid sequences, called riboswitches that control and modulate either transcription or translation (Mandal & Breaker, 2004). The binding of B_{12} is normally associated with the regulation of either the uptake of B_{12} or the biosynthesis of the molecule. A number of transcription factors have also been identified that bind B_{12}. In the best studied case, the CarH transcription factor has been shown to act as light sensor, employing AdoCbl as its light-sensitive pigment. In its repressor form, CarH is a tetramer that binds AdoCbl. However, when exposed to light, which leads to the scission of the cobalt ─ adenosyl bond, the tetramer disassociates and is released from the DNA resulting in transcription (Bridwell-Rabb & Drennan, 2017; Jost et al., 2015; Padmanabhan et al., 2017). CarH is a transcriptional repressor for carotenoid biosynthesis and the light deactivation of the repressor therefore results in the production of cellular light-protecting carotenoids.

2.3 Biosynthesis of B_{12}

Enzymes using B_{12} are often very efficient and result in significant rate enhancements when compared to model systems or enzymes that are B_{12}-independent. However, the ability to use B_{12} comes at a cost. It is a structurally intricate molecule whose complexity is reflected in a similarly sophisticated biosynthetic pathway, involving somewhere around thirty enzyme-mediated steps (Warren et al., 2002). The ability to biosynthesise B_{12} is restricted to certain prokaryotes. Some bacteria have the ability to make their own B_{12}, others acquire it from their environment and some have evolved to live in a B_{12}-less world. Bacteria that make B_{12} generally synthesise it by one of two distinct pathways, which are referred to as the aerobic and anaerobic routes (Battersby, 1994; Moore et al., 2013) (Supplementary Figure S1). Both pathways have now been fully elucidated, and there is also quite a lot of molecular information

available concerning the mechanism of the individual steps associated with the pathway. The two pathways for the synthesis of AdoCbl from the common tetrapyrrole primogenitor uroporphyrinogen III diverge at an intermediate called precorrin-2 and converge with the formation of adenosylcobyric acid.

Many bacteria are able to acquire or salvage B_{12} from the environment in which they grow, even if they have the ability to make the nutrient *de novo* (Escalante-Semerena, 2007). The salvage pathway often involves the acquisition of just the corrin component of the molecule, and in these cases, the bacteria are able to attach the lower loop and add the upper ligand. In these cases, bacteria can take up molecules such as cobinamide or cobyric acid, assemble the lower nucleotide and attach it to the corrin ring (Supplementary Figure S1). However, as some bacteria are able to make cobalamin variants with different bases to DMB, other bacteria have evolved systems to take up these B_{12} variants, remove the lower nucleotide loop and replace it with another loop containing a more appropriate base. The synthesis and sharing of B_{12} in complex microbial communities is a growing area of interest, as the ability to make, portion and acquire such a valuable metabolic resource has significant effects in shaping microbial communities (Degnan et al., 2014; Escalante-Semerena, 2007).

The structural complexity of B_{12} means that the nutrient cannot be made commercially through chemical synthesis. In this respect, B_{12} is one of the few vitamins that is obtained through bacterial fermentation (Martens et al., 2002). High-producing bacterial strains were developed initially by Rhone-Poulenc in France although similar strains are now also used to produce the vitamin in China. It is estimated that about 35 tonnes of B_{12} are produced by bacterial fermentation on an annual basis, some of which is used to supply the human nutrient market but the majority is used as supplements for animal feed.

2.4 B_{12} in eukaryotes

In contrast to the broad range of enzymes and molecular processes involving B_{12} in prokaryotic systems, the picture is very different in eukaryotes where B_{12} is restricted to just methionine synthase and methylmalonylCoA mutase. The eukaryotic systems that use B_{12} include mammals, birds, fish, worms and protists such as algae. Many eukaryotes do not require B_{12}, including higher plants and yeasts/fungi. The acquisition of B_{12} in eukaryotic systems represents a very interesting challenge. Algae, for instance, gain their B_{12} through association with bacteria in either a symbiotic or mutualistic manner (Croft et al., 2005). Some mammals, especially ruminants, are able to absorb their B_{12} from the enteric bacteria that live

in their intestines (Girard et al., 2009). Although humans have a rich microbial fauna, present mainly in the large intestine, the human B_{12} uptake system is located in the small intestine. Humans therefore do not acquire B_{12} from their enteric bacteria and have to rely on B_{12} present in their diet (Stabler & Allen, 2004).

As humans require B_{12} for just two enzymes and as they tend to retain and recycle their B_{12}, the daily requirement for B_{12} is very low (Stabler & Allen, 2004). The RDA for B_{12} is around 2.5 µg per day, which is the lowest of all the vitamins. Good dietary sources of B_{12} include meat, in particular liver, fish, shellfish and dairy products. Vegetarians, and more specifically vegans, are prone to B_{12} deficiency due to the lack of B_{12} in higher plants (Rizzo et al., 2016). However, some macro-algae such as Nori do contain B_{12}. A number of foodstuffs such as breakfast cereals and energy drinks are fortified with B_{12}. Most humans on a healthy varied diet are not prone to frank B_{12} deficiency, but B_{12} insufficiency is very much more common, as we will discuss in Section 3. Sometimes bacteria growing in foods or beverages can provide B_{12}, so, counterintuitively, the greater the hygiene applied to the preparation of foods and drinks, e.g., through washing the food, clean water and refrigeration, the lower the B_{12} levels. This process may partly explain the finding that the median plasma B_{12} in urban middle-class Indian men was 89 pmol/L, compared with 145 pmol/L for men living in urban slums (Yajnik et al., 2006).

A lack of B_{12} gives rise to a complex series of signs and symptoms, most of which arise from metabolic problems associated with the two human enzymes that require B_{12} (Green et al., 2017). The symptoms of B_{12} deficiency range from megaloblastic anemia, neurological problems, cardiovascular symptoms, methylmalonic aciduria, etc. For instance, impairment of methionine synthase impedes the methionine cycle, whereby homocysteine is converted into methionine and then to SAM. SAM is used in a range of one carbon methylation reactions where it is broken down first to S-adenosylhomocysteine (SAH) and then into homocysteine and adenosine. A lack of B_{12} results in a build-up of homocysteine and folate being trapped as methyltetrahydrofolate, which in return leads to depletion of tetrahydrofolates used in thymidylate and purine synthesis. This is how B_{12} deficiency impedes cellular proliferation and protein synthesis, and thereby cause development of megaloblastic anemia. A lack of B_{12} has similar detrimental effects on methylmalonylCoA mutase, an enzyme that is associated with the degradation of odd chain fatty acids, certain amino acids and propionate metabolism. The enzyme allows the interconversion of MMA and succinate, and a lack of B_{12} results in the build-up of MMA causing acidemia in the blood and urine.

Inborn errors. B_{12} insufficiency in humans is caused not only by a lack of the vitamin in the diet or by B_{12} malabsorption but also by genetic disorders linked with the nutrient uptake and distribution system (Froese & Gravel, 2010; Gherasim, Lofgren, & Banerjee, 2013; Watkins & Rosenblatt, 2013). Our understanding of the processes involved in cellular uptake and subcellular distribution of B_{12} has come from a large volume of worked associated with inborn errors of metabolism linked to genetic mutations of specific proteins (Froese & Gravel, 2010; Watkins & Rosenblatt, 2013). Distinct components associated with human B_{12} metabolism were identified, and coupled with biochemical analysis of radioactive metabolites and B_{12}, it was possible to classify a range of different genotypes and phenotypes (Froese & Gravel, 2010). As a result, it was possible to identify a number of complementation groups, termed *cblA-G* and *mut*, that represented genes associated with B_{12} metabolism and transport. The roles of these different loci are shown in Table 1, and their function is outlined in Figure 2. A number of the genes associated with these complementation groups already had assigned functions (eg *cblG* = methionine synthase), whereas in other cases, the identification of the gene led to the discovery of a new functionality (eg *cblC* = decyanalase).

[Table 1 and Figure 2 near here]

B_{12} uptake and distribution. In humans, several different B_{12}-binding proteins facilitate the absorption of B_{12} from dietary sources (Fedosov, 2012; Nielsen, Rasmussen, Andersen, Nexo, & Moestrup, 2012). These proteins include the three carrier proteins called haptocorrin (HC), intrinsic factor (IF) and transcobalamin (TC). The structures of all three proteins have been determined (Furger, Frei, Schibli, Fischer, & Prota, 2013; Mathews et al., 2007; Wuerges et al., 2006), including several with the proteins bound to their cognate receptors (Alam et al., 2016; Andersen, Madsen, Storm, Moestrup, & Andersen, 2010). Both HC and IF are glycoproteins, but all three proteins share a degree of sequence similarity, which is reflected in a similar overall topology. Importantly, they have different affinities for B_{12} and B_{12} analogues and play different roles as outlined below.

The first of the B_{12} binding proteins in the uptake mechanism is HC, which is excreted into the saliva and upper part of the gastro-intestinal tract, and it is also found in blood serum. This protein has a high affinity for B_{12} and for B_{12} analogues and binds them as they are released from the food source through the initial stages of digestion (involving pepsin and acid) in the mouth and stomach. The HC and other B_{12}-bound protein complexes are further digested in the small intestine to release the B_{12}, where it is bound by IF.

IF is produced by the parietal cells in the stomach, and adverse conditions targeting these cells will lead to IF deficiency. Notably, pernicious anemia is a B_{12} deficiency due to lack of IF, and is usually caused by an autoimmune condition that targets the parietal cells (Toh, van Driel, & Gleeson, 1997). In contrast to HC, IF is much more specific for binding B_{12} and has limited affinity for cobalamin analogues. In ileum, the IF-cobalamin complex crosses the intestinal membrane by first binding to the apical side of epithelial cells. Here it binds to the cubam receptor, which facilitates endocytosis into the lysosome. In the lysosome, IF is degraded and the liberated cobalamin is released into the cytosol and then transported to the bloodstream via the multidrug resistance protein MRP1. Once in the bloodstream, the majority of B_{12} (80%) and all the cobalamin analogues are bound to the less specific HC, whereas the remainder is bound to the much more specific TC. Like IF, TC binds only B_{12}. Significantly, only TC is able to facilitate uptake into cells. The role of circulating HC is not fully understood, but it may help remove cobalamin analogues or act as a general store. Interestingly, patients who are unable to produce HC have low serum levels of B_{12} but do not display symptoms of B_{12} deficiency.

The TC-cobalamin complex is taken up into cells by receptor-mediated endocytosis after binding to the TC receptor (Quadros, 2010). In the lysosome, the TC is digested away and the free cobalamin is exported into the cytosol via the synergistic effort of two membrane proteins called ABCD4 (*cblJ*) and LMBD1 (*cblF*) (Quadros, 2010; Rutsch et al., 2009). In the cytosol, B_{12} is acted upon by CblC (Lerner-Ellis et al., 2006), which is a protein that plays a key role for subsequent compartmentalisation of B_{12} within the cell. Up to this stage, the delivery of B_{12} to the cell has been determined by the requirement for an intact lower ligand (i.e., DMB) with little attention paid to the nature of the upper ligand. Thus, the transported cobalamins are likely to have different upper ligands (adenosyl, methyl, hydroxyl, cyano). In the cytosol, CblC has the ability to de-alkylate and de-cyanalate the various forms via distinct processes that have been studied through biochemical and molecular approaches (Hannibal et al., 2009; J. Kim et al., 2008). Alkylated cobalamins (methyl and adenosyl) are cleaved by binding to CblC, which in the presence of glutathione mediates a nucleophilic displacement of the upper ligand to form a glutathione ester and a cob(I)alamin (J. Kim et al., 2009). The latter is able to react rapidly with water to form hydroxocob(II)alamin. The reaction of CblC with cyanocobalamin is slightly different in that it requires a reduced flavin as a cofactor, which encourages homolytic cleavage of the bond to give cob(II)alamin and cyanide (J. Kim et al., 2008). Significantly, this means that people with a defective CblC usually respond well to

treatment with hydroxocobalamin, but not to other cobalamins. Interestingly, the structure of CblC reveals that it has a similar structure to the enzyme BluB (Koutmos et al., 2011), which is involved in the biosynthesis of the DMB base (Taga et al., 2007). Structurally, these proteins have a flavoprotein fold. The structure is also similar to the reductive dehalogenases indicating that this fold has evolved to be involved in a broad range of cobalamin-related reactions (Payne et al., 2015).

CblC also interacts with the cytosolic protein CblD, which acts as a key chaperone protein in the transfer of the nutrient to either methionine synthase (CblG) in the cytoplasm, or to the mitochondrial transport system (Coelho et al., 2008). CblD has a similar structure to CblC but does not bind B_{12} (Yamada et al., 2015). Generation of functional methionine synthase requires reductive methylation of cob(II)alamin to methylcobalamin using methionine synthase reductase in which SAM acts as a methyl donor (Leclerc et al., 1998).

The import system for B_{12} into the mitochondrion has not yet been identified, but once inside mitochondria, the B_{12} is adenosylated by an adenosyltransferase (*cblB*). The adenosyltransferase binds cob(II)alamin in a 4-coordinate manner, which has the effect of minimising the reducing power required to convert the cob(II)alamin into cob(I)alamin in the presence of a suitable reductant such as a flavodoxin or ferredoxin system (Stich et al., 2005). In the presence of ATP, the cob(I)alamin displaces the triphosphate of the ATP and generates AdoCbl (Mera, et al., 2007). Significantly, the adenosyltransferase also chaperones AdoCbl to the methylmalonylCoA mutase and thereby allows direct delivery of the coenzyme (Padovani et al., 2008). A further complementation group, *cblA*, was initially thought to be caused by mutations in the adenosyltransferase, but, when the gene was isolated, it was found to encode for a protein that was a member of the P-loop GTPases (Dobson et al., 2002). These proteins are linked with the assembly and protection of metal centres. An orthologous protein in bacteria, MeaB, has been shown to form a complex with the methylmalonylCoA mutase and to protect the enzyme from inactivation (Padovani & Banerjee, 2009).

In summary, the identification of a broad range of human inborn errors of metabolism has allowed us to piece together the complex uptake and delivery mechanisms required for cobalamin biochemistry (Froese & Gravel, 2010; Gherasim et al., 2013; Watkins & Rosenblatt, 2013).

3. POOR B_{12} STATUS– DEFICIENCY OR INADEQUACY?

3.1 How do we define B_{12} deficiency?

To answer this question, we need to specify the biological or clinical outcomes of relevance and to decide which marker will be measured. Since megaloblastic anemia was the first B_{12}-deficiency syndrome discovered, it has been traditional to use hematological signs, such as a raised mean corpuscular volume or megaloblastic anemia as markers (Green, 2017). However, in the classic paper by Lindenbaum and colleagues (1988), it was shown that neurological signs of B_{12} deficiency are often present in the absence of anemia. Even in patients with clinical pernicious anemia, 28% do not have anemia and up to 33% have normal mean corpuscular volume (Carmel, 2000). In fact, anemia is just one possible outcome of deficiency and should not therefore be used as a sole marker. Ideally, a marker should be associated with most, if not all, the known outcomes. For this reason, biochemical markers, such as serum total B_{12}, holoTC or the functional markers MMA or tHcy, are usually preferable.

Part of the problem in defining B_{12} deficiency lies in the measurement of serum total B_{12} levels. Serum levels reflect B_{12} that is bound to either HC or TC. Although HC binds the majority of serum B_{12}, up to 60% of bound material comprises incomplete corrinoids or cobalamin analogues (Morkbak, Poulsen, & Nexo, 2007). In this respect, an estimate of total serum B_{12} levels is not very reliable when trying to assess whether patients are B_{12} deficient, since a 'normal' reading using some assay methods can mask deficiency if serum contains relatively large amounts of cobalamin analogues. However, since TC only binds biologically-active cobalamin (Hardlei & Nexo, 2009), measurement of holoTC, i.e., TC-cobalamin, will give a more accurate diagnosis (Fedosov, 2012). Another advantage in measuring holoTC is in subjects who have markedly elevated total serum B_{12}, found in about 8% of tests; the high B_{12} is usually due to an excessive amount of HC and so could mask any deficiency (Arendt & Nexo, 2013).

But then the question arises, what value of the biochemical marker defines the cut-off for deficiency? Traditionally, the cut-off for serum/plasma B_{12} has been 148 pmol/L (200 pg/mL) or 150 pmol/L (203 pg/mL), according to WHO and the US Institute of Medicine. However, just as with cardiovascular disease risk factors, it is now recognised that there is no sharp cut-off for identifying harmful B_{12} status but that there is a continuous inverse relationship between serum B_{12} and a variety of undesirable outcomes (Smith & Refsum,

2012). Many undesirable outcomes have been associated with serum B_{12} levels that are well above the traditional cut-off value of 150 pmol/L, as will be discussed in Section 4. Distinct biological and biochemical outcomes have different relationships to the serum B_{12} level (see Figure 2 in (Smith & Refsum, 2016)), which makes it difficult to define 'deficiency'. For this reason, we prefer the terms 'B_{12} inadequacy' or 'B_{12} insufficiency'.

Insufficiency of B_{12} is also revealed by measurement of metabolic markers such as MMA and tHcy, whose blood levels start to rise as the serum B_{12} level falls below about 400 pmol/L (see Section 3). Recently, a new approach to defining B_{12} inadequacy has been proposed, which incorporates data on serum B_{12}, holoTC, tHcy and MMA to give a 'combined B_{12} indicator' (Fedosov et al., 2015). Although promising, this indicator requires extensive facilities for the assays and needs further validation, especially regarding the cognitive outcomes. For the time being, it remains a research tool. For instance, in a study on cognitive development in children, plasma B_{12} levels in infancy were not related to cognition at 5 y, but the combined indicator was related, as were MMA and tHcy (Kvestad et al., 2017).

As can be seen, the diagnosis of B_{12} deficiency/insufficiency is far from satisfactory, and we agree with the following statement by the British Society for Haematology in their Guidelines: '*The clinical picture is the most important factor in assessing the significance of test results assessing cobalamin status because there is no 'gold standard' test to define deficiency*.' (Devalia, et al., 2014).

3.2 How prevalent is B_{12} deficiency and inadequacy? (see Supplementary Table 1S)

There are several valuable reviews of the prevalence of B_{12} deficiency (Allen, 2004, 2009; Allen et al., 2017; Baik & Russell, 1999; Green et al., 2017; Hunt et al., 2014; McLean et al., 2008). In Supplementary Table 1S, we have summarised observational studies, mainly since 2010, on this question. In 62 of these reports, B_{12} deficiency was defined as serum/plasma levels < 148 or < 150 pmol/L. In 5 studies, the cut-off was lower (ranging from 111 to 130 pmol/L); in 12 studies the cut-off was higher (ranging from 160-221 pmol/L). We have amalgamated the data from Table 1S into summary tables (Tables 2 and 3) to make it easier to see the overall picture.

There is a tendency for the prevalence of *B_{12} deficiency* (Table 2) to increase with age, but this is not always so clear, as can be seen in one of the NHANES studies (Bailey et al.,

2011). A more marked effect of age was reported in a study from Liechtenstein (Koenig et al., 2014), reproduced in Supplementary Figure S2.

It is noteworthy that the prevalence of *B_{12} inadequacy* (Table 3) is high in all subgroups of the population, ranging from 30 to 60%. B_{12} inadequacy is 5 times more prevalent than deficiency in adults, 3-fold higher in non-pregnant women, twice as high in pregnant women and in children, and 3-fold higher in the elderly. Since B_{12} inadequacy is associated with impaired B_{12} function as judged by elevation of MMA and tHcy, and probably also with impaired health outcomes (see Section 4 and Table 4), the high prevalence is of concern to public health. Other matters of concern can be deduced from Tables 2, 3, and 1S, notably that about half of women have a poor B_{12} status, with one-quarter of pregnant women being classed as deficient. Infants, on average, appear to have poorer status than older children and adults. The latter result may be due to low B_{12} status usually observed in breastfed infants. Furthermore, many of these studies are from regions (e.g., parts of South Asia, Africa, Middle and South America) with a general poor B_{12} status. In parts of China, the prevalence of deficiency in the elderly is very high indeed, with 75% having concentrations below 185 pmol/L (J. Zhang et al., 2016; W. Zhang et al., 2014). In Hong Kong, 35% of institutionalised elderly had B_{12} below 150 pmol/L (Wong et al., 2015). As expected, high proportions of vegetarians and of vegans have poor B_{12} status, with deficiencies ranging from 11 to 90% (Gilsing et al., 2010; Pawlak et al., 2013, 2014; Rizzo et al., 2016). In countries like India, where life-long vegetarians form a high proportion of the population, up to 71% of pregnant women are B_{12}-deficient (Yajnik et al., 2008), with major implications for the health of the mother and child (see Section 5).

We would like to draw attention to a novel observation from a study in Chile: the prevalence of B_{12} deficiency in the elderly varied from 19.1% in the north of the country to 5.7% in the south (Cabrera et al., 2014). The authors found that the prevalence of B_{12} deficiency was inversely associated with geographical latitude and was positively related to solar radiation, and they speculated that solar radiation increases the degradation of B12. In Europe, a much less marked gradient in median serum B_{12} has been observed from 330 pmol/L in the north to 290 pmol/L in the south ($P<0.001$), and this was attributed to differences in diet (Eussen et al., 2013). In China, the gradient is in the opposite direction, with a much higher prevalence of low B_{12} status in the north (21% deficiency in adults) than in the south (4% deficiency) (Hao et al., 2007); this may also be due to differences in diets.

The likely causes of B_{12} deficiency and inadequacy are reviewed in a book chapter (Green & Miller, 2014), reviews (Green, 2017; Hunt et al., 2014; Stabler, 2013), a recent primer (Green et al., 2017) and a BOND report (Allen et al., 2017) and will not be covered here. However, regarding pernicious anemia, it should be recognised that the diagnosis and treatment of this disease is far from ideal, as revealed in a survey of patients in the UK (Hooper et al., 2014). We recommend the interested reader to the web site of the Pernicious Anaemia Society (https://pernicious-anaemia-society.org/). The clinical consequences of frank B_{12} deficiency are well reviewed in the articles cited above and will not be covered here, but we will examine the consequences of low-normal B_{12} status.

4. CLINICAL RELEVANCE OF B_{12} INDEQUACY/ INSUFFICIENCY

4.1 Concept of 'Subclinical cobalamin deficiency' (SCCD)

This concept was introduced by Carmel (2011) and is defined as 'a state of mild metabolic abnormalities without clinical signs or symptoms'. By 'mild metabolic abnormalities', Carmel meant that plasma tHcy and/or MMA are elevated and/or holoTC is low. While such changes very often occur without overt hematological signs, this does not in our view make them necessarily 'subclinical'. We have pointed out that the zone of 'metabolic insufficiency', as defined in Figure 3, may be associated with one or more clinical signs and symptoms, which are listed in Table 4 (Smith & Refsum, 2012). This zone lies well above the typical cut-off for B_{12} deficiency of 148 pmol/L and extends up to about 350 – 400 pmol/L.

[Figure 3 near here]

A threshold of between 350 and 400 pmol/L separates optimal from suboptimal B_{12} levels, based on metabolic B_{12} markers, and is consistent with several population studies. A detailed statistical analysis on data from 7,260 Americans from NHANES (1999-2000) used segmented regression to obtain two B_{12} cut-points for tHcy of 200 pmol/L (CI 187, 214) and 426 pmol/L (CI 367, 485) pmol/L (Bang et al., 2006). Vogiatzoglou et al. (2009) used segmented regression to obtain B_{12} cut-points of 334 pmol/L (SE 33) and 393 pmol/L (SE 11) for MMA and tHcy, respectively for 6,946 middle-aged and elderly Norwegians. A detailed analysis of data from 12,683 adults (≥ 19 y) in NHANES (1999-2004) used 5 different modelling methods to obtain cut-points related to raised MMA (Bailey et al., 2013). The

derived cut-point varied according to the model used. A linear splines model best fitted the data and gave two cut-points of 126 pmol/L (CI 117, 182) and 287 pmol/L (CI 253, 434), with a marked increase in MMA in the group below 126 pmol/L, and a more modest increase in those with B_{12} in the range 126-287 pmol/L. Accordingly, these authors divided the US population into three groups: Group 1 (B_{12} < 126 pmol/L) represented about 1% of adults, while Group 3 (B_{12} > 287 pmol/L) represented almost ~ 68% of adults. The middle Group 2, about 31% of adults, had B12 cut-off values between 126 and 287 pmol/L. This group corresponds most closely to our category of 'metabolic insufficiency' and would be likely to show a higher prevalence of clinical outcomes listed in Table 4.

[Table 4 near here]

Notably, in a study examining median and upper limits for MMA according to creatinine, age and B_{12} status, it appears that it is mainly the upper MMA limit that changes according to B_{12} status (see Figure 4S in Vogiatzoglou et al., (2009)), suggesting that the MMA increase occurs in a subgroup. Thus, we believe that the two NHANES thresholds of 126 and 287 pmol/L may distinguish between a level where a majority of the subjects have metabolic defects (<126 pmol/L), while the MMA increase in the intermediate levels (126-287) is mainly confined to a subgroup of people requiring higher B_{12} level to obtain optimal metabolic function.

A good example of how low-normal B_{12} status can lead to potentially serious harm is that the rate of atrophy of the whole brain in the normal elderly, measured by serial MRI scans, is linearly inversely related to the serum B_{12} level over almost the entire normal range (Hooshmand et al., 2016; Vogiatzoglou et al., 2008). We will discuss this further in Section 7.

4.2 Clinical implications of low-normal B_{12} status

The range and variety of the clinical outcomes in Table 4 suggest that it can no longer be stated that metabolic insufficiency related to low-normal B_{12} status is without clinical consequence. We therefore do not agree with the recent 'Primer' on B_{12} deficiency (Green et al., 2017), which states that B_{12} inadequacy is 'of no clinical relevance'.

Our conclusion has several important implications and *we make the following recommendations:*

1. Patients with symptoms or signs consistent with B_{12} deficiency and with low-normal B_{12} levels in the range above the cut-off value of 148 pmol/L, extending up to around 350 pmol/L should not be told that their B_{12} is 'normal', but that they have 'low-normal' levels.
2. Patients with any one of the outcomes listed in Table 4 should have their B_{12} status measured and possible causes for their low B_{12} status should be identified.
3. If the B_{12} falls in the range 150 to about 350 pmol/L, then these patients should be offered high-dose oral B_{12} (1 mg daily) for several months, or regular i.m. injections, to see if their symptoms are reduced and metabolic function markers are normalised. One month of treatment is unlikely to be sufficient (Favrat et al., 2011). *The aim of the treatment, whatever form or dose used, should always be the relief of signs and symptoms, especially neurological signs.*
4. Clinicians should look out for other unexpected clinical signs or symptoms in patients who have B_{12} status in this borderline range. Clinicians need to look outside the conventional hematological and neurological signs, and also consider conditions such as infertility, birth defects, orthostatic hypotension, skin changes and psychiatric and cognitive problems, as listed in Table 4.

4.3. What oral dose of B_{12} is needed to obtain optimal B_{12} status?

The literature on population dietary intake of B_{12} is surveyed in the recent BOND report (Allen et al., 2017) and so will not be reviewed here. However, we must note that the bioavailability of B_{12} from food sources is far from uniform and that the blood levels of B_{12} first reach a plateau when about 10 μg per day is consumed from food sources (Tucker et al., 2000;Vogiatzoglou et al., 2009). Our purpose here is to discuss the supplemental dose of B_{12} that will correct the signs of metabolic insufficiency. First, we must point out that the widespread use of intramuscular B_{12} treatment is often not necessary. Parenteral B_{12} is only required to correct the initial very poor B_{12} status when pernicious anemia is diagnosed. Later, although some patients prefer regular injections, oral high-dose (1 - 2 mg per day) maintenance treatment is adequate for most patients with pernicious anemia (Berlin et al., 1968; Kuzminski, et al., 1998; Vidal-Alaball et al., 2005). For subjects with low B_{12} status caused by poor dietary intake, or by food malabsorption, it will be simpler and cheaper to use

oral B_{12} preparations. In Sweden, a national survey found that in the year 2000, 2 out of 3 B_{12} prescriptions were for oral tablets (Nilsson et al., 2005). This statistic does not include the small proportion (< 1%) of oral B_{12} tablets freely purchased over the counter.

The normal mechanism of absorption of oral B_{12}, *via* the IF pathway, is easily saturated: While ~ 70% of B_{12} is absorbed from doses of 0.1 to 0.5 μg, this falls to 56% for 1 μg, 16% for 10 μg, and 3% for doses of 25-50 μg (Chanarin, 1979). High oral doses will very largely be absorbed passively: the classical study of Berlin and colleagues (1968) using radiolabelled cyanocobalamin showed that an upper limit of 2 μg can be absorbed via the IF pathway, while at higher doses, from 100 to 100,000 μg, the absorption is passive with 1.2% of the dose being absorbed. The authors recommended that an oral dose of 1000 μg of cyanocobalamin per day should be given to patients with pernicious anemia since they found that this doses was equivalent in every respect to the traditional intramuscular dose regime. Modern studies on the relation between oral B12 dose and blood levels have been conflicting. Notably, these studies have been performed in general populations or subjects with low B12, i.e., only few of them will suffered from pernicious anemia. A Danish study on 98 postmenopausal women found that an intake of ~ 6 μg per day from food and supplements was adequate to bring the plasma B_{12} and holoTC to a plateau and to maximally lower MMA and tHcy. The median plasma B_{12} at this level of intake was 380 pmol/L. The effectiveness of a relatively low dose was also found a randomised trial over one year in India (Deshmukh et al., 2010), where B_{12} status is very poor due to the largely vegetarian diet: 300 adults and children (48% were B_{12} deficient, i.e. < 148 pmol/L) were given either 2 or 10 μg of B_{12} and the B_{12} level rose, respectively, from 168 to 242 pmol/L and from 159 to 307 pmol/L. There were also significant decreases in tHcy levels. Other reports have suggested that much higher doses might be needed to normalise the biomarkers. Rajan et al. (2002) recruited 23 elderly Americans with B_{12} deficiency due to food-malabsorption and administered daily oral doses of 25, 100 and 1000 μg of cobalamin (form not stated) and they found that almost all participants required 1000 μg to normalise the serum MMA level. This result was confirmed by a dose-finding study in the Netherlands (Eussen et al., 2005) in which oral cyanocobalamin in doses of 2.5, 100, 250, 500 and 1000 μg was administered for 16 weeks to elderly people with a baseline serum B_{12} of 208 (IQR 113-362) pmol/L. The median serum B_{12} at the end of the trial was 404 (293) for those receiving 500 μg/day and was 574 (418) pmol/L for those receiving 1000 μg/day. The lowest MMA values were obtained after 500

and 1000 μg, while 1000 μg was required to maximally lower tHcy. The authors concluded that 'much higher doses of cyanocobalamin are required to normalize vitamin B_{12} deficiency than were previously believed'. A similar conclusion was reached by Hill et al., (2013), who found that in elderly subjects with marginal plasma B_{12} status (median 201, range 107-249 pmol/L), a dose of 10 μg/d for 8 weeks was sufficient to bring plasma B_{12} up to > 200 pmol/L in all but 15% of subjects but > 40% still had elevated plasma MMA. A dose of 500 μg/d still left 10% with B_{12} below 200 pmol/L and 23% with elevated MMA. The discrepancy between the studies showing that low B_{12} doses (< 10 μg/day) and those finding that only high doses (500 μg/day or higher) are effective may appear puzzling, but can probably be explained: First, if the supplement is taken with food, it will have a higher absorption due to the secretion of IF; this would also apply if several small doses were repeated throughout the day, e.g., by enriching food sources (Winkels et al., 2008). Second, the form of the supplementary B_{12} might be important, whether it is cyano-, hydroxo-, methyl- or some other form of cobalamin. Third, the effect of supplementary B_{12} may differ if the cause of low B_{12} status is inadequate diet compared with malabsorption of food-bound B_{12}, or if it is due to frank pernicious anemia.

Two excellent reviews on this topic (Dullemeijer et al., 2013; Hoey et al., 2009) have not fully resolved the above discrepancies, although the elegant meta-analysis by Dullemeijer et al. (2013) has provided a deeper understanding of the relationship between oral B_{12} intake and serum/plasma B_{12} levels. These authors examined outcomes from 19 observational studies on 12,570 subjects and 37 randomised controlled trials (RCT) on 3,398 subjects and obtained a pooled estimate of the dose-response relationship between B_{12} intake (x) and serum or plasma B_{12} (y) that satisfied the equation**:**

$$\log_e(y) = 0.15 \times \log_e(x) + 5.31$$

This estimate means that the doubling of B_{12} intake increases the B_{12} concentration in serum or plasma by $2^{0.15}$ fold, which corresponds to 11% (95% CI 9.4%, 12.5%). 'This calculation meant that a person with an intake of 2 μg B_{12}/day would have a serum/plasma concentration that was 11% higher than that of a person with and intake of 1 μg/day; or a person with an intake of 8 μg/day would have a B_{12} serum/plasma concentration that was 11% higher than that of a person who had an intake of 4 μg/day.' (Dullemeijer et al., 2013).

[Figure 4 near here]

The authors used the equations derived from the observational and RCT reports to derive a graph showing the relationships; this important outcome is shown in Figure 4. It is instructive to compare this graph with the results from the VITACOG trial, in which subjects with mild cognitive impairment (MCI) were given a supplement containing 500 μg per day of cyanocobalamin for 2 years: the geometric mean plasma B_{12} before treatment was 330 (CI 303, 360) pmol/L and was 672 (95% CI 626, 722) at the end of the study. The value of 672 pmol/L is very close to that predicted for an intake of 500 μg from the plot for RCTs in Figure 4.

We recommend that an oral dose of 500 μg/d should be the minimum used to treat those with B_{12} in the low-normal range.

5. B_{12} STATUS AND NEUROLOGICAL AND COGNITIVE FUNCTIONING IN ADULTS

5.1 Background

An association between B_{12} deficiency and impaired neurological or cognitive function in adults has been known since Addison's description of pernicious anemia in the 19th century (McCaddon, 2006; McCaddon, 2013; Stabler, 2013). Lindenbaum and colleagues (1988) described neurological and neuropsychiatric impairments in patients with pernicious anemia who did not display the classical hematological signs; the patients' neurological signs improved upon treatment with B_{12}. In another study of patients with B_{12} < 148 pmol/L, it was shown that a beneficial neuropsychiatric response to B_{12} treatment was largely confined to subjects with elevated tHcy or MMA (Stabler et al., 1990). In relation to normal aging, an important concept was introduced in 1992 by Rosenberg and Miller (1992), who stated "For most people, including elderly people, overt vitamin deficiencies are unlikely; it is more likely that mild or 'subclinical' vitamin deficiencies may play a role in the pathogenesis of declining cognitive function in aging". Thus, as discussed in Section 3.1, we need to consider the status of B_{12} over a range of values, not just focus on deficiency, when looking at cognitive outcomes. A good example is that in non-demented subjects, plasma holoTC was correlated ($r = 0.25$, $P = 0.04$) with global cognitive test scores over the entire normal range (35 to 200 pmol/L), higher concentrations being associated with better test scores (Refsum & Smith, 2003) – illustrated in Smith & Refsum (2009).

The topic has been reviewed extensively (Doets et al., 2013; Health Quality, 2013; Li et al., 2014; McCaddon, 2013; McCaddon & Miller, 2015; E. Moore et al., 2012; O'Leary, Allman-Farinelli, & Samman, 2012; Reynolds, 2006; Smith, 2008; Smith & Refsum, 2009, 2016; Venkatramanan et al., 2016; Werder, 2010; D. M. Zhang et al., 2017). Several of these reviews come to the conclusion that there is no convincing evidence for a link between B_{12} status and cognitive impairment or dementia. In our view, there are flaws in the analyses in some of these reviews, which we have not got space to discuss in detail here. We outlined some of the problems in our earlier review (Smith & Refsum, 2009) and in particular we pointed out that the association of poor B_{12} status with impaired cognition often depended on how B_{12} status was assessed and also upon the context. We will now summarise these aspects.

5.2 Use of markers of B_{12} function and the question of power

Since only about 20% of the total serum B_{12}, that which is bound to TC, is available to the tissues, the use of total B_{12} may mask an association with cognition. For example, in a case-control study on 51 patients with histopathologically-confirmed Alzheimer's disease (AD) and 65 control subjects, the serum total B_{12} levels were not significantly different, whereas the holoTC levels (41.1 and 57.1 pmol/L, respectively) were different ($P<0.001$) (Refsum & Smith, 2003). It was also found that both MMA and tHcy were significantly elevated in the AD cases compared with the controls, so it was concluded that a poor B_{12} status was associated with AD. Logistic regression confirmed this conclusion, with low holoTC showing a significant odds ratio for AD of 2.82 and raised MMA an odds ratio of 3.83. Total serum B_{12} showed a tendency to be associated with AD, but the confidence intervals for the odds ratio included 1.0. However, this appeared to be a power issue since when the same 51 subjects were combined in an analysis with the whole cohort (76 AD cases, 108 controls), there was a highly significant association between low serum B_{12} and AD with an odds ratio of 4.3 for those in the bottom tertile of serum B_{12} versus those in the top tertile (Clarke et al., 1998). In a cross-sectional study from Chicago, while total serum B_{12} was not related to global cognitive test scores, the markers of B_{12} status (tHcy, MMA) were significantly related to poorer scores (Tangney et al., 2011). These results indicate that it could be an error to conclude that there is no association between B_{12} status and a cognitive outcome, either because there were not enough subjects in the study or because a more sensitive marker was not used. A recent striking finding may also reflect the importance of sufficient power: a data mining study on 5,821 patients in the placebo arms of several AD clinical trials found that the

strongest biomarker, out of a total of 51 biomarkers, for a high score in the MMSE was serum vitamin B_{12} (Szalkai, Grolmusz, & Grolmusz, 2017).

Further examples of the importance of using markers of B_{12} function and not relying on total B_{12} are two large community studies in Oxfordshire, UK. In the first, cross-sectional study, the odds ratios for cognitive impairment in 830 elderly people were significant only for those in the bottom quartile of total serum B_{12}, but were significant for the bottom three quartiles of holoTC (Hin et al., 2006). The second, prospective, study on 692 elderly subjects over 10 y found that low baseline concentrations of holoTC and high baseline concentrations of MMA and of tHcy were associated with an increased rate of cognitive decline, whereas there was no relationship to baseline concentrations of total B_{12} (Clarke et al., 2007). The effects were highly significant: a doubling of the baseline concentration of holoTC was associated with a 30% slower rate of cognitive decline, while doubling of the MMA or of tHcy concentrations were each associated with a 50% increased rate of cognitive decline. It is noteworthy that in this study there was no association of serum folate with cognitive decline. The authors suggested that "the variability in the rates of cognitive decline were primarily explained by the reduced vitamin B_{12} status".

5.3 Importance of the context in which B_{12} is measured

Some of the discrepancies between different studies might have arisen because the populations studied had different characteristics. There are several reports that the relationship between B_{12} status and cognition in older people is stronger in, or even unique to, those who carry the ε4 allele of *ApoE* (Bunce et al., 2004; Feng *et al*., 2009; Vogiatzoglou et al., 2013). The relationship between B_{12} status and cognition is also more marked in people with higher depression scores (Bell et al., 1990; Garrod et al., 2008; Vogiatzoglou et al., 2013). Thus, the prevalence of *ApoE* ε4 and/or of depression in a population could influence the observed association of B_{12} status with cognition. Another contextual effect has been observed in a study on > 2,000 Norwegian elderly people, where there was an interaction between plasma choline and B_{12} (Nurk et al., 2012): subjects with either low choline (8.36 μmol/L) or low B_{12} (< 257 pmol/L) had no significant risk of cognitive impairment, but subjects with both low choline and low B_{12} had a 2- to 3-fold increased risk in several cognitive domains. Thus, if a population under study has a very good choline status, it may not be possible to demonstrate an association between B_{12} status and cognition. The final contextual effect we should mention is that of high folate status. There are reports that in

elderly subjects with high folate status the cognitive impairment due to low B_{12} status is made worse (Castillo-Lancellotti et al., 2015; Morris et al., 2007), and that this effect is related to circulating levels of unmetabolized folic acid (Morris et al., 2010). The folate/B_{12} interaction is controversial and has recently been reviewed (Selhub & Rosenberg, 2016; Smith & Refsum, 2016).

We conclude from cross-sectional and prospective observational studies that low B_{12} status, especially when assessed by functional markers, is often associated with cognitive impairment in elderly subjects, but that this association is context-dependent and so may not be detected in some populations.

5.4 Intervention trials

The crucial question is whether neurological and cognitive impairments in adults due to low B_{12} status can be reversed. The classical case study of Lindenbaum et al. (1988) showed that the neurological and neuropsychiatric impairments due to B_{12} deficiency in pernicious anemia could largely be reversed by treatment. Relatively few modern studies, mainly on elderly subjects, have tested whether such impairments can be reversed by administering B_{12}. It should be obvious, but nevertheless has to be stated, that randomised trials of this nature are meaningful only if carried out on subjects with low B12 status who show clear, and ideally progressive, deficits in neurological and/or cognitive functioning (Smith & Refsum, 2016). If the placebo group does not show impairment, what effect can be expected in the group treated with B_{12}? The published randomised trials are listed in Table 5. It can be concluded from this table that all of the trials listed have quite serious weaknesses that make it difficult to draw any conclusions about the possible benefit of B_{12} supplementation. Eight out of the ten trials either had no placebo group or there was no decline in scores in the placebo group over time. In one trial (Hvas, Ellegaard, & Nexo, 2001), there was neurological improvement in the B_{12} group in subjects with poor B_{12} status at baseline, but this was not confirmed in the larger OPEN trial (Miles et al., 2017). The OPEN trial (Dangour et al., 2015) was on asymptomatic older people (aged 80 y) who had low-normal serum B_{12} (median 223 pmol/L) and were given 1 mg oral B_{12} daily for 12 months. In the placebo group, there was no change in B_{12} over 1 year, whereas in the B_{12} treated group the B_{12} increased to 641 pmol/L. The placebo group showed no significant change in the various neurological parameters tested, and so it is hardly surprising that no

effect of B_{12} treatment was found. The OPEN trial has been criticised (Kalita & Misra, 2015) for the choice of neurological endpoints, which are not very sensitive to poor B_{12} status. The primary neurological endpoint was the posterior tibial compound muscle action potential, whereas more sensitive endpoints would have been sensory- and motor-evoked potentials (Kalita & Misra, 2015). Furthermore, there was no decline over time in the placebo group in any of the several cognitive tests used; indeed, in the primary outcome (memory) there was a tendency for the performance to get better in the placebo group (Dangour et al., 2015), probably due to learning effect. Clearly, slowing of cognitive decline by B_{12} treatment cannot be expected if there is no cognitive decline in the placebo group.

An open trial in 51 asymptomatic participants (age 73 y) with B_{12}-deficiency (median = 120 pmol/L) in Chile used a combination of B_{12} (10 mg), B6 (100 mg) and thiamine (100 mg) in a single intramuscular injection given 4 months before the second neurological assessment. At the end of the trial, the sensory latency of both sural nerves improved in parallel with improvement in the B_{12} status (Brito et al., 2016). Interpretation of this trial must be seen in light of the combination treatment and the lack of a placebo group.

In view of the paucity of well-designed trials to test the effect of B_{12} treatment on neurological and cognitive deficits, we will briefly summarise the VITACOG trial in which three B vitamins (0.8 mg folic acid; 0.5 mg B_{12}; 20 mg B6) were given over 2 y to older people with MCI. In this trial (reviewed in references (Smith & Refsum, 2016, 2017)), the placebo group showed worsening performance in scores for global cognition (MMSE), episodic memory, semantic memory and Clinical Dementia Rating (CDR sum-of-boxes) but only in those with tHcy above the median (11.3 μmol/L) at baseline. Participants with tHcy below the median did not show significant cognitive decline. B vitamin treatment markedly slowed cognitive decline in those with tHcy above the median and led to an improvement in clinical status (CDR score) in those with baseline tHcy > 13 μmol/L (de Jager et al., 2012). A similar beneficial effect of B vitamin treatment was found on the rate of whole brain atrophy, which was slowed by 30% compared with the placebo group. The slowing of brain atrophy rate was greater the higher the baseline tHcy concentration, reaching 53% for participants with tHcy in the top quartile (Smith et al., 2010). Thus, B vitamin treatment that includes B_{12} can slow the brain atrophy and cognitive decline that occurs in people with MCI, but only in those with relatively poor B vitamin status as revealed by high tHcy (above the median). The results of this trial show that modification of the disease process underlying cognitive decline in MCI (a prodromal stage of AD) is feasible using nutritional interventions in those with

low-normal B vitamin status (Smith & Refsum, 2017). No specific neurological tests were done in VITACOG, but it was observed that 13 participants (9.8%) taking the placebo showed a loss of vibration sense over 2 y, whereas only 3 subjects (2.2%) taking B vitamins showed such a loss (P = 0.019). VITACOG will be further discussed in Section 7.2.

6. POOR B_{12} STATUS IN MOTHER AND CHILD: HEALTH OUTCOMES

6.1 Background

This important topic requires a detailed review on its own, and we will only summarise a few findings here. As pointed out in Section 2.2, there is a high prevalence of poor B_{12} status in pregnant women in many parts of the world. This has major implications for maternal health, for example increasing the risk of gestational diabetes (Knight et al., 2015; Krishnaveni et al., 2009; Lai et al., 2017; Sukumar, Venkataraman, et al., 2016) and obesity (Sukumar, Venkataraman, et al., 2016). For the embryo and infant, low maternal B_{12} status is associated with slowing of embryonic growth rate (Parisi et al., 2017), increasing the risk of low birth weight (Rogne et al., 2017; Sukumar, Rafnsson, et al., 2016), of preterm birth (Rogne et al., 2017) and of neural tube defects (Molloy et al., 2009; Tang, Li, & Wang, 2015). The mother's B_{12} status may also be an important determinant of the later health of the child, as shown in the Pune Maternal Nutrition Study in which low B_{12} and high folate in the mother were risk factors for the development of obesity and insulin resistance in the child at age 6 y (Yajnik et al., 2008). This topic has recently been reviewed (Krishnaveni & Yajnik, 2017). Several studies have shown that maternal B_{12} status is an important determinant of the B_{12} status of the infant (Hay et al., 2010; Ueland & Monsen, 2003; Bjorke-Monsen & Ueland, 2011; Finkelstein et al., 2017). Furthermore, infants who are breastfed have a lower B_{12} status than non-breastfed infants. In Norway, breastfed infants at 6 months had a mean serum B_{12} of 242 pmol/L while those not breastfed had a B_{12} of 365 pmol/L (Hay et al., 2008). In India, the respective figures were medians of 184 and 334 pmol/L (Taneja et al., 2007). Notably, in the Norwegian study of healthy infants of omnivore mothers, the lower B_{12} status was found in all breastfed infants, not only in those that were exclusively breastfeeding (Hay et al., 2008). Thus, a critical question is whether the low B_{12} status in breastfeeding is solely explained by low B_{12} content in breast milk or whether metabolic effects of breast milk or breastfeeding cause a change in B_{12} homeostasis. At this stage, we do not know whether the low B_{12} in

healthy breastfed infants is a major concern in relation to later health and development, or if it just reflects different 'normal' B_{12} levels in breastfed vs. non-breastfed infants.

6.2 Severe B_{12} deficiency in exclusively breastfed infants

To further understand the potential harmful effect of low B_{12} status in infancy, we need to recognize that most data on major health concerns are from case studies of exclusively breastfed infants of mothers with poor B_{12} status (Goraya et al., 2015), or in infants with poor B_{12} status combined with poor health, e.g., low birth weight or stunted growth.

In a recent report of a case series in India, the literature of neonatal B_{12} deficiency in exclusively breastfed infants was also reviewed (Goraya et al., 2015). Briefly, the infants usually develop normally in the first 4 to 6 months. Initial symptoms include failure to thrive, lethargy, feeding difficulties, refusal of solid foods and pallor, followed by involuntary movements in the form of tremors, myoclonus, or choreoathetosis. Upon examination, anemia with macrocytosis and megaloblastic bone marrow is usually present and the infants have a low B_{12} combined with elevated tHcy and MMA. CT or MRI of the brain reveals cerebral atrophy and delayed myelination. If diagnosed early and treated promptly, the infants improve rapidly and usually catch up within some few months. Even reversal of cerebral atrophy has been observed (Casella et al., 2005; Lövblad et al., 1997; Goraya et al., 2015) (see also Section 6.2 and Figure 6, below). Nevertheless, long-term neurocognitive symptoms in later childhood may occur (Bhate et al., 2008; Zengin, Sarper, & Caki Kilic, 2009), which is probably due a combination of severity of the conditions and delayed treatment. Hence, it is critically important to discover B_{12} deficiency before severe symptoms arise.

6.3 B_{12} in children's motor and cognitive development and intervention trials

It is notable that inborn errors of remethylation (see Section 1, Table 1) have revealed several crucial roles for B_{12} in development (Huemer et al., 2017). In the general population, as shown in Supplementary Table 1S, the B_{12} status in infants and children is often poor, especially in less developed countries, with deficiencies of up to 30% (Nepal), 40% (Kenya), 58% (Honduras) and 68% (India). At this critical stage in childhood development, it is crucial to know if low B_{12} status observed in infants has harmful effects and also if there is beneficial effect of treatment. There are several reviews (Bjorke-Monsen & Ueland, 2011; Dror & Allen, 2008; Goraya et al., 2015; Venkatramanan et al., 2016) that suggest that a good B_{12} status may also improve health and development in a more general population of infants. A

good example of these findings is a study in New Delhi on 538 infants 12 to 18 months old in which cognitive performance was tested, using Bayley's Mental Development Index, 4 months after blood sampling (Strand et al., 2013). The mean plasma B_{12} was 221 pmol/L and 27% were B_{12} deficient (< 150 pmol/L): there was a positive association between plasma B_{12} and cognitive performance and an inverse association between tHcy and MMA and the cognitive scores. It is noteworthy that the associations with cognition were approximately linear over the whole normal range of B_{12} and its markers (Supplementary Figure S3). An important prospective study in Nepal has shown that B_{12} status in the infant is positively associated with development and performance on social perception tasks and visuospatial abilities at 5 y of age (Kvestad et al., 2017).

Table 6 summarises some of the associations between poor B_{12} status and childhood development according to observational studies cited in the above reviews (see also (Zengin et al., 2009; Demir et al., 2013; Goraya et al., 2015).

Unfortunately, there are relatively few randomised trials in this area. In Norway, 79 infants (mean age 4 months) referred for feeding difficulties and who had elevated tHcy were given a sham injection or a single intramuscular injection of hydroxocobalamin (0.4 mg): one month later, those treated with B_{12} showed a marked fall in tHcy and MMA and clinically a larger improvement than the placebo group in a test for gross motor development and also in regurgitation (Torsvik et al., 2013). Another randomised trial by the same authors showed that 6 month-old infants with low or low-normal birth weight (2000-3000 g) and modestly elevated tHcy (> 6.5 μmol/L) and who had been exclusively breastfed for > 1 month, had a lower serum B_{12} (median 321 pmol/L) than formula-fed infants (497 pmol/L) and had poorer gross motor development. A single injection of hydroxocobalamin (0.4 mg) in the breastfed infants led to an improvement in motor performance (Torsvik et al., 2015). These studies are important because they raise the idea that exclusive breastfeeding in susceptible groups (e.g., low birth weight, failure to thrive, elevated tHcy) might lead to impaired motor development through an effect on B_{12} status. Unfortunately, these trials did not further evaluate whether the beneficial effect was confined to certain subgroups, e.g., such as infants with more marked elevation of tHcy. Furthermore, the results cannot be extrapolated to healthy infants of mothers with normal B_{12} status.

An important trial has been carried out in New Delhi, in which 1,000 children (aged 6 – 30 months) were randomised in a factorial design to four groups: a paste containing 2 RDAs of B_{12}, folic acid, both, or placebo (folic acid 150 μg; B_{12} 1.8 μg; doses were halved

for infants < 12 months). The vitamins were administered for 6 months after which several outcomes were assessed, including common infections (Taneja et al., 2013), growth {(Strand et al., 2015), hemoglobin (Kumar et al., 2017) and, in a 422 children, cognitive and motor skills were also assessed (Kvestad et al., 2015). Notably, this is a study in a population where mothers often have adhered to a lifelong vegetarian diet, have low B_{12} status before, during and after pregnancy, and where malnutrition is common. Furthermore, the vast majority of mothers were still breastfeeding at the time of inclusion. In the analyses of the effect of treatment on infections and hemoglogin, there was no clear benefit of B_{12} treatment. In terms of growth, the effect of B_{12} was confined to children that were stunted, underweight or wasted. In tests of gross motor performance and problem solving skills, children who received both folic acid and B_{12} showed significantly better scores than those receiving placebo. B_{12} on its own improved gross motor scores, but folic acid alone had no effect. The effect was largest in those with high tHcy (>10 μmol/L), with no apparent effect on those with lower tHcy (Kvestad et al., 2015). It should be noted that in these trials in India, the dose of B_{12} was only twice the RDA for infants. Nevertheless, the treatment lasted for 6 months, and there was a marked improvement in B_{12} status with increase in B_{12} of about 180 pmol/L in those receiving B_{12}. Interestingly, the findings are quite similar to the Norwegian studies in that the effect seems to be largely confined to infants with poor B_{12} status and who have signs of growth impairment.

We conclude that the maternal B_{12} status during pregnancy is an essential determinant of B_{12} status at birth and in infancy. Furthermore, there is no doubt that breastfed infants have lower B_{12} status than non-breastfed infants. However, given the evidence that breastfeeding confers clear health benefits to both mother and child (Bar et al., 2016; Victora et al., 2016), it would be a serious error to recommend against breastfeeding, even exclusive breastfeeding for the recommended time period of 6 months. Rather, *we recommend that a public health initiative should be taken world-wide to provide B_{12} oral supplements to women throughout pregnancy and during the breastfeeding period to women, in particular to those adhering to vegetarian or vegan diet or in regions with overall low intake of animal-derived foods. Furthermore, in children that are exclusively breastfed for a prolonged time, B_{12} status (including tHcy) should be assessed if they had low birth weight or show failure to thrive.*

7. HOW DOES THE BRAIN MEDIATE THE EFFECTS OF B_{12} ON COGNITION?

Deficits in cognition due to B_{12} insufficiency could be due directly to impairment in B_{12}-dependent processes in the brain, or indirectly following an increase in brain homocysteine (Smith & Refsum, 2016), or to a combination of both. It is difficult to distinguish between these alternatives and we will not attempt to do so in what follows. Two main avenues through which low B_{12} or raised tHcy can influence cognition are directly by damage to nerve cells and neuronal networks or less directly by damage to the cerebrovascular system.

7.1 Damage to the cerebrovascular system

Functioning of the nervous system depends upon a well-functioning cerebrovascular system, as shown by the catastrophic consequences when blood supply to a brain region is compromised in ischemic stroke. Raised tHcy is a well-known risk factor for ischemic stroke (Casas et al., 2005), and low-normal B12 is also a risk factor for cerebral ischemia (ischemic stroke or transient ischemic attack) (Spence, 2016; Weikert et al., 2007). Animal studies have thrown light on possible mechanisms: in mice given a B-vitamin deficient diet for 10 weeks, there was a 7-fold increase in tHcy and the mice showed impaired spatial memory and learning (Troen et al., 2008). The cognitive impairment was highly correlated with a rarefaction of the vasculature in the hippocampus, specifically with a shortening of the length of the blood capillaries. Reduced capillary length was also correlated with higher tHcy. These changes in the vasculature occurred without any obvious signs of neurodegeneration, such as gliosis, leading the authors to suggest that changes in the vasculature may be more important mediators of cognitive impairment than effects on nerve cells. The vascular mediation of cognitive impairment caused by raised tHcy has recently been reviewed (Smith & Refsum, 2016; Hainsworth et al., 2016). The latter authors concluded that at physiological concentrations there are important vascular effects of homocysteine on myocyte proliferation, vessel wall fibrosis, impairment of nitric oxide signalling, generation of superoxide, and on pro-coagulant actions. We will focus here on the effects of low B_{12} on the nervous system.

7.2 Damage to the nervous system

While there may be controversy about the extent to which homocysteine-lowering treatments can prevent vascular disease (Spence, 2016), it is clear that raised homocysteine and low B_{12} have unique effects directly on the nervous system, and that many of these effects are probably independent of vascular disease.

There are currently four distinct hypotheses to account for the harmful effects of low B_{12} status on the nervous system, apart from the view that all the harmful effects of low B_{12} are mediated by increased tHcy: (i) accumulation of unusual fatty acids as a result of inactivation of methylmalonyCoA mutase; (ii) inhibition of methylation reactions; (iii) accumulation of toxic cytokines and decline in protective cytokines (Hathout & El-Saden, 2011; Scalabrino, 2009); (iv) increase in cellular prion levels (Veber & Scalabrino, 2015). We only have space to deal with the first two hypotheses and refer the reader to the comprehensive reviews cited for the last two hypotheses.

If we consider that B_{12} insufficiency might cause damage to the nervous system independent of homocysteine, then the obvious pathway is that involving AdoCbl as a cofactor in the conversion of methylmalonylCoA to succinylCoA (Section 1). Impairment in this pathway leads to the accumulation of MMA and to increased amounts of atypical fatty acids, such as odd-chain and branched-chain fatty acids. For a long time, it was thought that these unusual fatty acids might cause neuropathy and cognitive impairment by being incorporated into myelin lipids, but in his landmark review, Metz dismissed this idea: "...a large body of evidence can now be marshalled against the hypothesis that decreased function of AdoCbl is the mechanism of the cobalamin neuropathy." (Metz, 1992). As Metz pointed out, neuropathy is not found in those genetic diseases that lead to accumulation of MMA without effect on methionine synthase function, such as *cblA, cblB* and *Mut/MCM* (Table 1). However, although neurological impairments are not characteristic of these diseases, they do occur in about 10% of such patients and may be due to metabolic intoxication (Nizon et al., 2013).

The alternative hypothesis, proposed by Gandy and Jacobson in 1973 (Gandy et al., 1973), that neurological deficits in B_{12} deficiency are due to impaired methylation reactions was strongly supported by the observation of Scott and colleagues in 1981 that administration of methionine could prevent the subacute combined degeneration of the spinal cord produced in monkeys by exposure to N_2O, which inactivates methionine synthase (Scott et al., 1981).

Low B_{12} status impairs the formation of methionine and so decreases the supply of SAM, the body's main methyl donor. Low B_{12} status also causes increased levels of homocysteine, which, in turn, lead to increased levels of SAH, a potent inhibitor of methylation reactions throughout the body (Deguchi & Barchas, 1971). Thus, we propose the that most of the harmful effects of low B_{12} or high tHcy on the brain are initially caused by impaired methylation due to one or both of these biochemical events. We will distinguish four consequences of deficient methylation in the nervous system: (i) impairment of transmission of signals from one brain region to another, thus disrupting neural networks; (ii) impairment of synaptic mechanisms that mediate cognition; (iii) regional brain atrophy; (iv) formation of Alzheimer-type neuropathology.

Impairment of transmission of signals in the brain. A cardinal sign of B_{12} deficiency is the disorganisation of myelin in the long tracts of the spinal cord. This leads to the phenomenon called 'subacute combined degeneration'. It presumably also underlies the neuropathy found in sensory and motor nerves. A plausible hypothesis to account for the disruption of myelin is that myelin basic protein, which is essential to maintain the compact structure of myelin, needs to be methylated at one of its 18 arginine residues (residue 107) to facilitate the compaction of myelin (Kim et al., 1997). The enzyme that methylates this residue is protein methylase-1, and inhibition of this enzyme in embryonic neuronal cultures leads to loss of compact myelin structure (Amur et al., 1986). Deficient methylation due to B_{12} deficiency or in the presence of elevated tHcy would therefore lead to disruption of the compact structure of myelin that is essential for its normal function. This effect on myelin structure could explain the changes observed in the cerebral white matter (i.e., areas of the brain mainly made up of myelinated axons) that are revealed as regions of hyperintensity in MRI scans. These hyperintensities are often attributed to small vessel disease, but it appears that they can also be caused by high tHcy (reviewed in (Smith & Refsum, 2016) or by low B_{12} status (de Lau et al., 2009; Graber et al., 2010; van Overbeek et al., 2013). It was found in the Rotterdam scan study that such white matter damage could be detected over the entire normal range of holoTC (Supplementary Figure S4) (de Lau et al., 2009). The white matter lesions in people who are B_{12}-deficient can in part be reversed by B_{12} treatment, as shown in Supplementary Figure S5 (Vry et al., 2005), so it is unlikely that they are caused only by small vessel disease in these patients. Reversibility of MRI changes in the white matter of the spinal cord after B_{12} treatment has also been reported in 15 out of 29 cases of subacute combined degeneration (Ertan et al., 2002).

A more direct way of revealing the integrity of white matter tracts in the brain is the MRI technique called Diffusion Tensor Imaging (DTI), based on restrictions in the random movements of water molecules by macromolecules and myelin. Application of DTI to patients with B_{12} deficiency has revealed widespread abnormalities in many white matter tracts, notably corpus callosum and fornix; these abnormalities were partly reversed upon treatment with B_{12}. Furthermore, they were correlated with cognitive impairment in several domains (Gupta et al., 2014a, b). Importantly, one of the abnormalities found in B_{12}-deficient patients was an increase in radial diffusivity in white matter tracts (Supplementary Figure S6) (Gupta et al., 2014b), which reflects disruption of myelin (Song et al., 2002).

Since white matter tracts link regions of the brain involved in networks that underlie cognition, it can be predicted that the operation of these networks will be compromised in B_{12} insufficiency. This outcome has been demonstrated in a resting state functional MRI study from India (Gupta et al., 2016). These authors showed that the regional homogeneity was lower overall in the cerebral cortex of patients with B_{12} deficiency (116 pmol/L) than in control subjects. Regional homogeneity correlated with cognitive test scores in several domains, which were lower in the patients with B_{12} deficiency. Regional homogeneity was particularly low in regions that are components of networks important for cognition, such as the default mode network, where the hippocampus and the medial prefrontal cortex showed deficits. It is notable that several of these deficits were reversed upon B_{12} treatment for 6 weeks (Supplementary Figure S7), as were the deficits in neuropsychological tests. This elegant study shows that B_{12} deficiency leads to impairment in the functioning of neural networks in the brain, notably the network that includes the hippocampus, a structure that is crucial for memory.

Another fine-structural MRI study (Kobe et al., 2016) has examined the hippocampus in subjects with MCI using DTI to assess the mean diffusivity of water molecules in this region: a high mean diffusivity indicates impaired structural integrity of nerve cells and their processes in gray matter. Two subgroups of MCI patients were compared: those with low-normal B_{12} status (mean 253 pmol/L; range 153 – 303) and those with high-normal B_{12} status (mean 463 pmol/L; range 306 – 934). Those with low-normal B_{12} status performed less well on tests of episodic memory (learning ability and recognition memory), and on DTI/MRI, they had an increased mean diffusivity specifically in a small subfield of the hippocampus (CA4 and dentate gyrus). Mediation analysis showed that the microstructural impairment in this specific subfield mediated 48% of the association between low B_{12} status and the deficit in learning ability (Kobe et al., 2016). This landmark study shows, firstly, that B_{12}-deficiency

is not essential for impairment of memory since it already occurs at low-normal B_{12} levels and, secondly, that the memory deficit is in part directly caused by damage to the neurons in the hippocampus; wider implications of these results have been discussed elsewhere (Smith, 2016).

A different way of looking at the functioning of neural networks in the brain is Single Photon Emission Computed Tomography (SPECT), which reveals regional blood flow. A SPECT study on 12 patients with low B_{12} status (185 pmol/L), most with cognitive impairment, showed impairments in blood flow bilaterally in frontotemporal regions and in some cases in the prefrontal cortex that correlated with cognitive impairments (Tu et al., 2015).

We can conclude that low B_{12} status has profound effects on the functioning of neural networks in the brain and that this can account for many of the associations with cognition.

Impairment of synaptic mechanisms that mediate cognition. Omega-3 fatty acids play a crucial role in the structure and function of synapses in the brain (Bazinet & Laye, 2014), and they are involved in the synaptic plasticity that underlies learning and memory (Thomazeau et al., 2017). The omega-3 fatty acids are mainly present in brain in an esterified form in phospholipids such as phosphatidylcholine where they are involved in maintenance of membrane structure.

[Figure 5 near here]

Importantly, phosphatidylcholine species that contain omega-3 fatty acids can be formed by methylation of phosphatidylethanolamine, using SAM as methyl donor. Hence, a deficit in B_{12} could lead to impaired synthesis of omega-3-containing phosphatidylcholine by decreasing the supply of methionine and SAM, leading to the build-up of homocysteine and so of SAH, which inhibits the methylation reactions (Figure 5). A seminal paper by Selley in 2007 (Selley, 2007) used this hypothesis to account for his findings in patients with AD. Selley found that the raised tHcy and plasma SAH in patients with AD were associated with the following changes: a decrease in erythrocyte membrane content of phosphatidylcholine, a decreased proportion of docosahexaenoic acid in erythrocyte phosphatidylcholine, but an increase in the erythrocyte content of phosphatidylethanolamine. In cognitively normal men, a similar relationship has been reported: high tHcy is associated with lower levels of omega-3 fatty acids in plasma phospholipids (Li et al., 2006). Significantly, the latter authors also found a positive correlation between serum B_{12} and plasma omega-3 fatty acids. A study on

chick embryonic brain is consistent with the hypothesis (Miller et al., 2003): exposure of chick embryos to homocysteine led to a decrease in brain phosphatidylcholine; an increase in brain phosphatidylethanolamine; and a 76% fall in brain docosahexaenoic levels. These striking changes would lead to changes in synaptic membrane fluidity and organisation (Shaikh & Teague, 2012). Thus, through its role in the methylation of phosphatidyl-ethanolamine, B_{12} is likely to play a key role in the structure and function of synaptic membrane lipids and proteins (Sidhu et al., 2016). It is therefore of interest that beneficial interactions have been observed between omega-3 fatty acids and B vitamins in relation to brain atrophy (Jernerén et al., 2015) and to cognitive decline (Oulhaj et al., 2016) in people with MCI.

Another possible role for B_{12} in synaptic mechanisms related to cognition has been revealed by the surprising finding that DNA methylation is required for memory formation and consolidation involving hippocampal synapses. Furthermore, the synaptic plasticity believed to underlie memory is impaired by inhibitors of DNA methylation, although the precise molecular mechanisms linking DNA methylation with memory consolidation are not known (Day & Sweatt, 2011). A similar role for the methylation of lysine residues of histones in the consolidation of hippocampal long-term memory formation has been described (Gupta et al., 2010). These findings provide a novel way in which alteration in B_{12} status might influence cognition *via* epigenetic mechanisms.

Regional brain atrophy. Descriptions of B_{12} deficiency in very young children often mention that the brain shows atrophy. For example, brain atrophy was found in 8 out of 15 infants in one study (Taskesen et al., 2012), in 7 out of 14 in a second study (Ekici et al., 2016) and in 9 out of 9 in another study (Goraya et al., 2015). In several reports on infants, it was shown that treatment with B_{12} (usually intramuscular) could reverse some or all of the signs of atrophy and some of the associated cognitive deficits (Casella et al., 2005; Glaser et al., 2015; Lövblad et al., 1997); an example is shown in Figure 6.

[Figure 6 near here]

In adults with B_{12} deficiency, reports in which neuroimaging methods have been used to examine the brain are rare, and as a result, there are few descriptions of atrophy. There are, however, many studies on brain atrophy in adults in relation to tHcy. These studies were reviewed recently (Smith & Refsum, 2016) and so will not be discussed here. There are some

reports on brain volume in adults with B_{12} in the normal range. In a study on 32 elderly volunteers, it was found that total B_{12} intake (diet plus supplements = mean of 62 μg/day) was related to the volume of the left and right superior parietal lobules, with a greater intake being associated with a larger volume (Erickson et al., 2008). A cross-sectional study in Chicago on 121 elderly people found that serum B_{12} (mean 331 pmol/L) was not associated with total brain volume, but that higher MMA and tHcy were associated with a reduced total brain volume (Tangney et al., 2011).

[Figure 7 near here]

Two longitudinal studies, in the UK and Sweden, have shown that B_{12} status, assessed either by total serum B_{12} or holoTC, is inversely associated with the rate of whole brain atrophy (Hooshmand et al., 2016; Vogiatzoglou et al., 2008). The important finding was that the association extended over the whole normal range of B_{12} (200 to 600 pmol/L) or holoTC (Figure 7). This result is consistent with the extension of some clinical associations with B_{12} over a greater range than the classical cut-off for deficiency, as discussed in Section 4. Despite normal serum levels of B_{12}, it appears that in the brain, the concentrations of some forms of B_{12} may is reduced in the elderly. A recent study showed that there was a striking 10-fold decline in methylcobalamin levels in aging (Zhang et al., 2016). This unexpected finding may partly explain the increased sensitivity of neurological functions to low B_{12} with ageing. The study needs replicating.

The most detailed study of brain atrophy in relation to B vitamins is the VITACOG trial, summarised in Section 4.4. In this trial in people with MCI, the placebo group showed a rate of whole brain atrophy of 1.08% per y while those treated with B vitamins for 2 y showed an atrophy rate of 0.76% per y. The slowing of atrophy depended upon baseline tHcy and for those in the top quartile (> 13 μmol/L) the rate was slowed by 53% in the B vitamin group (Smith et al., 2010). In the B vitamin treated group, the plasma B_{12} increased from a geometric mean of 330 pmol/L to 672 pmol/L after 2 y of treatment. The greater the improvement in B_{12} status (assessed by plasma B_{12}, holoTC or TC saturation), the slower the rate of atrophy. More detailed analysis showed that the rate of atrophy of those regions of the brain that shrink in Alzheimer's disease (such as the hippocampus) was slowed by almost 90% by the B vitamin treatment (Supplementary Figure S8) and that the likely driver of this effect was the supplementation with B_{12} (Douaud et al., 2013). By means of a Bayesian

directed acyclic graph analysis, causal links mediating the modifying effect of B_{12} on cognitive decline were revealed (Figure 8).

[Figure 8 near here]

Subsequent work showed that the effects of the B vitamins on brain atrophy (Jernerén et al., 2015) and on cognitive decline (Oulhaj et al., 2016) depended upon the omega-3 fatty acid status of the subject; only those participants with a good omega-3 status benefitted from the B vitamin treatment.

Formation of Alzheimer-type neuropathology. Cognitive impairment due to B_{12} insufficiency could be an early expression of the role of low B_{12} in the causation of AD (see Section 5.2). So is there any evidence that low B_{12} is related to the formation of the two cardinal histopathological markers of AD, beta-amyloid and phosphorylated tau? There is little direct evidence on this question, but a great deal of evidence that raised tHcy is indeed related to these markers, as reviewed elsewhere (Zhuo et al., 2011; Fuso, 2013; Smith & Refsum, 2016; Sontag & Sontag, 2014). The basic hypotheses are that low B_{12} (or folate) status leads to raised tHcy which, via its effect on SAH, (i) inhibits the methylation of the promotor regions of genes that generate beta-amyloid, so leading to increased expression of these genes; and (ii) inhibits the methylation of protein phosphatase 2A, which inactivates the enzyme that converts phosphorylated-tau into tau, thereby causing a build-up of neurofibrillary tangles.

In animal experiments where beta-amyloid formation is increased in hyperhomocysteinemia, increased deposition of beta-amyloid and the accompanying memory deficits can be reversed by administration of SAM (Fuso et al., 2012). Evidence more directly related to B_{12} is that the greater dietary intake of B_{12} over the range 1 to 13 μg per day in older humans, the lower the beta-amyloid load is in the parietal cortex, as revealed by PET scans (Mosconi et al., 2014). Plasma B_{12} was inversely related to the CSF concentration of amyloid-beta$_{1\text{-}42}$ in another study on normal elderly, with a regression coefficient almost as large as that for tHcy (Oikonomidi et al., 2016).

There is considerable evidence of a link between raised tHcy and the formation of phosphorylated tau and of neurofibrillary tangles (Sontag & Sontag, 2014). In patients with AD, it has been found that raised tHcy up to 10 y before death is associated with an increased density of neurofibrillary tangles in the cerebral cortex (Hooshmand et al., 2013), but not with beta-amyloid deposition. In normal elderly as well as in AD patients, there is a

correlation between the CSF concentrations of SAH and phosphorylated tau and an inverse correlation between the ratio of SAM/SAH and phosphorylated tau (Obeid et al., 2007; Popp et al., 2009). Experiments in rats have shown that hyperhomocysteinemia leads to deposition of hyperphosphorylated tau in the hippocampus and to spatial memory deficits, while also activating several protein kinsases and inhibiting protein phosphatase 2A. It is striking that all these effects of hyperhomocysteinemia were reduced or abolished by administration of folate and B_{12} to the rats (Wei et al., 2011). In humans, the formation of hyperphosphorylated tau and deposition of neurofibrillary tangles is strongly linked to the degree of cortical atrophy and to cognitive impairment (Xia et al., 2017).

We conclude that there are multiple possible mechanisms in the brain through which B_{12} status could influence cognition. It is significant that several of these mechanisms are reversible. These results are consistent with clinical trials in which treatments that include B_{12} can slow or prevent cognitive decline. More such trials are needed.

8. Conclusions

There is a considerable interest today in B_{12} even nearly 200 years since the first probable description of pernicious anemia (Combe, 1824) and just over 60 years since the structure of B_{12} was discovered (Hodgkin et al., 1956). One thing is clear: it is not appropriate to tell people that all they need is "a good, well-balanced diet". So far as B_{12} is concerned, tens of millions of people in the world do not get sufficient B_{12} for optimum health due to dietary inadequacy and/or to decreased ability to absorb B_{12}. More research is needed to identify the best way to rectify this situation and to understand better why B_{12}, '*Nature's most beautiful cofactor*' (Stubbe, 1994), is so important for health. In the meantime, we agree with the consensus statement in the recent Primer on B_{12} deficiency: 'Universal improvement of B_{12} status… seems to be a nutritional imperative with possibly profound beneficial effects on the nervous system, particularly at the bookends of life.' (Green et al., 2017).

Table 1 Genes associated with B_{12} metabolism and transport

Complementation group	Protein name	Phenotype	Function
cblA/MMAA		Adenosylcobalamin deficiency in cells	P-loop GTP'ase – is thought to functions in the delivery of the adenosylcobalamin from the adenosyltranferase to the methylmalonylCoA mutase. Acts to protect the activity of the mutase.
cblB	Adenosyltransferase	Adenosylcobalamin deficiency in cells	Adenosylates cobalamin in ATP-dependent manner
cblC/MMACHC		Unable to convert cyanocobalamin into biological forms	Removes upper alkyl or cyano ligand from cobalamin.
cblD		Improper targeting of cobalamin to cognate enzymes	Branching of cobalamin within the cell to either the cytosol or mitochondrion
cblE/MTRR	Methionine synthase reductase	Inactive methionine synthase	Performs the reductive methylation of cobalamin to generate methylcobalamin from cob(II)alamin.
cblF/LMBRD1		Cobalamin accumulation within lysosome	Likely helps in the transport of cobalamin out of the lysosome.
cblG/MTR	5-methyl-tetrahydrofolate: homocysteine methyltransferase or methionine synthase	Build-up of homocysteine	Performs the transfer of a methyl group from methyltetrahydrofolate to homocysteine to produce methionine
cblJ/ABCD4		Cobalamin accumulation within lysosome	Transporter.
Mut/MCM	MethylmalonylCoA mutase	Accumulation of methylmalonic acid	The enzyme responsible for the reversible interconversion of methylmalonylCoA and succinylCoA.

Table 2. Summary of prevalence of B_{12} deficiency

(Defined as serum/plasma B_{12} < 148 or 150 pmol/L)

Derived from data in Supplementary Table 1S.

Category	Number of cohorts	Number of subjects	B_{12} deficiency (%, rounded)
Adults (under 60y)	18	81,438	6
Women (non-pregnant)	16	18,520	16
Women pregnant	11	11,381	27.5
Children	14	22,331	12.5
Elderly (over 60y)	25 23, excl. China 2 (China)	30.449 26,249 4,200	19 10 75

Table 3. Summary of prevalence of B_{12} inadequacy/insufficiency

(Defined as serum/plasma B_{12} < 220, < 258, or < 300 pmol/L, i.e., the group with deficient or low-normal B_{12} levels associated with signs of metabolic insufficiency with elevated tHcy and/or MMA).)

Derived from data in Supplementary Table 1S.

Category	Number of cohorts	Number of subjects	B_{12} inadequacy (%, rounded)
Adults (under 60 y)	18	66,529	33
Women (non-pregnant)	11	4,681	51
Women pregnant	4	2,848	60
Children	10	19,704	29
Elderly (over 60 y)	12 (excl. China)	19,023	31

Table 4. Clinical outcomes found in the zone of metabolic B_{12} insufficiency without deficiency

Outcome	Type of study	References
Neural tube defect	Prospective (in mothers)	(Kirke et al., 1993; Molloy et al., 2009)
Infantile tremor syndrome	Prospective (in mothers)	(Goraya et al., 2016)
Cognitive deficit in elderly	Cross-sectional	(Hin et al., 2006; Mizrahi, Lubart, & Leibovitz, 2017)
Cognitive decline in elderly	Prospective	(Clarke et al., 2007; Hooshmand et al., 2012; Tangney et al., 2009)
Alzheimer's disease	Cross-sectional	(Clarke et al., 1998; Refsum & Smith, 2003)
White matter damage	Cross-sectional	(de Lau et al., 2009; Graber et al., 2010; van Overbeek et al., 2013)
Impaired regional brain microstructure and memory impairment	Cross-sectional	(Kobe et al., 2016)
Whole brain atrophy	Prospective, Cross-sectional	(Hooshmand et al., 2016; Tangney et al., 2011; Vogiatzoglou et al., 2008)
Depression	Prospective	(J. M. Kim et al., 2008)
Response to treatment of depression	Prospective	(Hintikka et al., 2003)
Stroke	Prospective	(Spence, 2016; Weikert et al., 2007)
Cognitive and motor development in children	Prospective Intervention	(Strand et al., 2013; I. Torsvik et al., 2013; I. K. Torsvik et al., 2015; Kvestad et al., 2017; Kvestad et al., 2015)
Age-related macular degeneration	Prospective	(Gopinath et al., 2013)
Low bone mineral density in women	Cross-sectional	(Dhonukshe-Rutten et al., 2003)
Autonomic dysfunction (Orthostatic hypotension)	Clinical (head-up tilt) Treatment intervention	(Ganjehei et al., 2012; A. Moore et al., 2004; Oner et al., 2014)
DNA damage in lymphocytes	Treatment intervention	(Fenech,et al., 1998)
Uracil mis-incorporation into DNA	Cross-sectional	(Kapiszewska et al., 2005)

This Table is an expanded version of one given in reference (Smith & Refsum, 2012).

Table 5. Clinical trials of B_{12} in older adults in relation to neurological or cognitive outcomes

Trial subjects	N	Dose of B_{12}	Duration (months)	Outcome and *interpretation*	Reference
Patients with low B_{12} (112 pmol/L): Dementia Cogn. Impairment	132 22	Parenteral, but no details given	7.5 9	Dementia: no difference in cognition in B_{12} treated group cf. matched untreated group with normal B_{12}. Cogn. impairment: improved score on verbal fluency in B_{12} group cf. matched untreated group with normal B_{12}. *Cannot be interpreted since no information about dose and timing of B_{12} treatment.*	(Eastley et al., 2000)
High plasma MMA (≥ 0.4 μmol/L) Median B_{12}: 254 and 278 pmol/L. Randomised	140	1 mg i.m. weekly for 4 weeks	Follow-up at 3	Overall no improvement in neurological scores in B_{12} group, but significant improvements in neurological symptom scores in subjects with baseline MMA ≥ 0.6 μmol/L; tHcy ≥ 15 μmol/L and trend for B_{12} ≤ 250 pmol/L. *Treatment period very short but possible beneficial effect in subjects with worse baseline B_{12} status*	(Hvas et al., 2001)
High plasma MMA (same subjects as Hvas 2001) Randomised	140	1 mg i.m. weekly for 4 weeks	Follow-up at 3	Cognitive scores improved in both placebo and B_{12} groups. *Improved scores likely to be a learning effect. Treatment period too short*	(Hvas, et al., 2004)
Mild B_{12} deficiency (100-200 pmol/L; or MMA ≥ 0.32 μmol/L) Randomised	111	1 mg orally, daily	6	Cognitive scores in several domains 'improved slightly' in both placebo and B_{12} groups; improvement in memory in placebo was significantly better than in B_{12} group. *No decline, and often improvement, in placebo group so difficult to conclude anything about treatment.*	(Eussen et al., 2006)
Dementia (serum B_{12} < 200 pmol/L)	21	1 mg i.m., then daily orally	10	No cognitive improvement, slight improvement in delirium. *Open trial, no placebo group*	(Kwok et al., 2008)
Dementia (serum B_{12} < 184 pmol/L)	28	1 mg i.m., repeated	4	No significant clinical benefits. *Open trial, no placebo group*	(van Dyck et al., 2009)
Adults with neurological	33	1 mg i.m., repeated	6	Functional neurological recovery in the 'majority'.	(Kalita et al., 2013)

syndrome (B_{12} < 155 pmol/L)				Cognitive improvement in several domains. P3 improved. *Lack of placebo group and imprecise reporting*	
Asymptomatic elderly (80y) with low-normal B_{12} (median 228 pmol/L). 'OPEN' randomised trial	201	1 mg daily oral	12	Neurological measure: primary outcome was tibial compound muscle action potential. Slight (non sign.) increase in placebo; no change B_{12} group. Cognitive measure: word-list memory. Slight increase in both placebo and B_{12} groups, but no difference. *Lack of decline in placebo group scores so difficult to conclude anything about treatment.*	(Dangour et al., 2015)
Asymptomatic elderly (80 y) with low-normal B_{12} (median 228 pmol/L). 'OPEN' randomised trial	201	1 mg daily oral	12	Analysis based on subgroups with differing baseline B_{12} status showed no significant effect of B_{12} treatment on primary outcome of tibial compound muscle action potential in those with low B_{12} status at baseline. *Difficult to interpret as placebo data not reported*.	(Miles et al., 2017)
Elderly diabetic patients with low B_{12} (Mean 227 pmol/L)	271	1 mg daily oral methylcobalamin	27	Both groups showed cognitive decline but no difference in CDR or cognitive tests between treated and placebo groups. However, placebo group cognitive scores tended to increase. *Lack of decline in placebo group scores, so difficult to conclude anything about treatment.*	(Kwok et al., 2016)

Trials were included if B_{12} was the only treatment administered

Table 6. Some associations of B_{12} deficiency and poor B12 status with childhood development [1]

Neurological development	*Cognitive development*
Hypotonia	Impairment in social perception
Hyopkinesia	Impaired visuospatial abilities
Lethargy	Lower mental development scores
Ataxia	Impaired attention
Insufficient head control	Impaired short-term memory
Involuntary movements	Impaired academic performance at school
Swallowing dysfunction	Absenteeism from school
Seizures	Grade repetition at school
Brain atrophy	
White matter disease	

[1]Signs of neurological development have mainly been seen in infants with severe B12 deficiency, while signs of impaired cognitive impairment also include children with low B12 status during early childhood.

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FIGURE LEGENDS

Figure 1. Structure of vitamin B_{12}

The structure of cyanocobalamin is shown. The main biological forms of cobalamin have the cyano group replaced with an adenosyl, methyl or hydroxyl group, which are found in adenosylcobalamin, methylcobalamin and hydroxocobalamin, respectively.

Figure 2. The trafficking of cobalamin within humans

B_{12} is absorbed from the diet and passed via to transcobalamin (TC) in the bloodstream. The TC-B_{12} complex binds to the cubam receptor, where it is transported into the lysosome, where the TC is degraded and the B_{12} released. The nutrient is then transported via the CblJ and CblF transporters into the cytosol, where the B_{12} is acted upon by CblC, and any upper alkyl or cyano group (X) is removed. For methionine synthase (MTR), the B_{12} is chaperoned by the action of CblD and CblE to ensure correct delivery within the cytoplasm. For methylmalonylCoA mutase activity (MMCM), CblD ensures delivery to the mitochondrion, where the molecule is transported to the inside of the organelle by an unknown transporter. Once inside, the action of CblB and CblA ensure the molecule is adenosylated and delivered to the enzyme.

Figure 3. Defining B_{12} inadequacy by use of markers of metabolic insufficiency

Relationship between plasma vitamin B_{12} and plasma total homocysteine (tHcy) or methylmalonic acid (MMA) in 3262 community-dwelling people aged 71–74 year in Norway. Based on Figure 1 from (Smith & Refsum, 2012).

Figure 4. Blood level of B_{12} in relation to oral intake

Serum or plasma vitamin B_{12} concentration (pmol/L) as a function of dietary vitamin B_{12} intake (μg/d) estimated by using random-effects meta-analyses of a combination of observational studies and randomised controlled trials. Based on Figure 3 from (Dullemeijer et al., 2013).

Figure 5. Interface between homocysteine (Hcy), B vitamins, and omega-3 fatty acids

Elevated total Hcy (tHcy) leads to elevated S-adenosylhomocysteine (SAH), which inhibits the methylation of phosphatidylethanolamine to phosphatidylcholine by phosphatidylethanolamine *N*-methyltransferase (PEMT). Lowering tHcy by B vitamins releases this inhibition and allows phosphatidylcholine enriched in omega-3 fatty acids to be synthesized. *Abbreviations*: Met, methionine; SAM, S-adenosylmethionine; THF, tetrahydrofolate.

Figure 6. Brain atrophy in a child with B_{12} deficiency and its reversal by B_{12} treatment

A six-month old girl of vegetarian parents had serum B_{12} of 92 pmol/L and serious neurological impairment. MRI of the brain showed marked atrophy, especially in the frontal and temporal lobes (A). After 5 months of daily treatment with B_{12}, there was regression of atrophy and neurological signs were normal (B). Figures 2 and 5 from (Lövblad et al., 1997).

Figure 7. Rate of brain atrophy in elderly in relation to serum B_{12} concentration

Annual rate of brain atrophy in 107 volunteers (mean age 73.2 y) measured over 5 years. There was no obvious threshold, and the linear association between atrophy rate and B12 concentration indicates that up to 13% of the variance in atrophy rate can be explained by B12. Solid line mean, dashed lines show the 95% confidence intervals. Based on Figure A from (Vogiatzoglou et al., 2008).

Figure 8. VITACOG trial: pathway mediating the effect of B vitamin treatment on cognition

A directed acyclic graph analysis of B vitamin treatment and consequential changes in brain structure and function in mild cognitive impairment. The mediating pathway (causal links indicated by arrows) shows the optimal Bayesian network that explains the findings from the VITACOG trial. From data in (Douaud et al., 2013).