EFFECTS OF SHMT1 HETEROZYGOSITY ON MOTOR COORDINATION AND PERIPHERAL NEUROPATHY

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Folates, in the form of tetrahydrolates, serve as cofactors for enzymes in a biological network of metabolic pathways that are necessary for the *de novo* synthesis of purines, thymidylate, and the vitamin B_{12} dependent-remethylation of homocysteine to methionine. Impaired folate-mediated one-carbon metabolism has been identified as a risk factor for neurodegeneration and disrupted nerve function, however the pathway(s) are not clearly defined. Serine hydroxymethyltransferase 1 (SHMT1) is a folate-dependent enzyme that serves as a scaffold protein for the assembly of a metabolic multi-enzyme complex that regulates *de novo* thymidylate synthesis. Disrupted *Shmt1* expression sensitizes folate-deficient mice to develop folic-acid responsive neural tube defects. Therefore, studying the consequences of impairments in both *Shmt1* expression and one-carbon metabolism may clarify the mechanisms by which folate and vitamin B_{12} deficiencies contribute to peripheral neuropathy.

This research used a mouse model of decreased *Shmt1* expression to identify underlying mechanisms linking impaired folate and vitamin B_{12} metabolism and neuropathy. First, a mouse model of disrupted *Shmt1* expression was used to elucidate

the role of impaired *de novo* thymidylate (dTMP) synthesis in vitamin B_{12} deficiency-induced neurodegeneration in the peripheral and central nervous system. Second, the mouse model of disrupted *Shmt1* expression leading to disrupted *de novo* dTMP synthesis and a second diabetic model of diabetes was used to investigate the role of uracil misincorporation and DNA damage in peripheral neuropathy.

Differences in motor coordination and sensory function in aged *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed vitamin B_{12} and folic acid replete and deficient diets were measured using the accelerated rotarod, staggered rotarod, and tail flick test. Multiple linear and logistic regressive analyses indicated that *Shmt1*$^{+/-}$ mice fed vitamin B_{12} and folic acid deficient diets, as well as dietary folic acid and nucleoside supplemented diets, had significantly lower staggered rotarod motor performance and delayed latencies to tail flick compared to *Shmt1*$^{+/-}$ mice on a control diet. Additionally, dietary deoxyuridine and uridine supplementation did not rescue the impaired motor and sensory effects of vitamin B_{12} and folic acid deficiency in *Shmt1*$^{+/-}$ mice.

Motor and sensory nerve conduction was measured in peripheral nerve tissue of *Shmt1*$^{+/+}$, *Shmt1*$^{+/-}$, *db/db*, and *Shmt1*$^{+/-}$; *db/db* mice fed vitamin B_{12} and folic acid deficient and replete diets. Surprisingly, *Shmt1* heterozygosity significantly impaired motor nerve conduction velocity in the sciatic nerve of mice fed both control and folic acid and vitamin B_{12} deficient diets.

Together, these experiments were novel in their approach of using mouse models of impaired folate mediated one-carbon metabolism to identify a causal relationship between disrupted *de novo* dTMP synthesis and neuropathy. These findings implicate

DNA damage and repair as potential targets of treatment for populations at risk of peripheral neuropathy.

BIOGRAPHICAL SKETCH

Eunice Awuah was born in East Lansing, Michigan where she spent the first 8 years of her life living in Spartan Village until her family moved to Syracuse, New York. In middle school and high-school she pursued her interest in music and had the opportunity to play as a principle flutist in wind ensemble, symphonic orchestra, and marching band. In high-school is also where she developed her interest in both science and math. At the end of her senior year in high-school, she became inspired to pursue a career in cancer research due to the tragic loss of a family member to the disease.

Eunice enrolled at Johns Hopkins University as an undeclared pre-medicine major. After taking a few courses in biology as well as cognitive and abnormal psychology, it became clear that she was interested in learning more about the science behind how the brain worked.

Upon graduating from college in 2009 with a B.A. in Systems Neuroscience, Eunice began working as the study coordinator for Dr. Susan Courtney at the Johns Hopkins Department of Psychological and Brain Sciences until 2012. During these three years, she had the opportunity to recruit and enroll study participants with Multiple Sclerosis for functional MRI studies investigating the role that brain lesions play in altering the recruitment of functional networks in the brain for short-term working memory. It was through this research opportunity that she developed her desire to learn more about research related to nutritional neuroscience in order to understand the role that nutrient deficiencies play in neurodegenerative disease.

Eunice then embarked on graduate training in the Division of Nutritional Sciences at Cornell University where she pursued a PhD in Molecular Nutrition in the fall of 2012. During her time in the Stover Lab, she has had the privilege to conduct research that is closely related to her interests of investigating pathways linking nutritional interventions to improved nerve function and regeneration. Outside of the lab, she has participated in leadership roles in student groups such as the Nutritional Graduate Student Organization, Cornell Multiple Sclerosis Society, and EARS (Empathy, Assistance and Referral Services).

After Cornell, her future career and life goals include becoming a director of a clinical research center, starting a podcast listening club, creating a foundation to raise money for individuals in need of assistive technology devices, and learning how to cook.

To my parents, Dr. Emmanuel and Dr. Agatha Awuah, who have instilled in me the desire to serve others with kindness, compassion, and love. Thank you for all the sacrifices you have made to provide for me and Yaw. The emotional and spiritual guidance you have given over the years has taken me far along this journey called life. I love you and will continue to work hard to make you proud.

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CHAPTER 1: PERIPHERAL NEUROPATHY: MECHANISMS AND IMPLICATIONS FOR DIETARY INTERVENTIONS

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Abstract

Peripheral neuropathy is one of the most common neurological disorders in older patient populations. The current treatment options for peripheral neuropathy address pain management and not the cause or repair of nerve damage. In this review, we present an overview of folate and vitamin B_{12}-mediated pathways linking impaired *de novo* thymidylate (dTMP) synthesis to DNA damage and neuronal cell death in peripheral neuropathy. We review environmental exposures and chronic diseases associated with impaired folate-mediated one-carbon metabolism and peripheral neuropathy. We also discuss studies that support a distinctive nutritional requirement

for high-dose B-vitamin interventions in patients with peripheral neuropathy. Additional clinical studies are needed to identify distinctive tissue-specific and whole-body nutritional requirements for B-vitamin therapy in peripheral neuropathy.

The metabolic origins of peripheral neuropathies

Peripheral neuropathy (PN) is one of the most common neurological disorders in the United States, affecting 2.4% of the general population and approximately 8% of individuals 55 years and older (1). The clinical manifestations of peripheral neuropathy include numbness, tingling, burning sensations, autonomic dysfunction, and muscle weakness. The most common progression of the disorder is symmetric (occurring on both sides of the body), length-dependent neuropathy that begins in the terminals of the longest nerves in the body (i.e. distal foot). Symptoms of numbness or painful tingling ascend to the legs and hands while motor function is affected as the severity of neuropathy progresses (2,3).

Peripheral neuropathies are categorized as either axonal or demyelinating. Approximately 80% of peripheral neuropathies are axonal, which involves the deterioration of both small sensory nerve fibers and motor neurons. The remaining 20% of peripheral neuropathies are demyelinating, meaning that the protective myelin protein surrounding the axon is degraded (2).

Although the etiology of PN is not well understood, many cases have been linked to metabolic pathways involving B-vitamins that participate in folate-mediated one-carbon metabolism (FOCM). Most available treatments for PN only address pain

management and not the specific cause or nutritional needs of this patient population. Increasingly, there is a recognition that disease processes can alter cellular metabolism and nutrient needs, resulting in distinctive nutritional requirements (DNR) for patients that may not be attainable from a normal natural food-based diet. The literature reviewed herein will address the nutritional, pharmacological and environmental exposures, as well as the chronic disease states that lead to PN and support impaired FOCM leading to impaired *de novo* thymidylate (dTMP) biosynthesis as a potential biological etiology for PN. Current evidence in support for altered nutrient needs in this population is also summarized.

Overview of folate-mediated one-carbon metabolism

FOCM is a network of interconnected metabolic and anabolic pathways that require the interaction of several B-vitamin cofactors including folate, vitamin B_{12} and vitamin B_6 (**Figure 1**). Tetrahydrofolate (THF) serves as a cofactor that activates, carries and transfers one-carbon units among anabolic and catabolic pathways (4) (Figure 1). Once bound to THF, one-carbon units are interconverted among three oxidative states which include: 5-methyl-THF, 10-formyl-THF, and 5,10-methylene-THF (5). Each of these forms of THF serve as cofactors that play distinct roles in the *de novo* synthesis of purines and dTMP, and the vitamin B_{12}-mediated remethylation of homocysteine to methionine (5). As such, FOCM is fundamental to DNA synthesis, protein synthesis and cellular methylation reactions.

FOCM occurs in the cytosol, mitochondria, and nucleus (6,7). Mitochondria are responsible for the THF-dependent catabolism of serine, glycine, sarcosine and dimethylglycine to formate, which serves as a source of one-carbon units for cytosolic and nuclear FOCM. Both the nuclear and cytosolic compartment support the THF-dependent, *de novo* synthesis of dTMP, which is necessary for DNA synthesis and stability (5). The folate-dependent enzymes that generate THF-activated one-carbons include serine hydroxymethyltransferase 1 and serine hydroxymethyltransferase 2α (SHMT1 and SHMT2α) which generate 5,10-methyleneTHF, and methylenetetrahydrofolate dehydrogenase 1 (MTHFD1) which generates both 10-formylTHF and 5,10-methyleneTHF. Methylenetrahydrofolate reductase (MTHFR) reduces 5,10-methyleneTHF to 5-methylTHF. Serine catabolism supplies the one-carbon unit in the SHMT catalyzed reactions whereas formate is the one-carbon source for the MTHFD1 catalyzed synthesis of 5,10-methylene-THF.

De novo deoxythymidine monophosphate (dTMP) synthesis is catalyzed by thymidylate synthetase (TYMS), which transfers a one-carbon unit from 5,10-methylene-THF to deoxyuridine monophosphate (dUMP) to form dTMP. The *de novo* dTMP biosynthetic pathway consists of SHMT, TYMS, MTHFD1 and dihydrofolate reductase (DHFR) and dTMP biosynthesis occurs in both the cytosol and the nucleus of mammalian cells. The enzymes that constitute this pathway undergo cell cycle-specific covalent modification with small ubiquitin-like modifier (SUMO) protein, referred to as SUMOylation, which enables nuclear translocation during S-phase. SHMT1 and SHMT2α have redundant functions and serve as scaffold proteins that

enable the formation of a multienzyme complex with DNA and nuclear lamin proteins, such that the *de novo* dTMP biosynthesis pathway is localized to sites of DNA synthesis (8,9).

In the cytosol, folate cofactors, in the form of 10-formyl-THF support *de novo* purine synthesis, whereas 5-methyl-THF supports the vitamin B_{12}-dependent remethylation of homocysteine to methionine (7,10). Methionine is converted to the cofactor S-adenosylmethionine (SAM) through the activity of methionine adenosyltransferase (MAT). SAM is the major methyl donor in the cell that is involved in numerous methylation reactions for metabolic and anabolic pathways, as well as the regulation of gene expression through epigenetic processes such as DNA and histone methylation (11).

B-vitamin deficiency and neuropathy

Natural folates in the diet and in the body are mostly present in the form of 5-methyl-THF (4). This form of folate is then converted to THF by the vitamin B_{12}-dependent enzyme methionine synthetase (MTR) during the conversion of homocysteine to methionine. This reaction is essential both to remethylate homocysteine to methionine as well as to ensure THF is generated to support nucleic acid synthesis. Hence, reductions in MTR activity, as occur in vitamin B_{12} deficiency, can compromise the entire FOCM network through a trapping of folate cofactors as 5-methylTHF, leading to impairments in DNA synthesis and subsequent genome instability (12,13,14).

The demand for folate-activated one-carbon units is highest during proliferative states such as fetal growth and development. Impairments in FOCM during fetal development lead to birth defects known as neural tube defects (15,16,17). These defects are characterized by the failure of neural tube closure during early pregnancy, which results in a range of severe clinical outcomes including spina bifida. In adults, folate and vitamin B_{12} deficiency can result in megaloblastic anemia which is characterized by enlarged red blood cell precursors due to impaired DNA synthesis and cell division (18).

Vitamin B_{12} deficiency is the most common nutritional cause of megaloblastic anemia, followed by folate deficiency (18). In the stomach, dietary vitamin B_{12} binds to intrinsic factor, which is secreted by gastric parietal cells. The autoimmune destruction of gastric parietal cells, known as pernicious anemia, leads to the inhibition of vitamin B_{12} absorption due to low levels of intrinsic factor (19). Vitamin B_{12} deficiency is caused mainly by low dietary intake, as in the case of vegans and vegetarians, and malabsorption due to the depletion of intrinsic factor. The range of neurological manifestations of vitamin B_{12} deficiency range from peripheral neuropathy to subacute combined degeneration of the spinal cord, which affects both the peripheral and central nervous systems (20, 12). Neurodegeneration of both the dorsal and ventral columns of the spinal cord results in painful and abnormal sensations in the lower and upper extremities and in severe cases, the loss of muscle control and balance.

Risk factors for B-vitamin related peripheral neuropathy

Chronic Disease: Diabetes

Peripheral neuropathy is the most common co-morbidity of diabetes with up to 50-60% of patients with Type 2 diabetes eventually developing diabetic peripheral neuropathy (DPN) (19). The prevalence and severity of DPN is dependent on the duration of hyperglycemia. The uptake of glucose in neurons is facilitated by their high expression of glucose transporters and is not tightly regulated (20). During periods of hyperglycemia, there is an excessive increase in the neuronal uptake of glucose. This leads to an increase in tissue damage due to the production of reactive oxygen species as well as an inflammatory response (21,22).

Reactive oxygen species (ROS)

(ROS) such as superoxide, hydrogen peroxide, and nitric oxide are produced by the mitochondria as a by-product of electron transport. Due to their high demand for energy and elevated lipid content, neural tissues are sensitive to impaired mitochondrial function, which leads to decreased energy production and the formation of ROS (23,24). Biological molecules that are susceptible to being chemically attacked and damaged by ROS include DNA, protein, and lipids (23,24). Oxidative stress leads to the activation of poly ADP-ribose polymerase (PARP), which facilitates DNA repair (25). ROS-mediated overactivation of DNA repair pathways eventually leads to apoptosis and neuronal cell death, which is one proposed causal mechanism linking neuronal tissue damage and PN (26).

Inflammation

Oxidative stress, DNA damage, and inflammation are closely associated with PN. DPN is often preceded by an inflammatory response. A recent systematic review found that patients with Type 2 diabetes with PN have elevated serum levels of tumor necrosis factor-alpha (TNF-alpha) compared to Type 2 diabetic patients without PN (27). TNF-alpha is a pro-inflammatory cell signaling cytokine that plays a role in the acute phase reaction.

Inflammatory neuropathies are characterized by pathogenic leukocyte infiltration of peripheral or dorsal/ventral root nerves. This inflammatory reaction eventually leads to demyelination, axonal degeneration, and is associated with a heterogenous class of disorders that include Gullain-Barre syndrome, chronic inflammatory demyelinating polyradiculoneuropathy and peripheral vasculitis (28). These disorders further support the link between inflammation and PN.

Inflammation is also associated with increased rates of folate and vitamin B_6 catabolism and degradation which leads to impairments in FOCM (29, 30). In a recent study by Ulvik et al, it was reported that the increased catabolism of vitamin B_6 during inflammation was associated with upregulation of enzymes that reduce oxidative and aldehyde stress (31).

Environmental exposure

Chemotherapeutics

Chemotherapeutic agents that impair DNA synthesis and cause genome instability, such as Cisplatin and 5-fluoruracil (5-FU), have also been linked to PN (32,33). Cisplatin serves as an effective treatment for solid tumors by crosslinking with the purine bases of DNA to block DNA repair mechanisms and eventually causes DNA damage and cell death. Up to 30% of patients that receive Cisplatin treatment experience paraneoplastic neuropathy, which refers to nerve damage accompanies cancer as a result of chemotherapy (34). When patients finish treatment, the neuropathy lessens. However, in most cases, the nerve damage is permanent.

5-FU is wildly used in the treatment of a range of cancers, including colorectal and breast cancer. It serves as an antimetabolite that inhibits the folate-dependent enzyme TYMS, which is required for the *de novo* synthesis of dTMP (35). Impaired synthesis of the dTMP base in DNA leads to the misincorporation of uracil in DNA and ultimately futile cycles of uracil excision and DNA repair. This cyclic pattern of uracil misincorporation and DNA repair eventually leads to DNA strand breaks and cell death, which is a proposed mechanism linking 5-FU to PN. dTMP can be salvaged from thymidine through thymidine kinase, which alleviates the effects of 5-FU mediated inhibition of TYMS (36). Although 5-FU associated PN is rare, it is usually associated with the deficiency of the 5-FU catabolizing enzyme, dihydropyrimidine dehydrogenase (23,36).

Metformin

Metformin is the most commonly prescribed medication for the control of blood glucose levels in patients with Type 2 diabetes. Several studies have shown an association between long term use of Metformin and decreased serum levels of vitamin B_{12}. Metformin use has also been associated with elevated levels of plasma homocysteine, indicating a functional deficiency of vitamin B_{12}. (37,38,39)
There is evidence linking Metformin use to symptoms associated with DPN. In a recent study, diabetic patients taking Metformin scored higher on two screening tools used for the assessment of DPN, the Toronto Clinical Scoring System (TCSS) and Neuropathy Impairment Score (NIS). Interestingly, these patients did not exhibit any significant differences in electrophysiological measurements of neuropathy although Metformin dose and duration did correlate with total NIS and TCSS scores (40). These findings highlight the need for the development and use of well-defined and highly sensitive assessment of peripheral nerve function.

Arsenic

Factors that affect DNA integrity and cell death and that also interact with B-vitamin metabolism include arsenic exposure, which is also associated with PN risk. Arsenic is a common contaminant of drinking water due to industrial and agricultural runoff and is a major environmental concern in some parts of the world. With the WHO provisional guideline for drinking water set at 10-50 parts per billion of arsenic, more than 100 million people worldwide are at risk of exposure with more than 45 million people, mainly in India and China, being exposed to the maximum concentration limit (41).

In a study conducted in two districts in West Bengal, India with arsenic-contaminated wells, 41% of 451 subjects displayed symptoms of PN and impaired nerve conduction (42). In another study conducted in the Toruku valley of Japan, many individuals were observed to be affected by some form of sensory neuropathy after exposure to arsenic trioxide gas that was released between 1920 and 1962 from an arsenic mining and refining operation (43).

The causal mechanism linking arsenic to the development of PN is still unknown, largely because arsenic has many biological targets, including folate-dependent enzymes. Arsenic trioxide has been shown to downregulate the expression of TYMS in lung adenocarcinoma cells, SHMT1 and MTHFD1 expression in ovarian cancer cells and increase genomic instability (44,45). Arsenic also increases SUMOylaytion of the enzymes involved in the *de novo* dTMP synthesis pathway, leading to increased degradation of these proteins, decreased dTMP synthesis, and genome instability (45).

Dietary Intake

Dietary sources of folate include foods fortified with folic acid, dietary supplements, and natural folates found in animal products and green vegetables. The recommended daily allowance (RDA) for adults is 240 μg per day. The RDA for vitamin B_{12}, which is found in animal products and dietary supplements, is 1-3 μg per day (18). Deficiencies in folate and vitamin B_{12} are caused by several factors, including insufficient dietary intake, medications (i.e. metformin), diseases that cause

malabsorption (i.e. inflammatory bowel syndrome, pernicious anemia), and increased nutrient requirements due to pregnancy or chronic disease (i.e. hemolysis) (18). Folate deficiency in the United States is less common than in other countries due to fortification of flour, grains, and cereals with folic acid, a synthetic form of folate. The prevalence of vitamin B_{12} deficiency in the United States in the population three years of age and older is approximately 7% (46). Subgroups at a higher risk of deficiency include vegetarians, vegans, and the elderly due to poor intake and malabsorption (46). Even with dietary intake levels that meet the RDA, there are certain individuals that are more susceptible to PN associated with impaired FOCM due to inborn errors of metabolism caused by genetic disorders such as serine, cobalamin C, and MTHFR mutations (47,48).

B-vitamin supplementation and peripheral neuropathy

Although the use of dietary B-vitamin supplementation in the treatment of PN has been the subject of several clinical studies, the evidence for efficacy of B-vitamin treatment in the literature is limited. Few studies have addressed the role of high-dose and long-term B-vitamin supplementation in ameliorating symptoms of PN. A 2008 Cochrane Review summarized the evidence for the use of B-vitamin as an intervention for PN in diabetic and alcoholic populations. Only thirteen studies involving 741 patients met the qualifying criteria (49). Studies that were included reported the short-term (three months or less) and long-term (more than three months) changes in clinical outcomes in relation to the dosage and duration of supplementation with a B-vitamin complex. The supplements included the combination of two or more of the following

water-soluble compounds: thiamine (B_1), riboflavin (B_2), niacin (B_3), pyridoxine (B_6), folic acid (B_9) or cobalamin (B_{12}). Clinical outcomes measured included pain intensity, vibration detection, and nerve conduction. Of the trials included, one trial showed significant short-term improvements in PN as the result of higher doses of B-vitamin complex (50).

These results suggest the need for further interventional studies to determine the efficacy of doses higher than the RDA for specific types and combinations of B-vitamins in the treatment of PN.

Interestingly, a recent clinical trial among elderly, asymptomatic Chileans with vitamin B_{12} deficiency showed significant improvements in peripheral nerve conduction four months after a single high dose intramuscular injection containing vitamin B_{12}, vitamin B_6, and vitamin B_1 (51). Additionally, in a recent study that measured nuclear abnormalities in the oral mucosa cells of diabetic patients, high-dose folic acid supplementation (15mg/day) over a period of one month led to a significant decrease in nuclear abnormalities associated with cell damage and cell death (52).

Conclusions

Although our knowledge of the mechanisms remains unclear, preclinical and clinical evidence supports a biological pathway for disease and environmental exposure-induced B-vitamin deficiency in PN. There are several risk factors that are associated with impaired FOCM such as low dietary intake, diabetes, metformin therapy, chemotherapeutic treatment, and arsenic exposure (**Table 1**). Impaired FOCM leading

to impaired *de novo* dTMP synthesis induces DNA damage that is associated with cell death, loss of nerve function, and PN (**Figure 2**).

Randomized controlled trial data on short and long-term improvements in PN with B-vitamin supplementation is limited due to differences in treatment duration, dosage, and the sensitivity of clinical outcome assessment tools. Since there is a need for identifying distinctive whole-body and neuronal tissue-specific nutritional requirements in patients with PN, more well-designed trials must be conducted in order to determine the efficacy and optimal dosage of B-vitamin supplementation in patient populations suffering from this disorder.

Table 1. Risk factors associated with peripheral neuropathy (PN) and impaired folate-mediated one-carbon metabolism (FOCM)

	Risk factors for PN	Outcomes and Biomarkers related to PN and FOCM	References
Chronic Disease	Diabetes	B-vitamin catabolism and deficiency, DNA damage, apoptosis, myelin degradation, nerve fiber loss and neuropathy	21-31
Environmental Exposure	Chemotherapeutics (5-FU, Cisplatin)	Impaired dTMP synthesis, DNA damage, apoptosis, axonal neuropathy	32-36
	Metformin Therapy	Low serum vitamin B_{12}, elevated homocysteine, and neuropathy	37-40
	Arsenic Exposure	Small and large fiber neuropathy, impaired dTMP synthesis and DNA damage	41-45
	Low Dietary Intake of Folate and Vitamin B_{12}	Elevated homocysteine, impaired dTMP, DNA damage, demyelination, and neuropathy	18, 46-48, 51

Figures

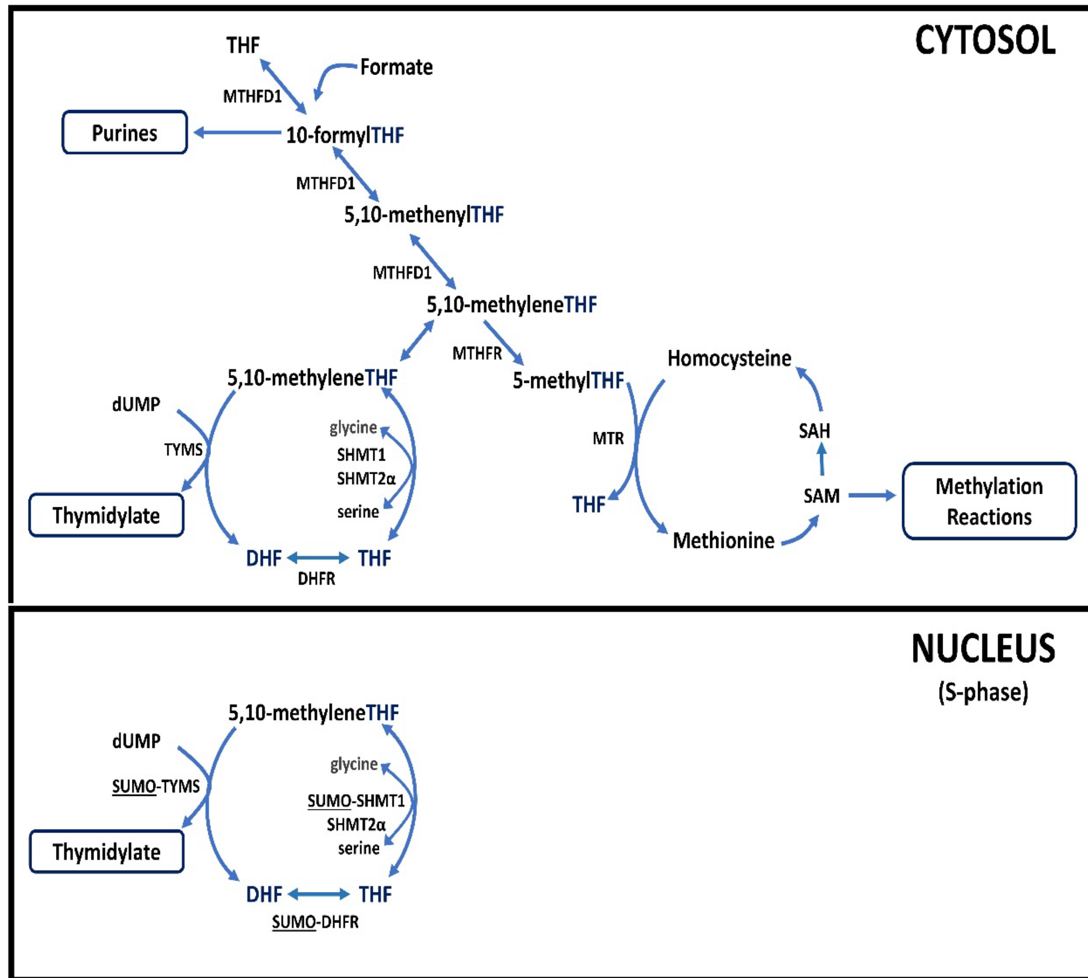

Figure 1. Folate-mediated one-carbon metabolism in the cytosol and nucleus. FOCM is required for the synthesis of purines, dTMP, and methionine. Mitochondrial-derived formate can enter the cytoplasm and function as a one-carbon unit for folate metabolism through the activity of MTHFD1. At S-phase, the enzymes of the *de novo* dTMP synthesis pathway undergo SUMO-dependent translocation to the nucleus. DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, thymidylate; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; SHMT1, serine hydroxymethyltransferase 1; SHMT2α, serine hydroxymethyltransferase 2 alpha; SUMO, small ubiquitin-like modifier; THF, tetrahydrofolate; TYMS, thymidylate synthase. Figure is adapted from *Field MS, Kamynina E, Watkins D, Rosenblatt DS, Stover PJ.* Human mutations in methylenetetrahydrofolate dehydrogenase 1 impair nuclear de novo thymidylate biosynthesis. *Proc Natl Acad Sci U S A. 2015; 112(2):400-5.*

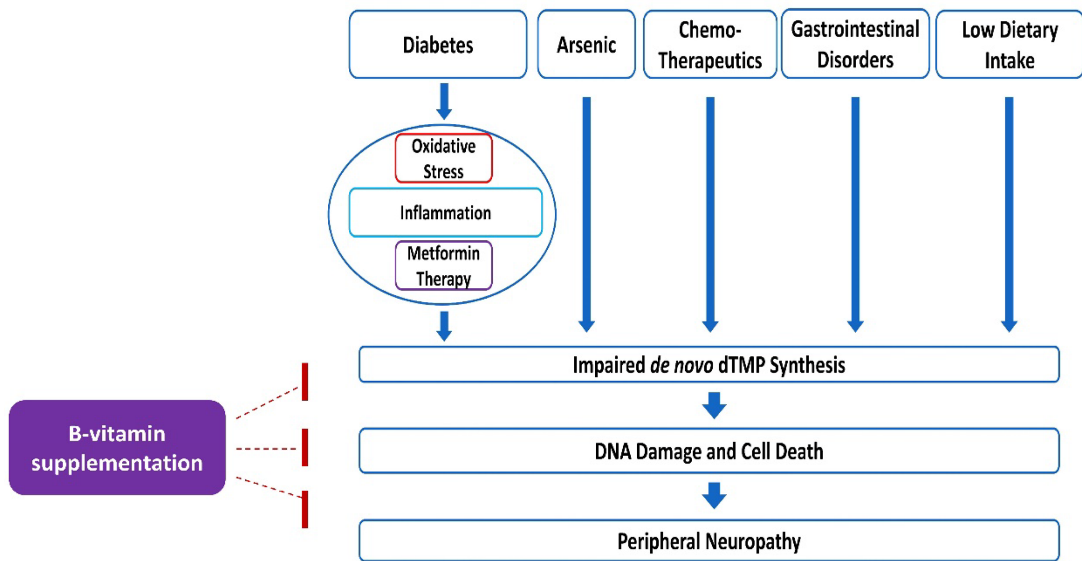

Figure 2. Proposed pathways linking risk factors of PN to impaired FOCM. Studies indicate a link between risk factors for PN, impaired dTMP synthesis, and DNA damage induced neuronal cell death.

References

1. Martyn CN, Hughes RAC. Epidemiology of peripheral neuropathy. J Neurol Neurosurg Psychiatry. 1997;62:310–318.
2. Hughes RAC. Peripheral Neuropathy. BMJ. 2002; 324(7335): 466–469
3. Watson JC, Dyck PJ. Peripheral Neuropathy: A Practical Approach to Diagnosis and Symptom Management. Mayo Clin Proc. 2015;90(7):940-51.
4. Wright AJA, Dainty JR, Finglas PM. Folic acid metabolism in human subjects revisited: potential implications for proposed mandatory folic acid fortification in the UK. Br J Nutr. 2007;98:667–675.
5. Ducker GS, Rabinowitz JD. One-Carbon Metabolism in Health and Disease. Cell Metab. 2017;25(1):27-42.
6. Tibbetts AS, Appling DR. Compartmentalization of Mammalian folate-mediated one-carbon metabolism. Annu Rev Nutr. 2010;30:57–81.
7. Ducker GS, Chen L, Morscher RJ, Ghergurovich JM, Esposito M, Teng X, Kang Y, Rabinowitz JD. Reversal of cytosolic one-carbon flux compensates for loss of the mitochondrial folate pathway. Cell Metab. 2016;23:1140–1153.
8. Anderson DD, Eom JY, Stover PJ. Competition between sumoylation and ubiquitination of serine hydroxymethyltransferase 1 determines its nuclear localization and its accumulation in the nucleus. J Biol Chem. 2012;287:4790–4799.
9. MacFarlane AJ, Anderson DD, Flodby P, Perry CA, Allen RH, Stabler SP, Stover PJ. Nuclear localization of de novo thymidylate biosynthesis

pathway is required to prevent uracil accumulation in DNA. J Biol Chem. 2011;286(51):44015–44022.

10. An S, Kumar R, Sheets ED, Benkovic SJ. Reversible compartmentalization of de novo purine biosynthetic complexes in living cells. Science. 2008;320:103–106.
11. Finkelstein JD. Methionine metabolism in mammals. J Nutr Biochem. 1990;1:228–237.
12. Stabler SP. Clinical practice. Vitamin B12 deficiency. N Engl J Med 2013;368:149–60.
13. Metz J, Kelly A, Swett VC, Waxman S, Herbert V. Deranged DNA synthesis by bone marrow from vitamin B_{12} deficient humans. Br J Haematol. 1968;14:575-592
14. Marshall RA, Jandl JH. Responses to “physiologic” doses of folic acid in the megaloblastic anemias. Arch Intern Med. 1960;105:352-360
15. Beaudin AE, Abarinov EV, Noden DM, Perry CA, Chu S, Stabler SP, Allen RH, Stover PJ. Shmt1 and de novo thymidylate biosynthesis underlie folate responsive neural tube defects in mice. Am J Clin Nutr. 2011; 93(4):789–798.
16. Bailey LB, Berry RJ. Folic acid supplementation and the occurrence of congenital heart defects, orofacial clefts, multiple births, and miscarriage. Am J Clin Nutr. 2005;81:1213S-1217S.
17. Beaudin AE, Stover PJ. Insights into metabolic mechanisms underlying folate-responsive neural tube defects: a minireview. Birth Defects Res A Clin Mol Teratol. 2009;85:274–284.

18. Nagao T, Hirokawa M. Diagnosis and Treatment of Macrocytic Anemia in Adults. J Gen Fam Med. 2017;18(5):200-204.
19. Park JY, Lam-Himlin D, Vemulapalli R. Review of autoimmune metaplastic atrophic gastritis. Gastrointest Endosc 2013;77:284–92.
20. Shipton MJ, Thachil J. Vitamin B12 deficiency-A21st century perspective. Clin Med (Lond). 2015;15:145–50.
21. Igbal Z, Azmi S, Yadav R, Ferdousi M, Kumar M, Cuthbertson DJ, Lim J, Malik RA, Alam U. Diabetic Peripheral Neuropathy: Epidemiology, Diagnosis, and Pharmacotherapy. Clin Ther. 2018;40(6):828-849.
22. Juster-Switlyk K, Smith AG. Updates in Diabetic Peripheral Neuropathy. F1000Res. 2016; 5: F1000 Faculty Rev-738.
23. Cashman CR, Hoke A. Mechanisms of distal axonal degeneration in peripheral neuropathies. Neurosci Lett. 2015;596:33-50.
24. Carelli V, Chan DC. Mitochondrial DNA: impacting central and peripheral nervous systems. Neuron 2014;84(6):1126-42.
25. Komirishetty P, Areti A, Yerra VG, Ruby PK, Sharma SS, Gogoi R, Sistla R, Kumar A. PARP inhibition attenuates neuroinflammation and oxidative stress in chronic constriction injury induced peripheral neuropathy. Life Sci. 2016;150:50-60.
26. Ross CA, Truant R. DNA repair: A unifying mechanism in neurodegeneration. Nature 2017; 541(7635): 34-35.
27. Mu Z, Wang YG, Li CQ, Lv WS, Wang B, Jing ZH, Song XJ, Lun Y, Qiu MY, Ma XL. Association Between Tumor Necrosis Factor-α

and Diabetic Peripheral Neuropathy in Patients with Type 2 Diabetes: a Meta-Analysis. Mol Neurobiol. 2017;54(2):983-996.

28. Ubogu EE. Inflammatory neuropathies: pathology, molecular markers and targets for specific therapeutic intervention. Acta Neuropathol. 2015;130(4):445-68.
29. Suh JR, Herbig AK, Stover PJ. New perspectives on folate catabolism. Annu Rev Nutr. 2001;21:255-82.
30. Herbert V. The five possible causes of all nutrient deficiency: illustrated by deficiencies of vitamin B 12. Am J Clin Nutr. 1973;26(1):77-86.
31. Ulvik A, Midttun Ø, Pedersen ER, Eussen SJ, Nygård O, Ueland PM. Evidence for increased catabolism of vitamin B-6 during systemic inflammation. Am J Clin Nutr. 2014;100(1):250-5.
32. Carozzi VA, Canta A, Chiorrazi A. Chemotherapy-induced peripheral neuropathy: What do we know about mechanisms? Neurosci Lett. 2015;596:90-107.
33. Saif MW, Wilson RH, Harold N, Keith B, Dougherty DS, Grem JL. Peripheral neuropathy associated with weekly oral 5-fluorouracil, leucovorin and eniluracil. Anticancer Drugs. 2001;12(6):525-31.
34. Briani C, Argyriou AA, Izquierdo C, Velasco R, Campagnolo M, Alberti P, Frigeni B, Cacciavillani M, Bergamo F, Cortinovis D, et al. Long-term course of oxaliplatin-induced polyneuropathy: a prospective 2-year follow-up study. J Peripher Nerv Syst. 2014;19(4):299-306.

35. Werbrouck BF, Pauwels WJ, De Bleecker JL. A case of 5-fluorouracil-induced peripheral neuropathy.Clin Toxicol (Phila). 2008;46(3):264-6.

36. Fox JT, Stover PJ. Folate-mediated One-Carbon Metabolism. Vitam Horm. 2008;79:1-44.

37. Jianbo L, Yuche C, Ming S, Jingrong T, Qing D, Yu Z, Jiawei C, Hongxing W. Association of homocysteine with peripheral neuropathy in Chinese patients with type 2 diabetes. Diabetes Res Clin Pract. 2011;93(1):38-42.

38. De Jager J. Long term treatment with metformin in patients with type 2 diabetes and risk of vitamin B-12 deficiency: randomised placebo controlled trial. BMJ. 2010 ;340:c2181.

39. Carpentier JL, Bury J, Luyckx A, Lefebvre P. Vitamin B 12 and folic acid serum levels in diabetics under various therapeutic regimens. Diabete Metab. 1976;2(4):187-90.

40. Wile DJ and Toth C. Association of metformin, elevated homocysteine, and methylmalonic acid levels and clinically worsened diabetic peripheral neuropathy. Diabetes Care. 2010;33(1):156-61.

41. Rodríguez VM, Jiménez-Capdeville ME, Giordano M. The effects of arsenic exposure on the nervous system. Toxicol Lett. 2003;145(1):1-18.

42. Mukherjee SC, Rahman MM, Chowdhury UK, Sengupta MK, Lodh D, Chanda CR, Saha KC, Chakraborti D. Neuropathy in arsenic toxicity from groundwater arsenic contamination in West Bengal, India. J Environ Sci Health A Tox Hazard Subst Environ Eng 2003;38(1):165-83.

43. Mochizuki H, Yagi K, Tsuruta K, Taniguchi A, Ishii N, Shiomi K, Nakazato M. Prolonged central sensory conduction time in patients with chronic arsenic exposure. J Neurol Sci. 2016 Feb 15;361:39-42.

44. Lam SK, Mak JC, Zheng CY, Li YY, Kwong YL, Ho JC. Downregulation of thymidylate synthase with arsenic trioxide in lung adenocarcinoma. Int J Oncol. 2014;44(6):2093–2102.

45. Kamynina E, Lachenauer ER, DiRisio AC, Liebenthal RP, Field MS, Stover PJ. Arsenic trioxide targets MTHFD1 and SUMO-dependent nuclear de novo thymidylate biosynthesis. Proc Natl Acad Sci U S A. 2017;114(12):E2319-E2326

46. Allen LH. How common is vitamin B-12 deficiency? Am J Clin Nutr. 2009;89(2):693S-6S.

47. D'Amico A, Bertini E. Metabolic neuropathies and myopathies. Handb Clin Neurol. 2013;113:1437-55.

48. Sedel F. Peripheral neuropathy and inborn errors of metabolism in adults. J Inherit Metab Dis. 2007;30(5):642-53.

49. Ang CD, Alviar MJM, Dans AL, Bautista-Velez GGP, Villaruz-Sulit MVC, Tan JJ, Co HU, Bautista MRM, Roxas AA. Vitamin B for treating peripheral neuropathy. Cochrane Database of Systematic Reviews. 2008; Issue 3. Art. No.: CD004573.

50. Abbas ZG, Swai ABM. Evaluation of the efficacy of thiamine and pyridoxine in the treatment of symptomatic diabetic peripheral neuropathy. East African Medical Journal. 1997;74(12):803-8.

51. Brito A, Verdugo R, Hertrampf E, Miller JW, Green R, Fedosov SN, Shahab-Ferdows S, Sanchez H, Albala C, Castillo JL, et al. Vitamin B12 treatment of asymptomatic, deficient, elderly Chileans improves conductivity in myelinated peripheral nerves, but high serum folate impairs vitamin B12 status response assessed by the combined indicator of vitamin B12 status. Am J Clin Nutr. 2016;103(1):250-7.
52. Gómez-Meda BC, Zamora-Perez AL, Muñoz-Magallanes T, Sánchez-Parada MG, García Bañuelos JJ, Guerrero-Velázquez C, Sánchez-Orozco LV, Vera-Cruz JM, Armendáriz-Borunda J, Zúñiga-González GM. Nuclear abnormalities in buccal mucosa cells of patients with type I and II diabetes treated with folic acid. Mutat Res Genet Toxicol Environ Mutagen. 2016;797:1-8.

CHAPTER 2: EFFECTS OF DIETARY VITAMN B_{12} INTAKE ON SENSORIMOTOR FUNCTION IN A MOUSE MODEL OF IMPAIRED DE NOVO THYMIDYLATE SYNTHESIS

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Eunice B. Awuah: developed the study design, conducted the research, analyzed the data, drafted and finalized the manuscript

Patrick J. Stover: developed the study design, drafted and finalized the manuscript

Abstract

Background: Impaired vitamin B_{12} metabolism has been associated with degeneration of sensory and motor neurons, but the underlying mechanisms remain poorly understood.

Objective: Here we investigated the interactions among the folate-dependent enzyme, serine hydroxymethyltransferase 1 (*Shmt1*), dietary vitamin B_{12} and folic acid, and nucleoside supplementation in sensorimotor function.

Methods: We used the accelerated and staggered rotarod test to measure motor coordination and the tail flick test to measure pain sensitivity in adult male *Shmt1*$^{+/-}$ mice fed vitamin B_{12} and folic acid-deficient diets with or without excess folic acid or nucleoside supplementation from weaning through the 19 month study duration.

Results: *Shmt1*$^{+/-}$ mice fed vitamin B_{12} and folic acid-deficient diets, as well as dietary folic acid and nucleoside-supplemented diets showed significantly lower staggered rotarod motor performance and delayed latencies to tail flick compared to *Shmt1*$^{+/-}$ mice fed the control diet. Additionally, dietary deoxyuridine and uridine supplementation did not rescue the observed motor and sensory defects found in *Shmt1*$^{+/-}$ mice fed vitamin B_{12} and folic acid-deficient diets.

Conclusion: *Shmt1* heterozygosity sensitizes mice to lower staggered rotarod performance and lower tail pain sensitivity when put on vitamin B_{12} and folic acid-deficient diets. This was the first study to use a mouse model of disrupted *Shmt1* expression to identify the role of impaired *de novo* thymidylate synthesis in the etiology of vitamin B_{12} related neurodegeneration. These findings implicate impaired *de novo* thymidylate biosynthesis in neuropathology in the *Shmt1*$^{+/-}$ mouse.

Introduction

Prolonged vitamin B_{12} deficiency causes neurodegeneration that affects both the peripheral and central nervous systems, but the underlying mechanisms are not well understood. Neurodegeneration of both the dorsal and ventral columns of the spinal cord results in painful and abnormal sensations in the lower and upper extremities and in severe cases, the loss of muscle control and balance (1,2).

Folate-mediated one-carbon metabolism (FOCM) consists of a network of pathways that are essential for the *de novo* synthesis of purines and thymidylate (dTMP), and the remethylation of homocysteine to methionine (**Supplemental Figure 1**) (3). Vitamin B_{12} and folate function in the remethylation of homocysteine to form methionine,

which is a substrate for the synthesis of S-adenosylmethionine (SAM), the primary methyl donor and regulator of epigenetic processes such as chromatin methylation.

Both folate and vitamin B_{12} deficiencies can impair *de novo* dTMP biosynthesis. Vitamin B_{12} deficiency impairs the activity of the vitamin B_{12}-depended enzyme thymidylate synthase, causing an accumulation of cellular folate cofactors as 5-methyltetrahydrofolate, thereby depleting other forms of folate required for nucleotide biosynthesis (2,4,5). Serine hydroxymethyltransferase 1 (SHMT1) is a folate-dependent enzyme that plays a key role in the regulation of *de novo* dTMP biosynthesis. Cell-cycle specific sumoylation of SHMT1 during S-phase leads to the nuclear translocation of SHMT1, which serves as an anchor for the assembly of the *de novo* dTMP biosynthesis pathway enzymes at sites of DNA synthesis (6,7,8) (Supplemental Figure 1). Disruptions in *Shmt1* expression, folate deficiency and vitamin B_{12} deficiency are each associated with elevated levels of uracil misincorporation into DNA, because DNA polymerases are unable to differentiate between deoxyuridine triphosphate (dUTP) and deoxythymidylate triphosphate (dTTP), therefore inadequate dTMP synthesis leads to dUTP misincorporation into DNA (9). Uracil misincorporation leads to DNA instability, which has been associated with neurodegeneration (9). Interestingly, *Shmt1* heterozygosity in mice has been associated with folate-responsive neural tube defects (NTDs) as well as impairments in neurogenesis in the hippocampus (10,11). The NTD phenotype exhibited by folate deficient *Shmt1*$^{+/-}$ and *Shmt1*$^{-/-}$ mice during embryogenesis was also prevented by maternal dietary supplementation with deoxyuridine whereas maternal uridine

supplementation increased the incidence of NTDs independent of maternal *Shmt1* genotype (12).

Dietary manipulation of vitamin B_{12} status in mice has not proven to be an effective model for investigating mechanisms of vitamin B_{12} deficiency-induced neuropathy (13). Because the *Shmt1* mouse model is sensitive to folate deficiency-induced NTDs through impaired *de novo* dTMP synthesis, we investigated the effects of *Shmt1* disruption on sensorimotor function as a model for identifying the underlying mechanisms connecting impaired vitamin B_{12} and folate metabolism to neurodegeneration. We hypothesized that disruption of dTMP biosynthesis in *Shmt1*$^{+/-}$ mice would decrease motor performance and pain sensitivity and predicted the rescue of sensorimotor deficits with dietary deoxyuridine, and exacerbated impairments with uridine supplementation as seen in the NTD phenotype.

Methods and Materials

Experimental mice and diets

The generation, genotyping, and characterization of the *Shmt1* mouse model have been previously described (10,11,14). All experiments were conducted using male *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice generated from *Shmt1*$^{+/-}$ breeding pairs that were maintained as a heterozygous breeding colony on a 129SvEv background (Jackson Laboratories). At weaning, littermates were randomly assigned to one of five diets: a standard AIN93G control diet (2mg/kg folic acid, 25μg/kg B_{12}); AIN93G diet lacking folic acid (FA) and vitamin B_{12} (-B12, -FA); AIN93G diet lacking vitamin B_{12} and supplemented with excess FA (8mg/kg) (-B12, +FA); or AIN93G FA and vitamin B_{12} deficient diet

supplemented with 0.1% uridine (-B12, -FA, + U) or 0.1% deoxyuridine (-B12, -FA, + dU) (Dyets, Bethlehem, PA). All mice were grouped according to diet and genotype and maintained on one of these 5 diets for the duration of the study. Mice were housed up to 5 to a cage in a temperature-controlled room (23 ± 1°C) with a 12-hour light/dark schedule (lights off at 8:00pm) and received ad libitum access to food and water. All animal experiments were approved by the Cornell University Institutional Animal Care and Use Committee according to the guidelines of the Animal Welfare Act and all applicable state and federal laws.

Experimental design

Behavioral tests were performed on each mouse following 14 months on diet by one female experimenter, in order to avoid unnecessary stress for the mice. Mice were given a habituation time of 1 hour in the testing space before handling. Accelerated and staggered rotarod tests trials were conducted once a month between 14-17 months on diet. For the accelerated rotarod test, mice were handled for 2 consecutive days for training and testing. One week after the rotarod test day, animals were handled for 4 consecutive days for training and testing of mice on the staggered rotarod. The accelerated and staggered rotarod tests were conducted on the same mice every 4 weeks in this pattern between 14-17 months on diet. The tail flick test was conducted at 18 months on diet followed by tissue and blood collection at 19 months post weaning.

Rotarod

The accelerated rotarod (4-40 rpm) and staggered rotarod (4, 16, 20, 24, 28, 32, and 40 rpm) were used to measure motor coordination, balance, and the ability of mice to improve motor performance with training. The rotarod apparatus (Med Associates, Fairfax, VT) had a rod of 3 cm in diameter and five lanes with a width of 5 cm each. The apparatus was designed to measure the amount of time that the mice were able to run on the rod. The time was automatically stopped when the animal fell off the rod. ***Accelerated*** Animals were tested 24 hours after an initial training session (4 one-minute trials at 4 rpm). If a mouse fell from the rod at any point during the training, it was placed back on the rod to complete the one minute. Each test session consisted of 4 trials per day with an intertrial period of 15 minutes to avoid mouse fatigue and exhaustion. Each trial lasted a maximum of 10 minutes, during which time the rotating rod underwent a linear acceleration from 4 to 40 rpm over the first 5 minutes of the trial and then remained at 40 rpm till the end of the trial. The mice were placed on the rotating rod and the latency to fall (in seconds) was recorded for each trial. In the event of a mouse passively rotating with the rod for 2 complete turns, the mouse was subsequently removed from the rod and the time was recorded. For analysis, the four trials were averaged for each mouse**. Staggered** Mice were initially trained (4 one-minute trials at 24 rpm) for three consecutive days. The test day took place 24 hours after the completion of the initial three-day training session. On the test day, each mouse received three trials (maximum of 5 minutes per trial) on each successive locked speed (4,16, 20, 24, 28, 32 and 40 rpm). The time spent on the rotating rod at

each of the different speeds (latency to fall) was measured in seconds. For analysis, the three trials on the test day were averaged for each speed.

Tail Flick

To assess tail pain sensitivity, a tail flick test was performed using an analgesic tail meter (Columbus Instruments, Columbia, Ohio). A shuttered controlled lamp (lamp intensity setting of 8) provided a constant heat source to the animal's tail. Before the test, mice were habituated to a transparent plastic restrainer for a period of 15-minutes. In order to obtain the mean latency of tail flicks, the tail was placed on the sensing groove above the heat source and the latency to flick the tail in response to the footswitch controlled heat stimulus was measured in seconds at three different locations from the end of the tail (5mm, 15mm, and 25mm) in order to prevent tissue damage. The heat stimulus was applied with an intertrial period of 10 minutes and a maximum cutoff time of 15 seconds.

Statistical analysis

A set of 5 *a priori* and predetermined comparisons between diets and genotypes were assessed for each behavioral test: 1. *Shmt1*$^{+/+}$ vs *Shmt1*$^{+/-}$ mice fed the control diet; 2. *Shmt1*$^{+/+}$ fed the control diet vs *Shmt1*$^{+/+}$ mice fed the vitamin B_{12} and folic acid-deficient diet; 3. *Shmt1*$^{+/+}$ vs *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet; 4. *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet vs *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} deficient diet supplemented with either excess folic acid (8mg/kg),

0.1% deoxyuridine (dU), or 0.1% uridine (U); 5. *Shmt1*$^{+/-}$ mice fed the control diet vs *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet supplemented with either excess folic acid (8mg/kg), 0.1% dU, or 0.1% U. Analyses of behavioral data were performed in JMP Pro version 13 (SAS Institute) using generalized linear and mixed models to determine the effects of mouse diet, genotype, the interaction between diet and genotype, and weight on the average latency to fall off the accelerated and staggered rotarod over the course of three test trials as well as the latency to tail flick. Log-transformation was applied to data that was not normally distributed. Statistical significance was determined by using a Student's *t* test followed by a Bonferroni correction for multiple comparisons (n=5). Differences were considered significant at $p \leq 0.05$.

Results

***Shmt1*$^{+/-}$ mice fed the control diet exhibited normal motor function on the accelerated rotarod compared to *Shmt1*$^{+/+}$ mice**

We compared the latency to fall off the accelerating rotarod between *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed either the control or vitamin B_{12} and folic acid-deficient diets (**Figure 1A, 1C, 1E**) for each test trial. This contrast revealed a significant effect of diet (p=0.018) for trial 1, indicating that the motor performance of mice fed the vitamin B_{12} and folic acid-depleted diet in trial 1 was worse than mice fed the replete diet (Figure 1A). However, this diet group difference was not significant among *Shmt1*$^{+/+}$ mice following a Bonferonni correction for multiple comparisons (unadjusted p=0.018; adjusted p=0.091) (Figure 1A). This diet group difference

among *Shmt1*$^{+/+}$ mice was also not significant for trial 2 (unadjusted p=0.166) (Figure 1C) and trial 3 (unadjusted p=0.868) (Figure 1E).

There was no significant main effect of genotype (trial 1: p=0.893; trial 2: p=0.943; trial 3: p= 0.657) or the interaction of diet and genotype (trial 1: p=0.114; trial 2: p=0.118; trial 3: p= 0.303) across all three test trials (Figure 1A, 1C, 1E). The analysis of the latency to fall for the contrast between *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed the control diet (trial 1: unadjusted p=0.536; trial 2: unadjusted p=0.552; trial 3: unadjusted p= 0.441) (Figure 1A, 1C, 1E) and between *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet (trial 1: unadjusted p=0.162; trial 2: unadjusted p=0.254; trial 3: unadjusted p= 0.176) (Figure 1A, 1C, 1E) revealed no significant differences across test trials, indicating that *Shmt1*$^{+/-}$ mice did not have a better or worse motor performance on the accelerated rotarod compared to *Shmt1*$^{+/+}$ mice independent of diet.

Shmt1*$^{+/-}$ *mice fed folic acid and vitamin* B_{12}*-deficient diets with folic acid or nucleoside supplementation did not exhibit impaired motor performance on the accelerated rotarod

Analysis of the latency to fall for the contrast between *Shmt1*$^{+/-}$ mice fed the control diet *vs Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet supplemented with either excess folic acid (8mg/kg), 0.1% dU or 0.1% U (**Figure 1B, 1D, 1F**) revealed a significant effect of diet in trial 2 (p=0.029) (Figure 1D). However, *Shmt1*$^{+/-}$ mice fed the control diet did not perform significantly better or worse on the

accelerated rotarod across all three trials compared to the other diet groups (trial 1: unadjusted p=0.315; trial 2: unadjusted p=0.883; trial 3: unadjusted p= 0.649) (Figure 1B, 1D, 1F). The significant main effect of diet in trial 2 (Figure 1D) indicated only a marginally better motor performance of *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-depleted diet compared to *Shmt1*$^{+/-}$ mice fed either the folic acid (8mg/kg) or nucleoside-supplemented diets (unadjusted p=0.016; adjusted p=0.053).

Overall, dietary supplementation with nucleosides or provision of excess folic acid (8mg/kg) in the diet did not improve or impair accelerated rotarod motor performance in *Shmt1*$^{+/-}$ mice fed vitamin B_{12}-deficient diets.

Shmt1*$^{+/-}$ *mice fed the control diet exhibited normal motor function on the staggered rotarod test compared to Shmt1*$^{+/+}$ *mice

We analyzed the latency to fall off the staggered rotarod across all seven set speeds (4, 16, 20, 24, 28, 32, and 40 rpm) for the contrast between *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed either the control or vitamin B_{12} and folic acid-deficient diets for each test trial (**Figure 2A, 2C, 2E**). This contrast revealed a significant main effect of diet for trial 1 ($p<0.0001$) and trial 2 (p=0.0001), indicating that the staggered rotarod performance of mice fed a vitamin B_{12} and folic acid-depleted diet was worse than mice fed the replete diet (Figure 2A, 2C). However, the contrast between the *Shmt1*$^{+/+}$ diet groups in trial 1 (unadjusted p=0.225), trial 2 (unadjusted p=0.016; adjusted p=0.079), and trial 3 (unadjusted p=0.242) did not demonstrate a significant difference following the Bonferonni correction for multiple comparisons (Figure 2A, 2C, 2E).

There was a significant main effect of genotype for trials 1 and 2 (trial 1: $p=0.009$; trial 2: $p=0.035$; trial 3: $p=0.101$) indicating that *Shmt1*$^{+/-}$ mice had higher latencies to fall off the rotarod across all speeds for trials 1 and 2. There was also a significant effect of the interaction of diet and genotype (trial 1: $p=0.009$; trial 2: $p=0.81$; trial 3: $p= 0.061$) indicating that *Shmt1*$^{+/-}$ mice fed either the control or folic acid and vitamin B_{12}-deficient diets had a better motor performance on the staggered rotarod test compared to the *Shmt1*$^{+/+}$ mice fed the same diets in trial 1. The analysis of the latency to fall across all speeds for the contrast between *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed the control diet was significant for trial 1 (unadjusted $p=0.0004$, adjusted $p=0.002$). However, the contrast between *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet did not reveal a significant difference across test trials (trial 1: unadjusted $p=0.989$; trial 2: unadjusted $p=0.16$; trial 3: unadjusted $p= 0.867$), indicating that *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet did not have a better or worse motor performance compared to *Shmt1*$^{+/+}$ mice for all three test trials. Overall, the *Shmt1*$^{+/-}$ mice fed the control or the vitamin B_{12} and folic acid-deficient diet did not show significant differences in motor performance compared to *Shmt1*$^{+/+}$ mice.

Shmt1*$^{+/-}$ *mice fed folic acid and vitamin* B_{12}*-deficient diets with folic acid or nucleoside supplementation exhibited decreased motor performance on the staggered rotarod

Analysis of the latency to fall for the contrast between *Shmt1*$^{+/-}$ mice fed the control diet vs. *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet supplemented with either folic acid (8mg/kg) or nucleosides revealed a significant main effect of diet for all three trials (trial 1: p<0.0001; trial 2: p<0.0001; trial 3: p<0.0001) (**Figure 2B, 2D, 2F**). This finding indicated that *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet, as well as *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet supplemented with either folic acid (8mg/kg), 0.1% dU, or 0.1% U performed significantly worse than *Shmt1*$^{+/-}$ mice fed the control diet across all test trials (trials 1, 2 and 3: unadjusted p<0.0001; adjusted p<0.0005).

Overall, *Shmt1*$^{+/-}$ mice fed vitamin B_{12} deficient diets supplemented with either folic acid (8mg/kg), 0.1% U, or 0.1% dU exhibited decreased motor performance compared to *Shmt1*$^{+/-}$ mice fed the control diet.

Neither Shmt1$^{+/-}$ genotype nor diet affected pain sensitivity using the tail flick test compared to Shmt1$^{+/+}$ mice

We analyzed the latency to tail flick for the contrast between *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed either the control or vitamin B_{12} and folic acid-deficient diets (**Figure 3A**). This contrast revealed a significant main effect of diet (p=0.008), indicating that mice fed a vitamin B_{12} and folic acid-depleted diet had a lower sensitivity to the pain stimulus than mice fed the replete diet. However, this diet group difference was not significant for *Shmt1*$^{+/+}$ mice (unadjusted p=0.363). Additionally, the effect of genotype (p=0.071) and the interaction of diet and genotype was not significant

(p=0.179). Although *Shmt1*$^{+/-}$ mice fed the control diet had a lower latency to tail flick, this effect was not statistically significant compared to the *Shmt1*$^{+/+}$ mice fed the control diet (unadjusted p=0.04; adjusted p=0.2) (Figure 3A). The contrast between *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet also did not reveal a statistically significant difference between groups (unadjusted p=0.693), indicating that *Shmt1*$^{+/-}$ mice did not exhibit better or worse pain sensitivity compared to *Shmt1*$^{+/+}$ mice (Figure 3A).

Shmt1*$^{+/-}$ *mice fed a folic acid and vitamin* B_{12}*-deficient diet supplemented with either folic acid, U, or dU exhibited decreased pain sensitivity on the tail flick test

The latency to tail flick for *Shmt1*$^{+/-}$ mice fed the control diet vs *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet supplemented with either folic acid (8mg/kg), 0.1% U, or 0.1% dU revealed a significant main effect of diet (p=0.010) (**Figure 3B**). This finding indicated that *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet as well as *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet supplemented with either folic acid or nucleosides exhibited pain sensitivity that was significantly lower than *Shmt1*$^{+/-}$ mice fed the control diet (unadjusted p=0.005; adjusted p=0.026).

The contrast between *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet vs. *Shmt1*$^{+/-}$ mice fed the folic acid and vitamin B_{12}-deficient diet supplemented with either folic acid (8mg/kg), 0.1% U, or 0.1% dU did not reveal any significant differences in pain sensitivity (unadjusted p=0.461) (Figure 3B).

Discussion

In this study, we investigated the effects of decreased expression of *Shmt1* on sensorimotor function using the rotarod and tail flick test. The *Shmt1*$^{+/-}$ mouse model is sensitized to pathologies associated with folate deficiency, including NTDs and adult hippocampal neurogenesis (10,11). We also investigated the effects of vitamin B_{12} and folic acid-deficient diets as well as supplemental excess folic acid (8mg/kg), 0.1% dU, and 0.1% U on sensorimotor outcomes in *Shmt1*$^{+/-}$ mice. These dietary components have been shown to affect NTD incidence in the *Shmt1* mouse model. Our study is novel in its use of the *Shmt1*$^{+/-}$ mouse model to directly investigate the role of impaired *de novo* dTMP synthesis on vitamin B_{12} associated deficits in sensory and motor function.

Our primary finding was a decrease in staggered rotarod motor performance and pain sensitivity in *Shmt1*$^{+/-}$ mice fed folic acid and vitamin B_{12}-deficient diets compared to *Shmt1*$^{+/-}$ mice fed the control diet (Figure 2, Figure 3). A previous study reported that vitamin B_{12} depletion in HeLa cells and mutations in human fibroblasts of the vitamin B_{12} dependent enzyme, methionine synthase, resulted in decreased rates of *de novo* dTMP synthesis and genome instability. This study suggests the link between vitamin B_{12} deficiency, impaired DNA synthesis, and genome instability as a possible mechanism underlying vitamin B_{12} deficient neuropathy (15).

Shmt1$^{+/-}$ mice fed folic acid and vitamin B_{12}-deficient diets supplemented with either 0.1% dU or 0.1% U also showed a decrease in staggered rotarod motor performance

and slower latencies to tail flick compared to *Shmt1*$^{+/-}$ mice fed the control diet. We predicted a protective effect of 0.1% dU on sensorimotor function in mice fed vitamin B_{12} and folic acid-deficient diets. However, the current literature regarding the role of supplemental nucleosides on the prevention of neurodegeneration is still not conclusive. Deoxyuridine, which provides a precursor for thymidine synthesis, prevented NTDs in embryos from folate deficient *Shmt1*$^{+/+}$ *and Shmt1*$^{-/-}$ dams in a previous study (12). Surprisingly, this same study reported that maternal thymidine supplementation did not prevent NTDs whereas uridine supplementation caused NTDs (12).

Further investigation into uracil levels in DNA as well as DNA damage in peripheral and central nervous tissue in vitamin B_{12} deficient *Shmt1*$^{+/+}$ *and Shmt1*$^{+/-}$ mice is needed in order to establish causal mechanisms by which vitamin B_{12} deficiency affects sensorimotor function. This study demonstrates that the *Shmt1* mouse model is sensitized to vitamin B_{12} deficiency and may be a model for other B-vitamin related neuropathies, which has been identified as a gap in advancing research in this area (13).

Acknowledgements

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E.B.A and P.J.S wrote the manuscript; and P.J.S had the primary responsibility for final content. All authors read and approved the final manuscript.

Figures

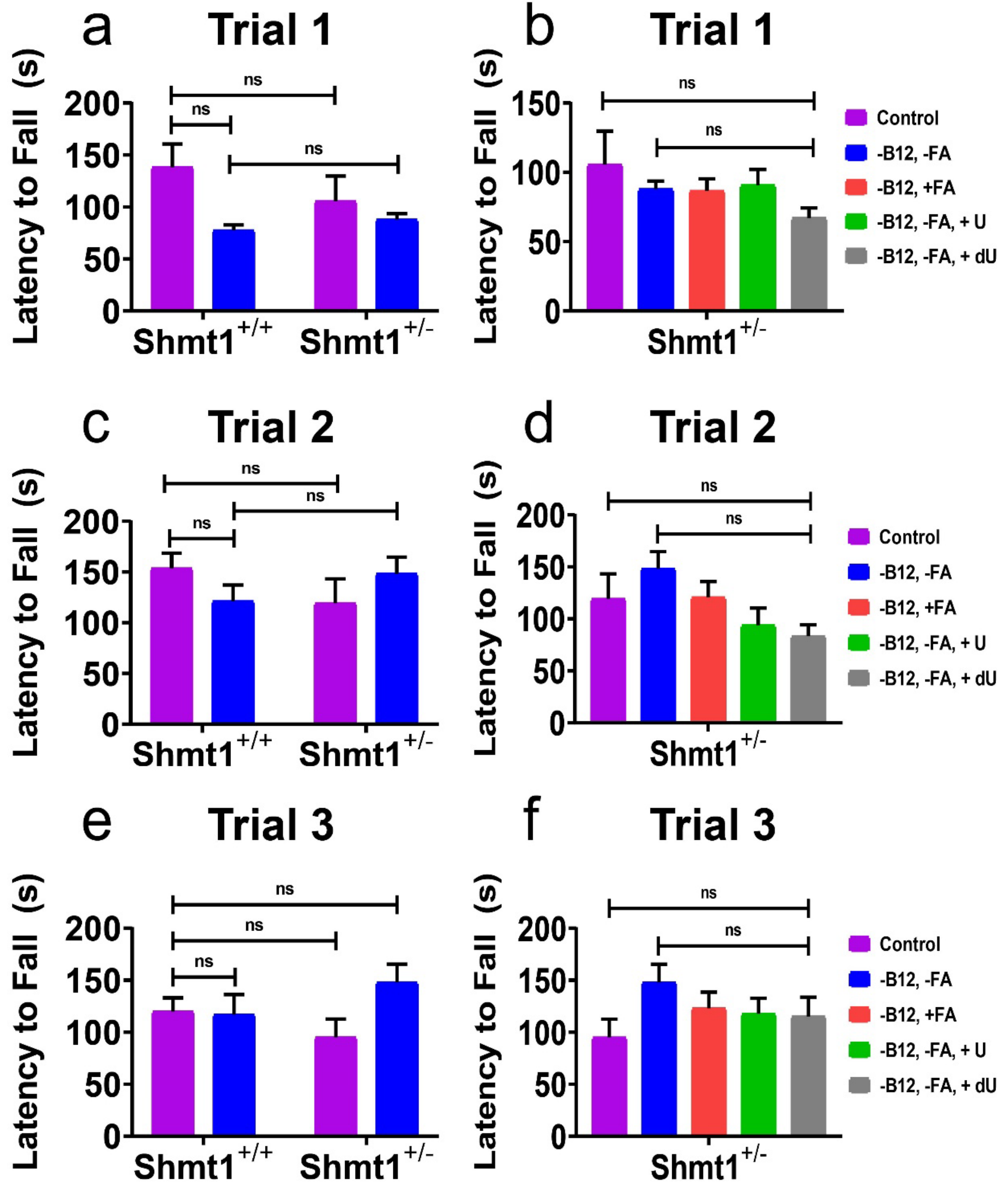
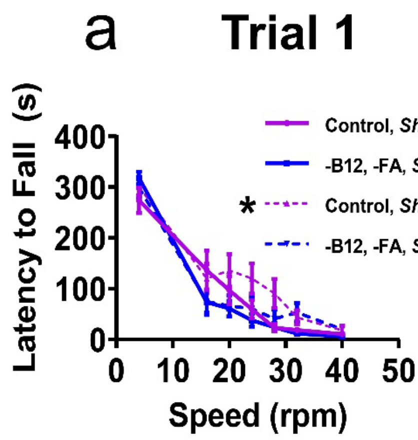
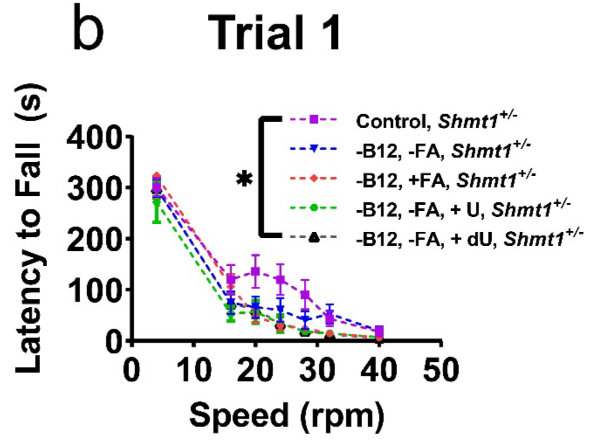
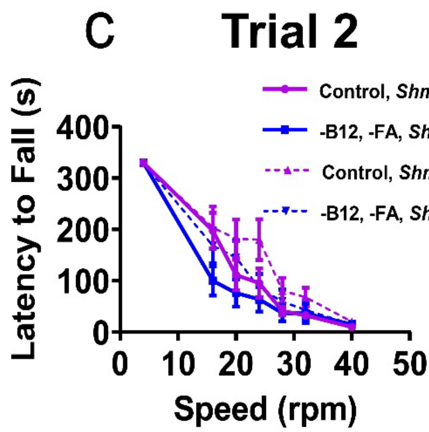
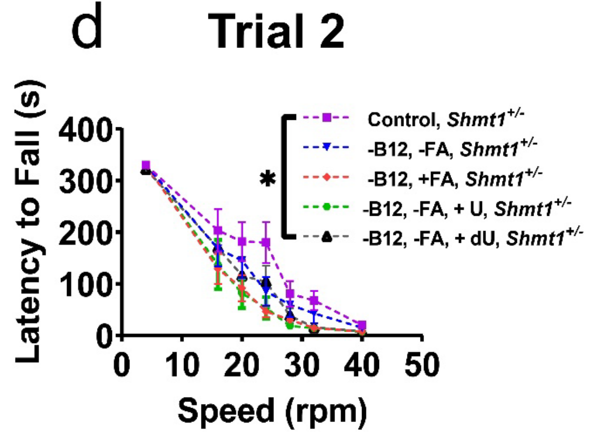


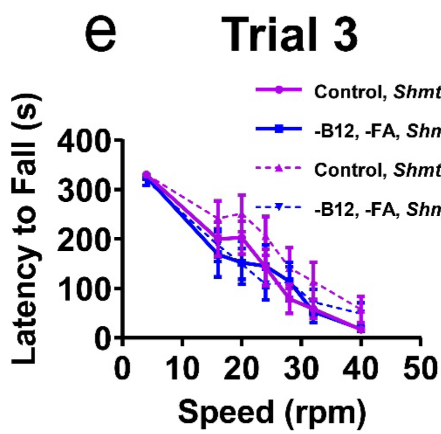
Figure 1. Assessment of motor function using the accelerated rotarod task. The latency to fall from an accelerating rotarod was measured for three test trials for *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed their respective diets. (A) For the comparison between *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed either the control or vitamin B_{12} and folic acid-deficient diet for test trial 1, the main effect of diet was significant. (B) For test trial 1, the comparison between *Shmt1*$^{+/-}$ mice fed the control diet vs *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet supplemented with either folic acid (8mg/kg), 0.1% dU, or 0.1% U did not reveal a significant effect of diet. (C) For test trial 2, the main effects of diet, genotype, and the interaction between diet and genotype were not significant. (D) The main effect of diet was significant for test trial 2 ($p=0.029$) for the comparison between *Shmt1*$^{+/-}$ mice fed the control diet vs *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet supplemented with either folic acid (8mg/kg), 0.1% dU or 0.1% U. (E,F) For test trial 3, there were no significant main effects of diet, genotype, or the interaction between diet and genotype. NS, not significant; FA, folic acid; U, uridine; dU, deoxyuridine. Data indicate means ± SEM (n=7-19 animals per group). <u>Eunice B Awuah collected and analyzed accelerated rotarod data from all study mice.</u>


a
Trial 1
Latency to Fall (s)
Speed (rpm)
Control, Shmt1+/+
-B12, -FA, Shmt1+/+
*
Control, Shmt1+/-
-B12, -FA, Shmt1+/-
0
100
200
300
400
0 10 20 30 40 50


b
Trial 1
Latency to Fall (s)
Speed (rpm)
*
Control, Shmt1+/-
-B12, -FA, Shmt1+/-
-B12, +FA, Shmt1+/-
-B12, -FA, + U, Shmt1+/-
-B12, -FA, + dU, Shmt1+/-
0
100
200
300
400
0 10 20 30 40 50


c
Trial 2
Latency to Fall (s)
Speed (rpm)
Control, Shmt1+/+
-B12, -FA, Shmt1+/+
Control, Shmt1+/-
-B12, -FA, Shmt1+/-
0
100
200
300
400
0 10 20 30 40 50


d
Trial 2
Latency to Fall (s)
Speed (rpm)
*
Control, Shmt1+/-
-B12, -FA, Shmt1+/-
-B12, +FA, Shmt1+/-
-B12, -FA, + U, Shmt1+/-
-B12, -FA, + dU, Shmt1+/-
0
100
200
300
400
0 10 20 30 40 50


e
Trial 3
Latency to Fall (s)
Speed (rpm)
Control, Shmt1+/+
-B12, -FA, Shmt1+/+
Control, Shmt1+/-
-B12, -FA, Shmt1+/-
0
100
200
300
400
0 10 20 30 40 50

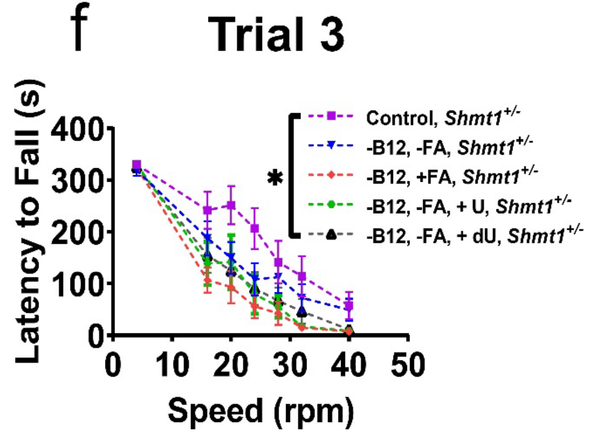

f
Trial 3
Latency to Fall (s)
Speed (rpm)
*
Control, Shmt1+/-
-B12, -FA, Shmt1+/-
-B12, +FA, Shmt1+/-
-B12, -FA, + U, Shmt1+/-
-B12, -FA, + dU, Shmt1+/-
0
100
200
300
400
0 10 20 30 40 50

Figure 2. Assessment of motor function using the staggered rotarod task. The latency to fall from the rotarod across seven different speeds (4, 16, 20, 24, 28, 32, and 40 rpm) was analyzed using a mixed model approach for the three test trials for *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed their respective diets. (A, C, E) The comparison between *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed either the control or vitamin B_{12} and folic acid-deficient diet revealed a significant effect of diet, genotype, and the interaction between diet in trial 1 and a significant effect of diet and genotype in trial 2. There were no significant main effects of diet and genotype in trial 3. In trial 1, *Shmt1*$^{+/-}$ mice fed the control diet had significantly higher latencies to fall in trial 1 compared to *Shmt1*$^{+/+}$ mice fed the control diet (unadjusted p=0.0004, adjusted p=0.002). (B, D, F) The comparison between *Shmt1*$^{+/-}$ mice fed the control diet vs *Shmt1*$^{+/-}$ mice fed the folic acid and vitamin B_{12}-deficient diet supplemented with either excess folic acid (8mg/kg), 0.1% dU, or 0.1% U showed a significant effect of diet for all three trials. *Shmt1*$^{+/-}$ mice fed the control diet had significantly higher latencies to fall than *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet with or without excess folic acid or nucleoside-supplementation (unadjusted p<0.0001, adjusted p<0.0005). FA, folic acid; U, uridine; dU, deoxyuridine. Data indicate means ± SEM (n=7-19 animals per group). *-indicates a significant difference (p<0.05) from the control group. Eunice B Awuah collected and analyzed staggered rotarod data from all study mice.

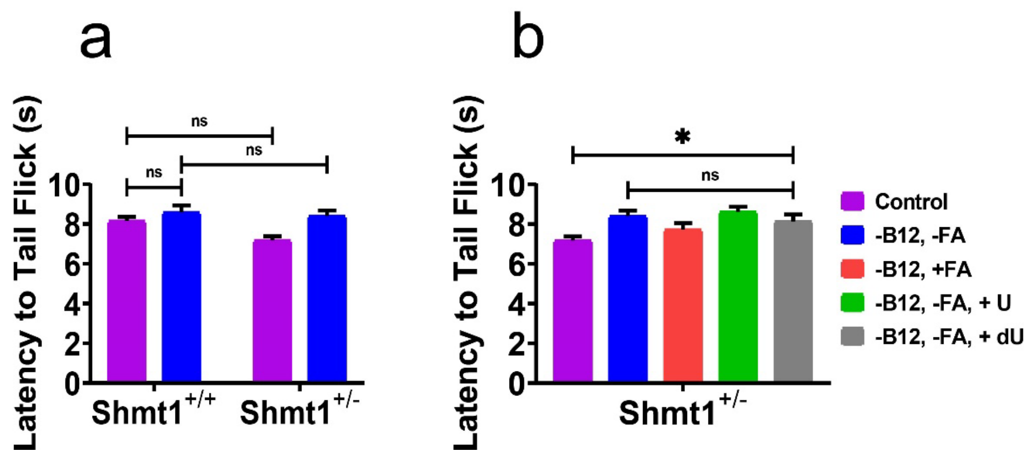


Figure 3. Assessment of pain sensitivity using the tail flick test. Mice were placed on a tail flick apparatus and the latency to tail flick in response to the painful stimulus was recorded for *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed their respective diets. (A) The main effect of diet was significant for the comparison between *Shmt1*$^{+/+}$ and *Shmt1*$^{+/-}$ mice fed either the control or vitamin B_{12} and folic acid-deficient diets (p=0.008), however there was no significant effect of genotype or the interaction between diet and genotype. (B) For the contrast between *Shmt1*$^{+/-}$ diet groups, there was a significant effect of diet (p=0.01). *Shmt1*$^{+/-}$ mice fed the control diet had significantly lower latencies to tail flick than *Shmt1*$^{+/-}$ mice fed the vitamin B_{12} and folic acid-deficient diet as well as *Shmt1*$^{+/-}$ mice fed supplemented the dietary folic acid and nucleoside-supplemented diets (unadjusted p=0.005, adjusted p=0.026). NS, not significant; FA, folic acid; U, uridine; dU, deoxyuridine. Data indicate means ± SEM (n=7-19 animals per group). *-indicates a significant difference ($p<0.05$). Eunice B Awuah collected and analyzed tail flick data from all study mice.

Supplemental Figure 1. Folate-mediated one-carbon metabolism in the cytosol and nucleus. FOCM is required for the synthesis of purines, dTMP, and methionine. Mitochondrial-derived formate can enter the cytoplasm and function as a one-carbon unit for folate metabolism through the activity of MTHFD1. At S-phase, the enzymes of the *de novo* dTMP synthesis pathway undergo SUMO-dependent translocation to the nucleus. DHF, dihydrofolate; DHFR, dihydrofolate reductase; dTMP, thymidylate; MTHFD1, methylenetetrahydrofolate dehydrogenase 1; MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; SHMT1, serine hydroxymethyltransferase 1; SHMT2α, serine hydroxymethyltransferase 2 alpha; SUMO, small ubiquitin-like modifier; THF, tetrahydrofolate; TYMS, thymidylate synthase. Figure is adapted from *Field MS, Kamynina E, Watkins D, Rosenblatt DS, Stover PJ.* Human mutations in methylenetetrahydrofolate dehydrogenase 1 impair nuclear de novo thymidylate biosynthesis. *Proc Natl Acad Sci U S A. 2015; 112(2):400-5.*

References

1. Shipton MJ, Thachil J. Vitamin B12 deficiency-A21st century perspective. Clin Med (Lond). 2015;15:145–50.
2. Stabler SP. Clinical practice. Vitamin B12 deficiency. N Engl J Med. 2013;368:149–60.
3. Field MS, Kamynina E, Watkins D, Rosenblatt DS, Stover PJ. Human mutations in methylenetetrahydrofolate dehydrogenase 1 impair nuclear de novo thymidylate biosynthesis. Proc Natl Acad Sci U S A. 2015; 112(2):400-5
4. Metz J, Kelly A, Swett VC, Waxman S, Herbert V. Deranged DNA synthesis by bone marrow from vitamin B_{12} deficient humans. Br J Haematol. 1968;14:575-592.
5. Marshall RA, Jandl JH. Responses to “physiologic” doses of folic acid in the megaloblastic anemias. Arch Intern Med. 1960;105:352-360.
6. Anderson DD, Stover PJ. SHMT1 and SHMT2 are functionally redundant in nuclear de novo thymidylate biosynthesis. PLoS ONE. 2009;4:e5839.
7. Anderson DD, Woeller CF, Stover PJ. Small ubiquitin-like modifier-1 (SUMO-1) modification of thymidylate synthase and dihydrofolate reductase. Clin Chem Lab Med. 2007;45:1760–3.
8. Woeller CF, Anderson DD, Szebenyi DM, Stover PJ. Evidence for small ubiquitin-like modifier-dependent nuclear import of the thymidylate biosynthesis pathway. J Biol Chem. 2007;282:17623–31.
9. McKinnon, PJ. Genome integrity and disease prevention in the nervous system. Genes Dev. 2017;31(12):1180-1194.

10. Beauding AE, Abarinov EV, Noden DM, Perry CA, Chu S, Stabler SP, Allen RH, Stover PJ. Shmt1 and de novo thymidylate biosynthesis underlie folate responsive neural tube defects in mice. Am J Clin Nutr. 2011; 93(4):789–798.
11. Abarinov EV, Beaudin AE, Field MS, Perry CA, Allen RH, Stabler SP, Stover PJ. Shmt1 impairs hippocampal neurogenesis and mnemonic function in mice. J Nutr. 2013;143(7):1028-35.
12. Martiniova, L, Field MS, Finkelstein JL, Perry CA, Stover PJ. Maternal dietary uridine causes, and deoxyuridine prevents, neural tube closure defects in a mouse model of folate-responsive neural tube defects. Am J Clin Nutr. 2015;101(4):860-9.
13. National Toxicology Program. NTP Monograph: Identifying Research Needs for Assessing Safe Use of High Intakes of Folic Acid. 2015;1-51.
14. MacFarlane AJ, Liu X, Perry CA, Flodby P, Allen RH, Stabler SP, Stover PJ. Cytoplasmic serine hydroxymethyltransferase regulates the metabolic partitioning of methylenetetrahydrofolate but is not essential in mice. J Biol Chem. 2008;283:25846–53.
15. Palmer AM, Kamynina E, Field MS, Stover PJ. Folate rescues vitamin B_{12} depletion-induced inhibition of nuclear thymidylate biosynthesis and genome instability. Proc Natl Acad Sci U S A. 2017;114(20): E4095–E4102.

CHAPTER 3: EFFECTS OF IMPAIRED ONE-CARBON METABOLISM IN A MOUSE MODEL OF TYPE 2 DIABETES

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Author Contributions:

Eunice B. Awuah: developed study design, collected and analyzed all behavioral data, drafted manuscript

Wilhelm H. Elmore: customized software and amplifier equipment for collection of nerve conduction velocity data

Martha S. Field: developed study design

Sally S. Stabler: performed GC-MS analysis on plasma samples to measure markers of the remethylation and transsulfuration pathway

Patrick J Stover: developed study design and drafted manuscript

Abstract

INTRODUCTION: It is increasingly recognized that DNA damage is a major driver of neurodegenerative disease. However, the effects of impaired one-carbon metabolism and *de novo* dTMP synthesis on peripheral neuropathy are not well

understood. In this study we investigated the effects of disrupted folate and vitamin B_{12} metabolism on peripheral neuropathy in a mouse model of Type 2 diabetes (*db/db*). We hypothesize that *Shmt1* heterozygosity, which induces DNA damage by increasing uracil misincorporation into DNA, will exacerbate peripheral neuropathy in *db/db* mice and that this effect can be modified by a vitamin B_{12} and folic acid deficiency.

METHODS: Shmt1$^{+/-}$ mice were crossed with *db/db* mice to form the following compound genotypes: *db*$^{+}$ *Shmt1*$^{+/+}$, *db*$^{+}$ *Shmt1*$^{+/-}$, *db/db Shmt1*$^{+/+}$, and *db/db Shmt1*$^{+/-}$. Mice were fed either folic acid and vitamin B_{12} sufficient or folic acid and vitamin B_{12} deficient diets from weaning through the duration of the study. Several tests were employed to assess sensory and motor nerve function to identify changes in peripheral neuropathy. Sensory loss was evaluated using tail flick latency as a measure of thermal sensitivity. Nerve conduction velocity in the sciatic and tail nerves was used as an electrophysiological measure of nerve impairment.

RESULTS: Deficits in peripheral nerve function in *db/db* mice were not significantly impacted by diet or *Shmt1* genotype. Sciatic motor nerve conduction velocities in *Shmt1*$^{+/-}$ mice were significantly lower compared to wildtype mice. The combined *db/db* genotype and diet groups had significantly higher plasma concentrations of cysteine and significantly lower concentrations of plasma homocysteine, methionine, serine, and glycine compared to wildtype mice on the control diet

CONCLUSION:

The results of this study support a role for impaired *de novo* dTMP synthesis in peripheral neuropathy. The results provide new insight into establishing guidelines for

folate and vitamin B_{12} level analysis and supplementation in diabetic patients leading to the prevention and treatment of peripheral neuropathy.

Introduction

Over the past several years, peripheral neuropathy has become one of the most common neurological disorders in the United States, affecting 8% of individuals 55 years and older (1). Peripheral neuropathy is also the most common co-morbidity of diabetes with up to 50-60% of patients with Type 2 diabetes eventually developing diabetic peripheral neuropathy (DPN) (2). DPN is characterized by symptoms of numbness, painful tingling sensations, autonomic dysfunction, and muscle weakness and neurodegeneration that progresses along the nerve fibers of the hands and legs with disease severity (3,4).

The most commonly prescribed medication for the treatment of Type 2 diabetes is Metformin. Long term use of Metformin has been associated with decreased serum levels of vitamin B_{12} (5). Vitamin B_{12} deficiency leads to symptoms of peripheral neuropathy that are clinically indistinguishable from DPN. Genome instability and DNA damage are closely associated with vitamin B_{12} deficiency and diabetes-induced peripheral neuropathy.

Elevated glucose levels lead to an increase in reactive oxygen species (ROS) and oxidative stress, which activates the DNA repair enzyme, poly ADP-ribose polymerase (PARP). ROS mediated overactivation of DNA repair pathways eventually leads to apoptosis and neuronal cell death which is the proposed causal mechanism linking neuronal tissue damage and peripheral neuropathy (6).

Vitamin B_{12} plays a role in folate-mediated one-carbon metabolism which consists of a network of pathways that generate *de novo* synthesis of purine and thymidylate (dTMP) and methionine (Appendix 1). Impairments in *de novo* dTMP synthesis have also been linked to increased DNA damage through the incorporation of uracil in DNA. The *de novo* dTMP biosynthesis pathway is regulated by the folate dependent enzyme, serine hydroxymethyltransferase 1 (SHMT1). During DNA replication in the S phase of the cell cycle, SHMT1 is sumoylated and translocated to the nucleus for dTMP synthesis (7,8) (Appendix 1). *Shmt1* heterozygosity leads to elevated genomic uracil misincorporation and has been associated with deficits in neural tube closure and hippocampal neurogenesis (9,10). Therefore, studying the consequences of *Shmt1* disruption and impaired folate- mediated one-carbon metabolism may elucidate the underlying metabolic pathways linking vitamin B_{12} deficiency and diabetes to peripheral neuropathy.

In this study, we used *Shmt1*$^{+/-}$ and *db/db* mice to identify causal mechanisms shared between impaired folate metabolism and diabetes induced peripheral neuropathy by measuring sensory and motor nerve conduction and pain sensitivity. We hypothesized that disruption of *de novo* dTMP biosynthesis in *Shmt1*$^{+/-}$ mice would exacerbate symptoms of peripheral neuropathy in a mouse model of type 2 diabetes.

Methods and Materials

Experimental mice and diets

C57BL/6 *Lepr*^{db}*/J* mice were purchased from Jackson Laboratories (Bar Harbor, Maine). *Shmt1*$^{-/+}$ mice were maintained as a congenic heterozygous breeding colony on a C57BL/6 background. Lepr$^{db/+}$ females were crossed with Shmt1$^{-/+}$ males. Double heterozygous male offspring were crossed with Lepr$^{db/+}$ females to achieve Lepr$^{+/+}$ *Shmt1*$^{+/+}$, Lepr$^{+/+}$ *Shmt1*$^{-/+}$, Lepr$^{db/db}$ *Shmt1*$^{+/+}$, or Lepr$^{db/db}$ *Shmt1*$^{-/+}$.

Genotyping for Lepr$^{db/+}$ mice was performed using the following primers recommended by the Jackson Laboratory: forward primer 5'-AGAACGGACACTCTTTGAAGTCTC-3', reverse primer 5'-CATTCAAACCATAGTTTAGGTTTGTGT-3'. Shmt$^{-/+}$ mice were genotyped using the forward primer 5'- GACACTGTTCACATCCCTC-3' and reverse primer 5'-CAAAACATTCGGGAGCCTC-3'.

Breeding colonies were established at Cornell University to provide the animals used in this study. Mice were genotyped 4 weeks after birth and housed in a pathogen-free environment with continuous access to food and water on a 12-hour light-dark schedule. All experimental procedures were approved by the Cornell University Institutional Animal Care and Use Committee (IACUC) according to the guidelines of the Animal Welfare Act and all applicable state and federal laws.

Diets

At 19-21 days of age, male mice were randomly weaned onto either an AIN-93G control diet (2mg/kg folic acid and 25μg/kg vitamin B_{12}) or a modified AIN-93G diet lacking folate and vitamin B_{12} (Dyets, Inc., Bethlehem, PA). Mice were maintained on the diet for the entire study duration, 6 months post induction of diabetes. Each group contained 15-18 animals.

Tail Flick Test

Mice were placed in an acrylic holder atop a tail flick analgesia meter (Columbus Instruments, Columbus, OH). The time from activation of the light beam to tail flick response was measured at three different locations from the end of the tail (5mm, 15mm, and 25mm) with an intertrial period of 15 minutes.

Nerve Conduction Studies

Measures of nerve conduction velocity (NCV) were done in compliance with protocols established by the AMDCC (http://www.amdcc.org). Mice were anesthetized with isoflurane according to protocols established by the Cornell IACUC. Body temperature was monitored and maintained at 32-34°C with a heating pad and overhead lamp. The concentric needle electrodes (Navus Appartus) were cleaned with 70% ethanol between animals to maintain a pathogen-free status.

For tail motor and sensory NCV measurements, recording/stimulating electrodes were placed 30mm apart on the tail. Tail sensory NCV (TSNCV) was an orthodromic measurement determined by stimulating the tail 30mm proximal to the recording electrode. Compound muscle action potentials were measured and peak latency was averaged for three trials. NCV was calculated by dividing the distance (30mm) by the

peak latency (msec) of the sensory nerve action potential. Tail motor NCV (TMNCV) was determined by stimulating the tail 30mm distal to the recording electrode at the base of the tail. Compound muscle action potentials were measured, and peak latency was averaged for three trials. NCV was calculated by dividing the distance (30mm) by the peak latency (msec) of the motor nerve action potential.

For the sciatic motor NCV measurement, the recording electrode was placed in the dorsum of the foot. Sciatic motor NCV (SMNVC) was determined by recording in the dorsum of the foot and stimulating with supramaximal stimulation first at the sciatic notch (15mm from the recording electrode) and then at the knee (5mm from the recording electrode). Compound muscle action potentials were measured and peak latency was averaged for three trials. The SMNCV was calculated by dividing the distance between the electrode placements (10mm) by the difference calculated by subtracting the peak latency at the knee from the sciatic notch.

The frequency band for sensory and motor NCV recordings included two 10Hz for muscle action potential and ten 2Hz for sensory action potential recordings.

Tissue Harvest

Blood (100ul) was collected into EDTA coated tubes centrifuged at 2,000 x g for 5min to isolate plasma for the determination of metabolites. Serum and blood were immediately flash frozen in liquid nitrogen and stored at -80°C. Plasma homocysteine metabolite concentrations were determined by stable isotope dilution capillary GS-MS. Prior to tissue extraction, mice were anesthetized with 0.15ml/10g tribromoethanol. Foot pad skin biopsies, sciatic nerve, brains and spinal cords were dissected and post-fixed in 4% paraformaldehyde for 48 hours.

Statistical Analysis

A set of 5 *a priori* comparisons between diets and genotypes were assessed for sensory and motor nerve conduction velocity, latency to tail flick, uracil concentration, and metabolic markers of homocysteine metabolism: 1. *Shmt1*$^{+/+}$ mice fed the control diet vs *db/db* and *Shmt1*$^{+/-}$ *db/db* mice fed control and vitamin B_{12} and folic acid deficient diets 2. *db/db* mice fed the control diet vs *db/db* mice fed the vitamin B_{12} and folic acid deficient diet*,* *Shmt1*$^{+/-}$ *db/db* mice fed the control and *Shmt1*$^{+/-}$ *db/db* mice fed the vitamin B_{12} and folic acid deficient diet 3. *db/db* mice fed the control diet vs *db/db* mice fed the vitamin B_{12} and folic acid deficient diet 4. *Shmt1* $^{+/-}$ *db/db* mice fed the control vs *Shmt1*$^{+/-}$ *db/db* mice fed the vitamin B_{12} and folic acid deficient diet 5. *Shmt1*$^{+/+}$ vs *Shmt1*$^{+/-}$ mice fed the control diet. Generalized linear and mixed models were used to determine the effects of mouse diet, genotype, and the interaction between diet and genotype on nerve conduction velocity, average latency to tail flick, hepatic uracil concentrations in DNA, and concentrations of plasma homocysteine metabolic off the accelerated and staggered rotarod over the course of three test trials as well as the latency to tail flick. Log-transformation was applied to normalize the data that was not normally distributed. LS means contrast was used to determine differences within main effects. A Bonferroni correction was applied for multiple comparisons. Analysis of tail flick latencies, nerve conduction velocity, metabolite measurements and genomic uracil content were conducted in JMP (JMP Pro 13.1).

Results

Shmt1 disruption and vitamin B_{12} and folic acid deficiency do not modify pain sensitivity in db/db mice

We analyzed tail flick latency for *Shmt1*$^{+/+}$ *db*$^{+}$, *Shmt1*$^{+/-}$ *db*$^{+}$, *Shmt1*$^{+/+}$ *db/db*, and *Shmt1*$^{+/-}$ *db/db* mice which revealed a significant effect of diet ($p<0.0001$). The contrast between *Shmt1*$^{+/+}$ *db*$^{+}$ mice on the control diet vs *Shmt1*$^{+/+}$ *db/db* and *Shmt1*$^{+/-}$ *db/db* mice on either the control or vitamin B_{12} and folic acid deficient diet revealed that nerve conduction in the tail motor nerve for each *Shmt1 db/db* genotype was significantly impaired, independent of diet (unadjusted $p<0.0001$, adjusted $p<0.0005$). The effect of genotype ($p=0.7655$) and the interaction between diet and genotype ($p=0.3324$) were not significant (Figure 2).

The contrast between *Shmt1*$^{+/+}$ *db*$^{+}$ mice on the control diet vs *Shmt1*$^{+/+}$ *db/db* and *Shmt1*$^{+/-}$ *db/db* mice on either the control or vitamin B_{12} and folic acid deficient diet revealed that nerve conduction in the tail motor nerve for each *Shmt1 db/db* genotype was significantly impaired, independent of diet (unadjusted $p<0.0001$, adjusted $p<0.0005$).

However, the contrast between *Shmt1*$^{+/+}$ *db/db* mice on the control diet vs *Shmt1*$^{+/+}$ *db/db* and *Shmt1*$^{+/-}$ *db/db* mice on either the control or vitamin B_{12} and folic acid

deficient diet revealed no significant differences in tail motor nerve conduction velocity (unadjusted p=0.5301).

The contrast between *Shmt1*$^{+/+}$ *db/db* mice on the control vs deficient diet and between *Shmt1*$^{+/-}$ *db/db* mice on both diets revealed no significant differences in nerve conduction velocity (unadjusted p=0.0874; unadjusted p=0.9840, respectively). These results indicate that neither the disruption of *Shmt1* or vitamin B_{12} and folic acid deficiency significantly impair the nerve conduction of *db/db* mice (Figure 2).

Shmt1 disruption and vitamin B_{12} and folic acid deficiency do not modify tail motor nerve conduction velocities in db/db mice

The analysis of tail motor nerve conduction velocities for *Shmt1*$^{+/+}$ *db*$^{+}$, *Shmt1*$^{+/-}$ *db*$^{+}$, *Shmt1*$^{+/+}$ *db/db*, and *Shmt1*$^{+/-}$ *db/db* mice revealed a significant effect of genotype (p<0.0001). The effect of diet (p=0.7655) and the interaction between diet and genotype (p=0.3324) were not significant (Figure 2A). The contrast between *Shmt1*$^{+/+}$ *db*$^{+}$ mice on the control diet vs *Shmt1*$^{+/+}$ *db/db* and *Shmt1*$^{+/-}$ *db/db* mice on either the control or vitamin B_{12} and folic acid deficient diet revealed that nerve conduction in the tail motor nerve for each *Shmt1 db/db* genotype was significantly impaired, independent of diet (unadjusted p<0.0001, adjusted p<0.0005).

However, the contrast between *Shmt1*$^{+/+}$ *db/db* mice on the control diet vs *Shmt1*$^{+/+}$ *db/db* and *Shmt1*$^{+/-}$ *db/db* mice on either the control or vitamin B_{12} and folic acid deficient diet revealed no significant differences in tail motor nerve conduction velocity (unadjusted p=0.5301).

The contrast between *Shmt1*$^{+/+}$ *db/db* mice on the control vs deficient diet and between *Shmt1*$^{+/-}$ *db/db* mice on both diets revealed no significant differences in nerve

conduction velocity (unadjusted p=0.0874; unadjusted p=0.9840, respectively). These results indicate that neither the disruption of *Shmt1* or vitamin B_{12} and folic acid deficiency significantly impair the nerve conduction of *db/db* mice (Figure 2A).

Shmt1 disruption does not impair tail sensory nerve conduction velocity in non-diabetic mice

We analyzed the sensory nerve conduction velocity of the tail nerve in non-diabetic and diabetic mice (Figure 2B). However, the amplitude of the action potentials for most of the *db/db* mice were too small to allow for the reliable detection of the peak latency. The contrast between *Shmt1*$^{+/+}$ *db*$^{+}$ and *Shmt1*$^{+/-}$ *db*$^{+}$ on the control diet revealed no significant effect of diet, genotype, and the interaction between diet and genotype (p>0.05). Shmt1$^{+/-}$ db^{+} mice on the control diet did not have any significant impairments in tail sensory nerve conduction compared to the wildtype group (unadjusted p=0.4975).

Shmt1 disruption impaired sciatic motor nerve conduction velocity in non-diabetic mice

We analyzed the effects of *Shmt1* heterozygosity and vitamin B_{12} and folic acid deficiency on sciatic motor nerve conduction in *db/db* mice (Figure 3). There was a significant effect of genotype (p=0.0012) but no significant effect of diet (p=0.6259) or the interaction between diet and genotype (p=0.5595). The contrast between *Shmt1*$^{+/+}$ *db*$^{+}$ mice on the control diet vs *Shmt1*$^{+/+}$ *db/db* and Shmt1$^{+/-}$ db/db mice on either the control or vitamin B_{12} and folic acid deficient diet revealed that motor nerve conduction in the sciatic nerve for each *Shmt1 db/db* genotype was significantly impaired, independent of diet (unadjusted p=0.0003, adjusted p=0.0015).

The contrast between *Shmt1*$^{+/+}$ *db/db* mice on the control diet vs *Shmt1*$^{+/+}$ *db/db* and *Shmt1*$^{+/-}$ *db/db* mice on either the control or vitamin B_{12} and folic acid deficient diet revealed no significant differences in sciatic motor nerve conduction velocity (unadjusted p=0.6512).

The contrast between *Shmt1*$^{+/+}$ *db/db* mice on the control vs deficient diet and between *Shmt1*$^{+/-}$ *db/db* mice on both diets revealed no significant differences in nerve conduction velocity (unadjusted p=0.0874; unadjusted p=0.9840, respectively). These results indicate that although neither the disruption of *Shmt1* or vitamin B_{12} and folic acid deficiency significantly impair the nerve conduction of *db/db* mice.

Interestingly, the contrast between *Shmt1*$^{+/+}$ *db*$^{+}$ and *Shmt1*$^{+/-}$ *db*$^{+}$ mice on the control diet revealed significantly impaired motor nerve conduction in the sciatic nerve of *Shmt1*$^{+/-}$ *db*$^{+}$ (unadjusted p=0.0016, adjusted p=0.0080). This finding indicates that disruption of *Shmt1* significantly impairs motor nerve conduction in the sciatic nerve of non-diabetic mice.

Shmt1 and Leprdb disruption as well as dietary vitamin B_{12} and folic acid deficiency influenced metabolic markers of homocysteine remethylation

Homocysteine: The effects of diet (p=0.0038) and genotype (p<0.0001) were significant on plasma concentrations of homocysteine indicating that: 1. mice fed the vitamin B_{12} and folic acid deficient diet showed higher homocysteine concentrations compared with those on the control diet and 2. *db*$^{+}$ mice had higher overall concentrations of homocysteine than *db/db* mice, independent of *Shmt1* genotype (Table 1). The contrast between *Shmt1*$^{+/+}$ *db*$^{+}$ mice on the control diet vs *Shmt1*$^{+/+}$ *db/db* and *Shmt1*$^{+/-}$ *db/db* mice on either the control or vitamin B_{12} and folic acid

deficient diet revealed that plasma homocysteine concentrations for each *Shmt1 db/db* genotype were significantly lower, independent of diet (unadjusted p=0.0068, adjusted p=0.0340). The effect of the interaction between diet and genotype was not significant (p=0.2661).

Methionine: The effect of diet (p=0.0001) was significant on plasma concentrations of methionine indicating that mice fed the vitamin B_{12} and folic acid deficient diet showed lower methionine concentrations for all genotypes, except for *Shmt1*$^{+/-}$ *db/db*, compared to those fed the control diet (Table 1). The contrast between between *Shmt1*$^{+/+}$ *db*$^{+}$ mice on the control diet vs *Shmt1*$^{+/+}$ *db/db* and *Shmt1*$^{+/-}$ *db/db* mice on either the control or vitamin B_{12} and folic acid deficient diet revealed that plasma methionine concentrations for each *Shmt1 db/db* genotype were significantly lower, independent of diet (unadjusted p=0.0015, adjusted p=0.0075). There was no significant effect of genotype (p=0.6153) or the interaction of diet and genotype (p=0.0709).

α-Aminobutyric acid: Plasma concentrations of α-Aminobutyric acid were significantly affected by diet (p=0.0032), genotype (p=0.0008), and the interaction between diet and genotype (p=0.0032). These results indicated that: 1. *db*$^{+}$ mice fed the vitamin B_{12} and folic acid deficient diet showed higher α-Aminobutyric acid concentrations compared to *db*$^{+}$ mice fed the control diet, independent of *Shmt1* genotype and 2. *db/db* mice fed the deficient diet showed the opposite effect 3. *Shmt1*$^{+/-}$ *db*$^{+}$ mice on the deficient diet had the highest plasma concentration of α-Aminobutyric acid whereas *Shmt1*$^{+/-}$ *db/db* mice fed the deficient diet had the lowest (Table 1).

Glycine: The effect of genotype (p=0.0006) was significant on plasma concentrations of glycine indicating that *Shmt1*$^{+/+}$ *db*$^{+}$ mice had higher levels compared to the other genotype groups. The contrast between *Shmt1*$^{+/+}$ *db*$^{+}$ mice fed the control diet and *Shmt1*$^{+/+}$ *db/db* and *Shmt1*$^{+/-}$ *db/d*b mice fed either the control or deficient diet revealed significantly higher glycine concentrations in *Shmt1*$^{+/+}$ *db/db* mice fed the control diet (unadjusted p=0.0001; adjusted p=0.0005). *Shmt1*$^{+/+}$ *db*$^{+}$ on the control diet had higher glycine concentrations compared to *Shmt1*$^{+/-}$ *db*$^{+}$ mice, however this difference was not significant after controlling for multiple comparisons (unadjusted p=0.0439, adjusted p=0.2195). The effect of diet (p=0.2446) and the interaction between diet and genotype (p=0.7062) was not significant (Table 1).

Serine: The effect of diet (p<0.0001) was significant on plasma concentrations of serine indicating that mice fed the vitamin B_{12} and folic acid deficient diet had lower serine concentrations for all groups, except for *Shmt1*$^{+/-}$ *db/db* mice, compared to those fed the control diet (Table 1). The contrast between *Shmt1*$^{+/+}$ *db*$^{+}$ mice fed the control diet and *Shmt1*$^{+/+}$ *db/db* and *Shmt1*$^{+/-}$ *db/db* mice fed either the control or deficient diet revealed that *Shmt1*$^{+/+}$ *db*$^{+}$ mice on the control diet had significantly higher serine concentrations than *db/db* mice, independent of *Shmt1* genotype (unadjusted p=0.0031, adjusted p=0.0155). *Shmt1*$^{+/+}$ *db/db* mice on the control diet had a higher serine concentration compared to the deficient diet group, however this difference was not significant when adjusted for multiple comparisons (unadjusted p=0.0165, adjusted p=0.0825). *Shmt1*$^{+/+}$ *db/db* mice on the control diet had a higher serine concentration compared to *Shmt1*$^{+/+}$ *db/db* mice on the deficient diet and *Shmt1*$^{+/-}$

db/db mice on either the control or deficient diet, however this effect was also not statistically significant (unadjusted p=0.0475, adjusted p=0.2375).

The effect of genotype and the interaction between diet and genotype was not significant on plasma concentrations of serine (p>0.05).

Cystathione: The effects of diet, genotype, and the interaction of diet and genotype were not significant on plasma concentrations of cystathione (p>0.05).

Cysteine: The effects of diet, genotype, and the interaction of diet and genotype were not significant on plasma concentrations of cysteine (p>0.05).

Leprdb disruption does not significantly elevate uracil content in hepatic nuclear DNA

The analysis of liver uracil content for *Shmt1*$^{+/+}$ *db*$^{+}$, *Shmt1*$^{+/-}$ *db*$^{+}$, *Shmt1*$^{+/+}$ *db/db*, and *Shmt1*$^{+/-}$ *db/db* mice revealed a significant effect of diet (p=0.0247), indicating that mice on vitamin B_{12} and folic acid deficient diets had higher genomic DNA levels of uracil compared to the control diet (Figure 4). The effect of genotype (p=0.5293) and the interaction between diet and genotype (p=0.1884) were not significant.

The contrast between *Shmt1*$^{+/+}$ *db*$^{+}$ mice on the control diet vs *Shmt1*$^{+/+}$ *db/db* and *Shmt1*$^{+/-}$ *db/db* mice revealed elevated concentrations of uracil content in *db/db* mice, however, this difference in liver DNA uracil concentrations was not significant (unadjusted p=0.5931). These results indicate that neither the disruption of *Shmt1* or vitamin B_{12} and folic acid deficiency significantly increase uracil misincorporation in the hepatic DNA of *db/db* mice (Figure 2).

Taken together, these results indicate that impaired sensory and motor nerve conduction in *db/db* mice may not be attributed to genome instability caused by elevated uracil misincorporation in DNA.

Discussion

In this study, we investigated the effects of decreased expression of *Shmt1* and impaired *de novo* dTMP synthesis in a mouse model of diabetic peripheral neuropathy. We measured differences in thermal pain sensitivity and tail and sciatic nerve conduction in *Shmt1*$^{+/+}$ *, Shmt1*$^{+/-}$, *db/db*, and *Shmt1*$^{+/-}$ *db/db* mice on control and vitamin B_{12} and folic acid deficient diets to determine whether *Shmt1* heterozygosity exacerbates diabetic neuropathy and whether these effects would be modified by diet. The disruptive effects of impaired *Leprdb* expression on motor and sensory peripheral neuropathy in the *db/db* mouse model of spontaneous type 2 diabetes have been well characterized (11-14). In this study*, db/db* mice on control and vitamin B_{12} and folic acid deficient diets exhibited significantly decreased sciatic and tail motor nerve conduction velocities as well as decreased sensitivity to pain. We predicted that Shmt1$^{+/-}$ *db/db* mice would show the most severe symptoms of peripheral neuropathy. However, deficits in peripheral nerve function in *db/db* mice were not significantly impacted by diet or *Shmt1* genotype.

Surprisingly, sciatic motor nerve conduction velocities in *Shmt1*$^{+/-}$ mice were significantly lower compared to wildtype mice. These findings suggest that *Shmt1* heterozygosity and impaired *de novo* thymidylate synthesis lead to peripheral neuropathy in the sciatic nerve that is comparable to diabetic peripheral neuropathy.

Interestingly, *Shmt1*$^{+/-}$ mice did not show significant impairments in sensory and motor nerve conduction or pain sensitivity in the tail motor nerve. Peripheral neuropathy can affect multiple nerves or one nerve at a time (15,16). These results indicate a progression pattern of mononeuropathy in *Shmt1*$^{+/-}$ mice. Future nerve conduction studies are needed to investigate the long-term patterns of peripheral neuropathy progression in *Shmt1*$^{+/-}$ mice over a longer study period.

Epidemiological studies in diabetic populations have shown an association between impaired glucose metabolism, homocysteine, and disruption of the transsulfuration pathway indicating a link between impaired folate- mediated one-carbon metabolism and diabetes associated pathologies (17). In this study, the combined *db/db* genotype and diet groups had significantly higher plasma concentrations of cysteine and significantly lower concentrations of plasma homocysteine, methionine, serine, and glycine compared to wildtype mice on the control diet. These results further implicate a role of impaired folate and vitamin B_{12} metabolism in diabetic peripheral neuropathy.

Interestingly, plasma α-aminobutyric acid concentrations were not increased in *db/db* and *Shmt1*$^{+/-}$*db/db* mice fed the vitamin B_{12} and folic acid deficient diets. α-Aminobutryic acid has been shown to regulate the synthesis and homeostasis of glutathione, which plays a protective role in the cell as an anti-oxidant (18). A recent epidemiological study in Kingston, Jamaica found that type 2 diabetic patients had significantly lower blood concentrations of glutathione (19). Together, these findings suggest a distinctive metabolic profile of impaired homocysteine metabolism in diabetic peripheral neuropathy.

We measured uracil content in DNA to investigate the potential causal mechanisms linking impaired *de novo* dTMP synthesis and peripheral neuropathy. We expected hepatic uracil concentrations in DNA to be elevated in *db/db* mice. Although the results were not significant, we did observe a 13% increase in uracil concentrations in *db/db* mice on the control diet compared to wildtype mice (0.902 pg Uracil/µg DNA, 0.798 pg Uracil/µg DNA; respectively) (Figure 4). Further investigation of the effects of *Leprdb* disruption on *de novo* dTMP synthesis is needed in order to elucidate causal mechanisms of impaired folate-mediated one-carbon metabolism in diabetic neuropathy.

This study is novel in its approach of using a mouse model of decreased expression of the folate-dependent *Shmt1* enzyme to investigate the role of impaired *de novo* dTMP synthesis. Further ongoing studies include immunohistochemical analysis of biomarkers of DNA damage and genome instability in sciatic nerves tissue of *Shmt1*$^{+/-}$ and *db/db*. Taken together, these findings suggest the potential need for folate and vitamin B_{12} supplementation in clinical populations at risk for peripheral neuropathy.

Metabolite	**Control Diet**				**Vitamin B_{12} and Folic Acid Deficient Diet**			
	Shmt1$^{+/+}$ *db*$^{+}$	*Shmt1*$^{+/-}$ *db*$^{+}$	*Shmt1*$^{+/+}$ *db/db*	*Shmt1*$^{+/-}$ *db/db*	*Shmt1*$^{+/+}$ *db*$^{+}$	*Shmt1*$^{+/-}$ *db*$^{+}$	*Shmt1*$^{+/+}$ *db/db*	*Shmt1*$^{+/-}$ *db/db*
Homocysteine *µmol/L*	3.07 ± 0.29	2.61 ± 0.28	1.68 ± 0.16	1.85 ± 0.17	4.42 ± 0.37	3.03 ± 0.30	2.2 ± 0.32	2.14 ± 0.38
Cystathione *nmol/L*	976 ± 71.3	869 ± 140.2	757 ± 83.3	909 ± 98.1	702 ± 94.6	531 ± 122.0	786 ± 96.9	949 ± 156.7
Cysteine *µmol/L*	153 ± 10.1	145 ± 10.32	161 ± 8.35	163 ± 16.0	152 ± 8.88	115 ± 11.02	153 ± 15.54	139 ± 13.76
Methionine *µmol/L*	96.3 ± 9.26	84.7 ± 15.3	70.2 ± 14.3	60.2 ± 10.9	43.9 ± 3.75	35.8 ± 5.95	43.3 ± 10.77	61.2 ± 7.65
α-Aminobutyric acid *µmol/L*	8.18 ± 0.99	8.22 ± 1.19	8.35 ± 0.90	6.78 ± 0.65	17.2 ± 3.49	19.4 ± 4.81	7.02 ± 0.67	5.72 ± 0.47
Glycine *µmol/L*	184 ± 10.4	154 ± 6.64	152 ± 9.05	136 ± 6.03	171 ± 12.5	159 ± 19.7	130 ± 13.6	129 ± 8.59
Serine *µmol/L*	173 ± 17.2	134 ± 17.4	149 ± 26.0	118 ± 11.0	90.0 ± 7.65	130 ± 13.6	91.7 ± 17.5	121 ± 10.8

Table 1. Metabolic profile of plasma isolated from *Shmt1*$^{+/+}$*, Shmt1*$^{+/-}$*, db/db*, and *Shmt1*$^{+/-}$ *db/db* mice on control and vitamin B_{12} and folic acid deficient diets. Data indicate means ± SEM (n=9-10 animals per group). Sally P Stabler performed GC-MS analysis on all plasma samples to measure markers of the remethylation and transsulfuration pathway. Eunice B Awuah collected plasma samples from all mice and analyzed GC-MS data.

Figures

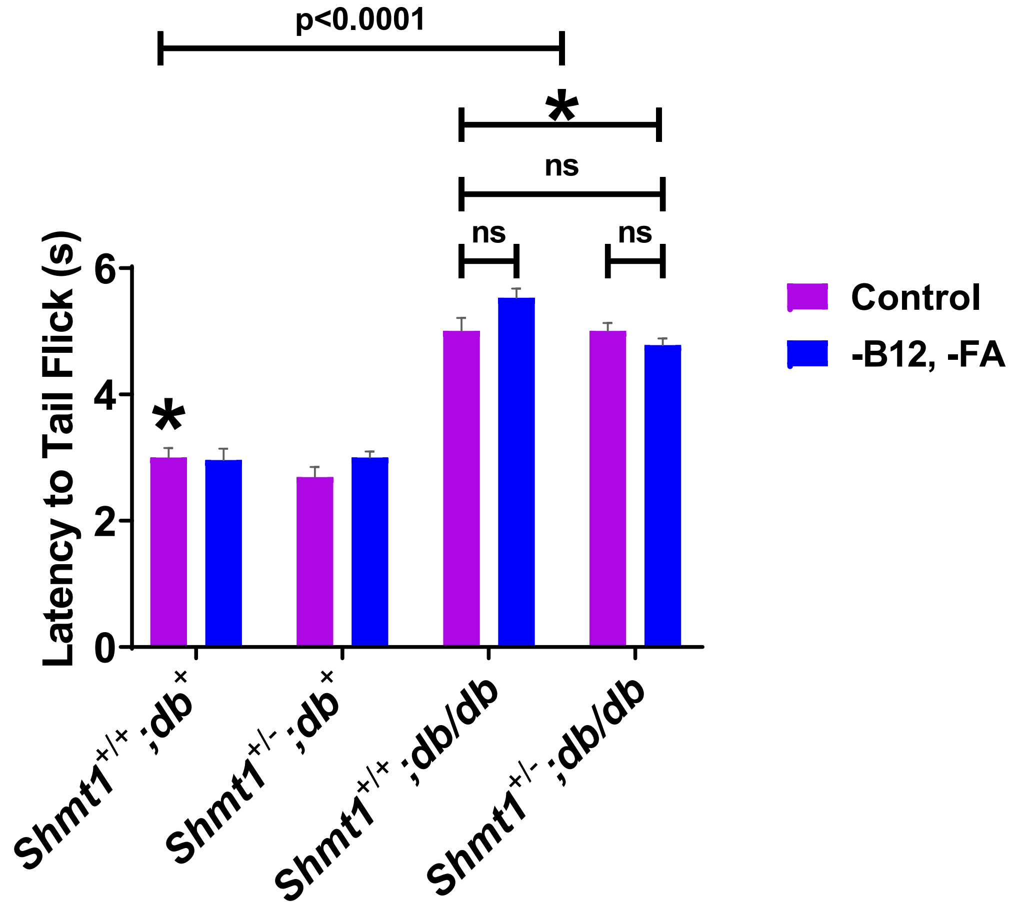


Figure 1. Assessment of latency to tail flick in *Shmt1*$^{+/-}$ and *db/db* mice. Mice were placed on a tail flick apparatus and latency to tail flick in response to a painful thermal stimulus was recorded for *Shmt1*$^{+/+}$, *Shmt1*$^{+/-}$, *db/db,* and *Shmt1*$^{+/-}$ *db/db* mice on their respective diets. The main effect of genotype was significant whereas the effects of diet and the interaction between diet and genotype were not. *db/db* mice on either the control or vitamin B_{12} deficient diet had significantly higher latencies to tail flick compared to *Shmt1*$^{+/+}$ mice on the control diet ($p<0.0001$). Latencies to tail flick were not significantly different between *Shmt1*$^{+/+}$ *db/db* mice on the control diet versus *Shmt1*$^{+/+}$ *db/db* mice on the vitamin B_{12} and folic acid deficient diet as well as *Shmt1*$^{+/-}$ *db/db* mice on either the control or vitamin B_{12} and folic acid deficient. Data indicate means ± SEM (n=10-15 animals per group). * indicates a comparison with *Shmt1*$^{+/+}$ *db*$^{+}$ mice on the control diet. Eunice B. Awuah performed behavioral tasks on all mice, collected and analyzed data.

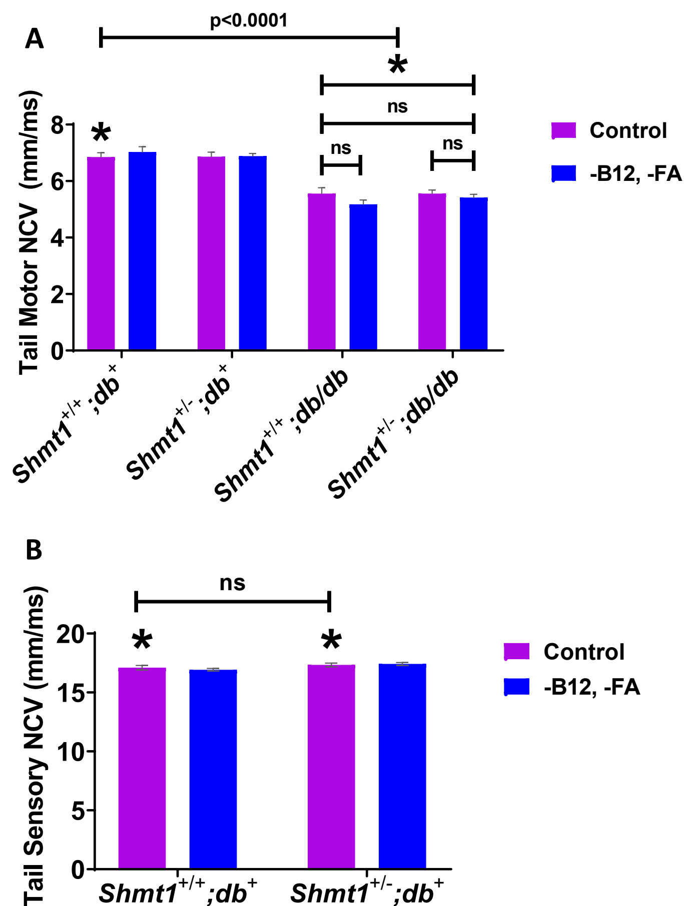


Figure 2. Assessment of tail motor and sensory nerve conduction velocity in *Shmt1*$^{+/-}$ and *db/db* mice. Nerve conduction velocity was recorded from the tail nerve for *Shmt1*$^{+/+}$*, Shmt1*$^{+/-}$*, db/db, and Shmt1*$^{+/-}$ *db/db* mice on their respective diets. A) The main effect of genotype was significant whereas the effects of diet and the interaction between diet and genotype were not. *db/db* mice on either the control or vitamin B_{12} deficient diet had significantly lower tail motor nerve conduction velocities compared to *Shmt1*$^{+/+}$ mice on the control diet ($p<0.0001$). Nerve conduction velocities for *Shmt1*$^{+/+}$ *db/db* mice on the control diet were not significantly different from *Shmt1*$^{+/+}$ *db/db* mice on the vitamin B_{12} and folic acid deficient diet as well as *Shmt1*$^{+/-}$ *db/db* mice on either the control or vitamin B_{12} and folic acid deficient. B) *Shmt1*$^{+/+}$ *db*$^{+}$ mice on the control diet did not have significantly different tail sensory nerve conduction velocities compared to *Shmt1*$^{+/-}$ *db*$^{+}$ mice on the same diet. Data indicate means ± SEM (n=10-15 animals per group). * indicates a comparison with *Shmt1*$^{+/+}$ *db*$^{+}$ mice on the control diet. <u>Wilhelm H Elmore designed and built the NCV amplifier and NCV data collection software. Eunice B Awuah collected NCV data from all mice and analyzed the data.</u>

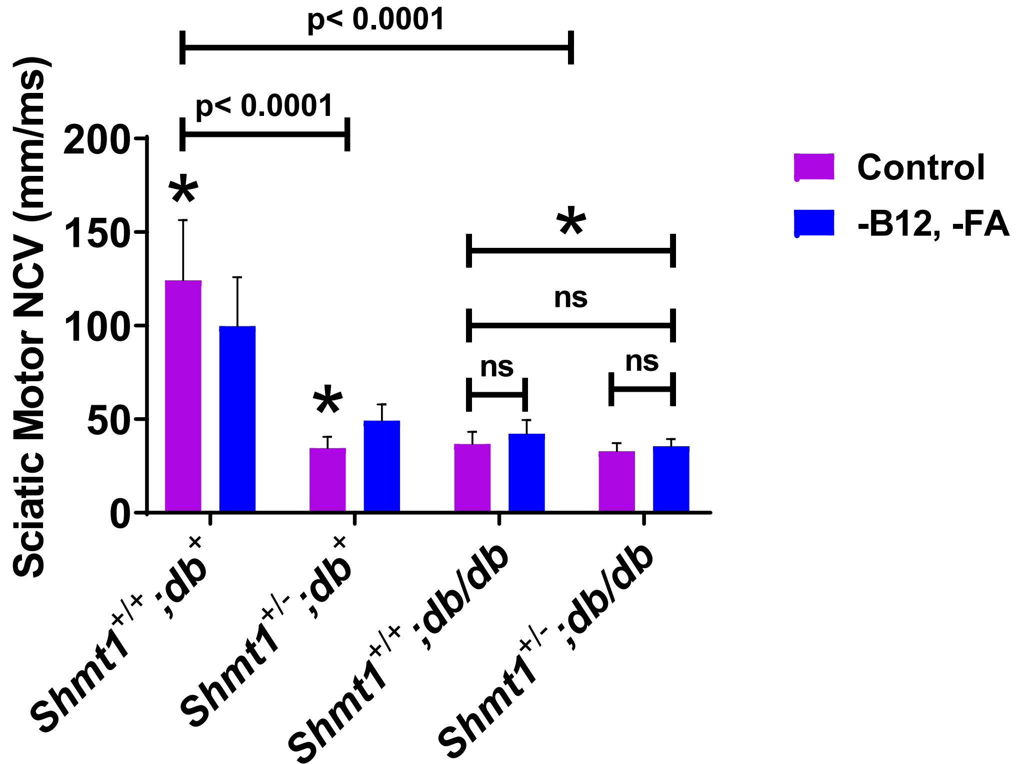


Figure 3. Assessment of sciatic motor nerve conduction velocity in *Shmt1*$^{+/-}$ and *db/db* mice. Motor nerve conduction velocity was recorded from the sciatic nerve for *Shmt1*$^{+/+}$, *Shmt1*$^{+/-}$, *db/db, and Shmt1*$^{+/-}$ *db/db* mice on their respective diets. Data indicate means ± SEM (n=10-15 animals per group). Wilhelm H Elmore designed and built the NCV amplifier and NCV data collection software. Eunice B Awuah collected NCV data from all mice and analyzed the data.

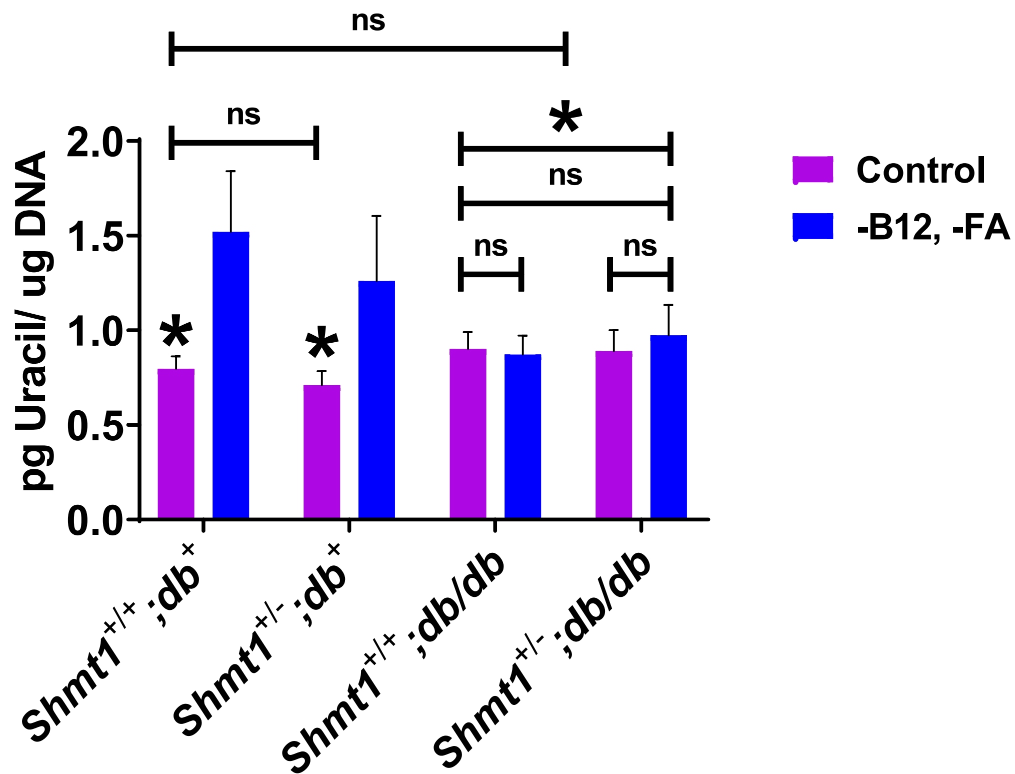


Figure 4. Assessment of liver uracil DNA concentration in *Shmt1*$^{+/+}$, *Shmt1*$^{+/-}$, *db/db*, and *Shmt1*$^{+/-}$ *db/db* mice on control and vitamin B_{12} and folic acid deficient diets. Data indicate means ± SEM (n=5 animals per group). Eunice B Awuah collected liver tissue from each mouse, isolated liver DNA, collected and analyzed GC-MS data.

References

1.Martyn CN, Hughes RAC. Epidemiology of peripheral neuropathy. J Neurol Neurosurg Psychiatry. 1997;62:310–318.

2.Igbal Z et al. Diabetic Peripheral Neuropathy: Epidemiology, Diagnosis, and Pharmacotherapy. Clin Ther. 2018 Jun;40(6):828-849.

3.Hughes RAC. Peripheral Neuropathy. BMJ. 2002 Feb 23; 324(7335): 466–469

4.Watson JC, Dyck PJ. Peripheral Neuropathy: A Practical Approach to Diagnosis and Symptom Management. Mayo Clin Proc. 2015 Jul;90(7):940-51

5.De Jager J. Long term treatment with metformin in patients with type 2 diabetes and risk of vitamin B-12 deficiency: randomised placebo controlled trial. BMJ. 2010 May 20;340:c2181

6.Komirishetty P et al. PARP inhibition attenuates neuroinflammation and oxidative stress in chronic constriction injury induced peripheral neuropathy. Life Sci. 2016 Apr 1;150:50-60

7. Anderson DD, Stover PJ. SHMT1 and SHMT2 are functionally redundant in nuclear de novo thymidylate biosynthesis. PLoS ONE 2009;4:e5839.

8. Anderson DD, Woeller CF, Stover PJ. Small ubiquitin-like modifier-1 (SUMO-1) modification of thymidylate synthase and dihydrofolate reductase. Clin Chem Lab Med 2007;45:1760–3

9.Beaudin AE, et al. Shmt1 and de novo thymidylate biosynthesis underlie folate responsive neural tube defects in mice. Am J Clin Nutr. 2011; 93(4):789–798.

10. Abarinov EV, Beaudin AE, Field MS, et al. Shmt1 impairs hippocampal neurogenesis and mnemonic function in mice. *J Nutr.* 2013 Jul;143(7):1028-35

11.Cheng HT et al. Nerve growth factor mediates mechanical allodynia in a mouse model of type 2 diabetes. J Neuropathol Exp Neurol. 2009;68:1229–1243.

12.Kan M, Guo G, Singh B, Singh V, Zochodne DW. Glucagon-like peptide 1, insulin, sensory neurons, and diabetic neuropathy. J Neuropathol Exp Neurol. 2012;71:494–510.

13.Dauch JR, Yanik BM, Hsieh W, Oh SS, Cheng HT. Neuron-astrocyte signaling network in spinal cord dorsal horn mediates painful neuropathy of type 2 diabetes. Glia. 2012 Sep; 60(9):1301-15.

14.Sullivan KA, Hayes JM, Wiggin TD, Backus C, Su Oh S, Lentz SI, Brosius F 3rd, Feldman EL.Mouse models of diabetic neuropathy. Neurobiol Dis. 2007 Dec; 28(3):276-85.

15.Hughes RAC. Peripheral Neuropathy. BMJ. 2002 Feb 23; 324(7335): 466–469

16.Watson JC, Dyck PJ. Peripheral Neuropathy: A Practical Approach to Diagnosis and Symptom Management. Mayo Clin Proc. 2015 Jul;90(7):940-51

17.Wijekoon EP, et al. Homocysteine metabolism in diabetes. Biochem Soc Trans. 2007. Biochem Soc Trans. 2007 Nov;35(Pt 5):1175-9.

18.Irino Y et al.2-Aminobutyric acid modulates glutathione homeostasis in the myocardium. *Scientific Reports*. 2016; (6): 36749

19.Lutchmansingh FK et al. Glutathione metabolism in type 2 diabetes and its relationship with microvascular complications and glycemia. PLoS One. 2018; 13(6): e019862

CHAPTER 4: FUTURE DIRECTIONS

The results from the above-mentioned studies indicate a role of impaired *de novo* thymidylate synthesis in peripheral neuropathy. However, motor coordination and balance, as assessed by the rotarod test, was not significantly impacted by the disruption of *Shmt1* expression. Long-term exposure to diets deficient in folic acid and vitamin B_{12} led to a decrease in latency to fall in the staggered rotarod tests as well as an increased latency to tail flick, suggesting a potential link between impaired folate-mediated one-carbon metabolism and damage to peripheral nerves involved with motor and sensory function. These behavioral and sensory tests alone were not sensitive enough to determine the causal mechanism by which impaired *de novo* dTMP synthesis and vitamin B_{12} and folic acid deficiency on neurodegeneration in the peripheral and central nervous system.

Future Histology Experiments

Impairments in *de novo* dTMP synthesis have been linked to uracil misincorporation, DNA damage, and disrupted proliferation of neural tissue. In addition to the accelerated and staggered rotarod test, follow up studies examining the causal role of impaired folate-mediated one-carbon metabolism on neurodegeneration should include immunohistochemical analysis of the expression of markers related to neuronal and DNA damage in peripheral and central nerve tissue including sciatic nerve, brain, and spinal cord. Quantifying immunofluorescence intensity of glial fibrillary acid protein, an indicator of neuron damage, in the sciatic nerve, spinal cord, and areas of the brain associated with motor coordination of *Shmt1*$^{+/-}$ mice (i.e. cerebellum) can also be used

to elucidate the progression pattern of neurodegeneration. Previous work in our lab has established a role of *Shmt1* heterozygosity in impaired hippocampal neurogenesis (1). In order to determine whether impaired *de novo* dTMP synthesis leads to DNA damage and strand breaks in proliferative cells in the brain, future studies should also include immunohistochemical analysis of gamma-H2AX in hippocampal tissue of *Shmt1*$^{+/-}$ mice.

In addition to measuring motor and sensory nerve conduction velocity in *Shmt1*$^{+/-}$ and *db/db* mice, future experiments should include immunohistochemical analysis of DNA damage in neuronal and Schwann cells of the sciatic nerve using gamma-H2AX and a Schwann cell marker, such as s100 to further investigate whether *Shmt1*$^{+/-}$ induced neuropathy is caused by demyelinating or axonal degeneration. Immunohistochemical analysis of neuronal and DNA damage in sciatic nerve, spinal cord, hippocampal, and cerebellar tissue in Shmt1$^{+/-}$ mice is part of ongoing studies in our lab.

Future *in vitro* and *in vivo* Experiments

Parp1 has been established as an essential enzyme in DNA repair. Its overactivation in response to DNA damage has been implicated in having a causative role in neurodegeneration (2). High levels of reactive oxygen species in diabetes have been linked to exacerbated DNA damage-induced Parp1 activation and neuronal cell death (3).

Impairments in sciatic motor nerve conduction velocity were observed in both *Shmt1*$^{+/-}$ and *db/db* mice, implicating a possible shared mechanism in peripheral neuropathy. However, it is not known whether uracil misincorporation caused by impaired *de novo* dTMP synthesis leads to Parp-1 activation-induced cell death.

Future *in vitro* studies using CRISPR technology to inhibit the expression of SHMT1 (Figure 1) are needed to determine whether uracil misincorporation into DNA leads to increased Parp1 activation and cleavage. This would help to elucidate common mechanisms linking peripheral neuropathy caused by diabetes and impaired folate-mediated one-carbon metabolism.

Parp1 inhibition has also been linked to improved nerve function in experimental animal models of neuropathy and neuropathic pain (4). These findings implicate the need for future experiments that measure nerve conduction velocity in *Shmt1*$^{+/-}$ and *db/db* mice with impaired *Parp1* expression in order to determine the role of *Parp1* activation in diabetic and impaired *de novo* dTMP synthesis-induced peripheral neuropathy (Figure 2).

<u>Future Supplementation and Treatment Experiments</u>

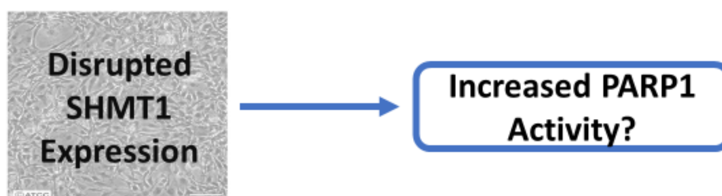
The analysis of metabolic markers of homocysteine metabolism in *db/db* and *Shmt1*$^{+/-}$*; db/db* mice revealed a distinctive metabolic profile of decreased plasma markers of functional folate and vitamin B_{12} status and increased activation of the trans-sulfuration pathway compared to wildtype mice. These findings implicate a distinctive deficiency in folate-mediated one-carbon metabolism nutrients in a mouse model of diabetic peripheral neuropathy. Future mouse model (Figure 3) and clinical studies (Figure 4) are needed to determine whether long-term dietary supplementation with folic acid and vitamin B_{12} increase nerve conduction in diabetic and impaired *de novo* dTMP synthesis-induced peripheral neuropathy.

Increased reactive oxygen species production and oxidative stress link diabetes-induced DNA damage to PARP1 activation. The results from the homocysteine

metabolism profile of *db/db* and *Shmt1*$^{+/-}$*; db/db* also showed a decrease in plasma α-aminobutryic acid. This implies a potential sequestering of α-aminobutryic acid within cells in order to increase the synthesis of glutathione, which plays a major role in reducing oxidative stress (5). A recent study conducted in Kingston, Jamaica found that type 2 diabetic patients had significantly lower blood concentrations of glutathione (6). In another study, mice treated with the chemotherapeutic drug oxaliplatin and administered glutathione showed reduced symptoms of peripheral neuropathy (7)

Since the effects of long-term exposure to PARP1 inhibition therapy in humans are not well understood, future clinical studies are needed to investigate the effects of inhibiting upstream molecular activators of DNA damage-induced PARP1 activation. Future clinical studies are needed to investigate both the effects of diabetes on markers of trans-sulfuration as well as the anti-oxidative effects of dietary glutathione supplementation on nerve conduction (Figure 4).

The investigation of the effects of impaired *de novo* dTMP synthesis as well as folic acid, vitamin B_{12}, and anti-oxidant supplementation in diabetic populations is necessary for determining new approaches for treatment of peripheral neuropathy.



Human Hela and A549 Cells

Cell Line	Modified Media	Effect Measured
Control Hela	+B12, +FA	Western Blot -Parp1 protein expression -Parp1 protein cleavage
CRISPR SHMT1 Hela	+B12, +FA	
Control A549	+B12, +FA	
CRISPR SHMT1 A549	+B12, +FA	

Figure 1. Future In vitro experiment to determine whether PARP1 is activated in response to impaired SHMT1 expression

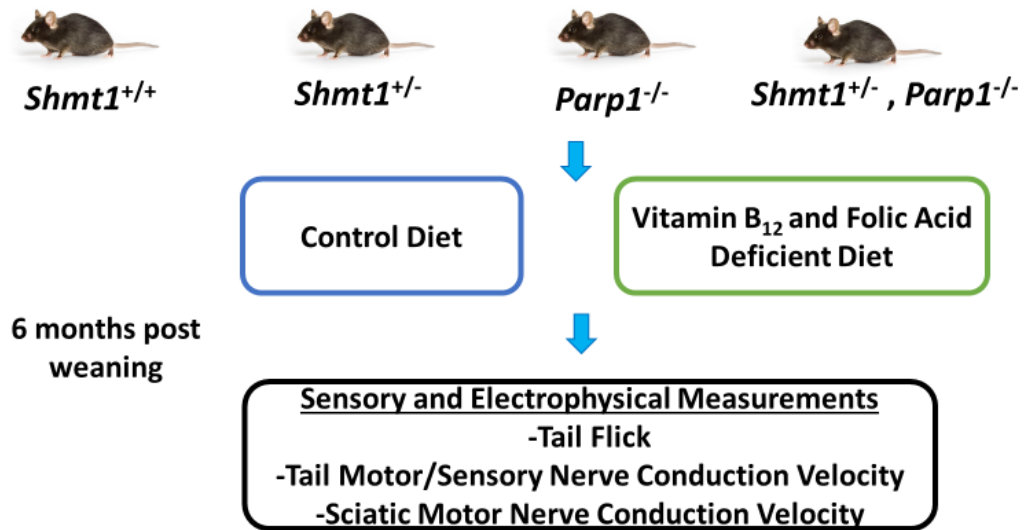


Figure 2. Future In vivo experiment to determine the role of PARP1 activation in peripheral neuropathy in Shmt1$^{+/-}$ mice

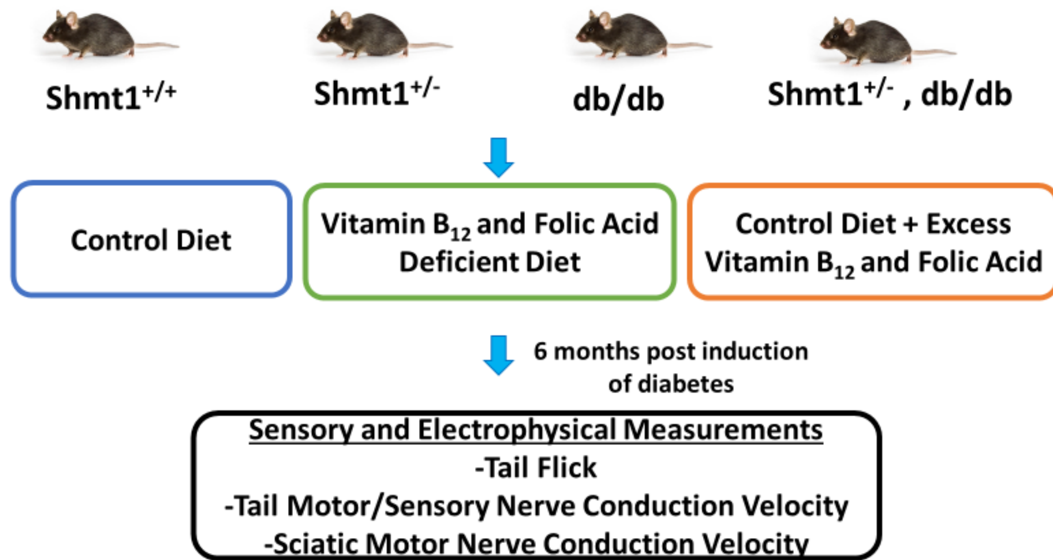


Figure 3. Future In vivo experiment to determine the role of folic acid and vitamin B_{12} supplementation in peripheral neuropathy

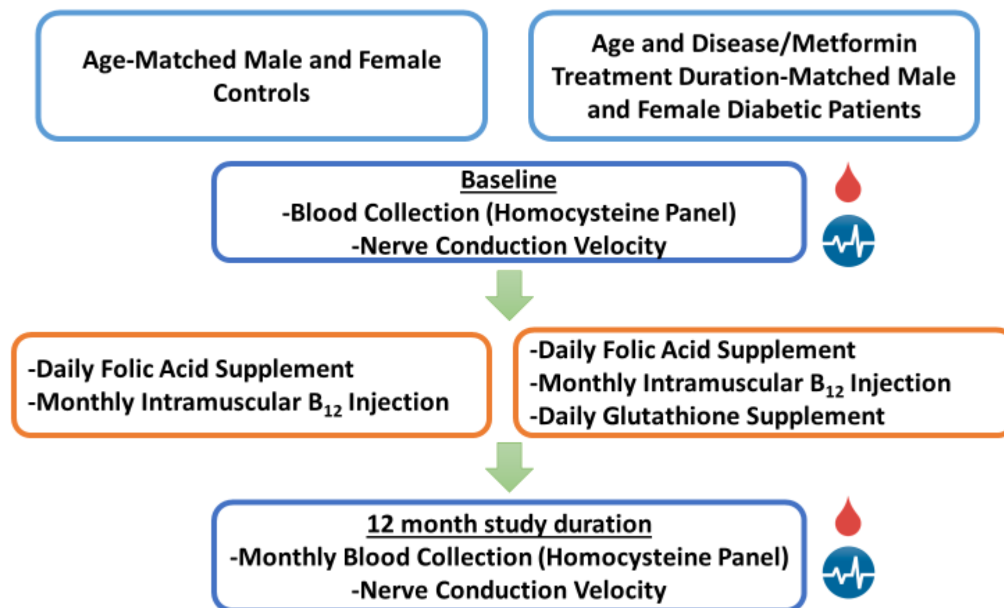


Figure 4. Future clinical experiment to determine the role of folic acid, vitamin B_{12}, and glutathione supplementation in peripheral neuropathy

References

1. Abarinov E et al. Disruption of *Shmt1* Impairs Hippocampal Neurogenesis and Mnemonic Function in Mice**.** J Nutr. 2013 Jul; 143(7): 1028–1035.

2. Martire S, Mosca L, d'Erme M. PARP-1 involvement in neurodegeneration: A focus on Alzheimer's and Parkinson's diseases. Mech Ageing Dev. 2015 Mar;146-148:53-64.

3. J. L. Edwards, A. M. Vincent, H. T. Cheng, and E. L. Feldman, "Diabetic neuropathy: mechanisms to management," Pharmacology & Therapeutics, vol. 120, no. 1, pp. 1–34, 2008

4. Komirishetty P et al. Poly(ADP-ribose) polymerase inhibition reveals a potential mechanism to promote neuroprotection and treat neuropathic pain. Neural Regen Res. 2016 Oct; 11(10): 1545–1548.

5. Irino Y et al.2-Aminobutyric acid modulates glutathione homeostasis in the myocardium. *Scientific Reports*. 2016; (6): 36749

6. Lutchmansingh FK et al. Glutathione metabolism in type 2 diabetes and its relationship with microvascular complications and glycemia. PLoS One. 2018; 13(6): e019862

7. Lee M et al. Glutathione alleviated peripheral neuropathy in oxaliplatin-treated mice by removing aluminum from dorsal root ganglia. Am J Transl Res. 2017; 9(3): 926–939.

APPENDIX 1: B-VITAMIN SUPPLEMENTATION IN SICKLE CELL DISEASE

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Background

Description of the condition

Sickle cell disease (SCD) is a group of genetic disorders that affect haemoglobin, the molecule in red blood cells that transports oxygen throughout the body, leading to a range of clinical manifestations and decreased life expectancy. Worldwide an estimated 312,000 children are born each year with sickle cell anaemia, the most common and severe form of SCD (Piel 2013), and although the majority of affected

individuals originate from Sub-Saharan Africa (Modell 2008), SCD also affects people of Mediterranean, Caribbean, Middle-Eastern, and Asian origin. In the U.S., life expectancy for people with SCD is 42 years and 48 years for males and females, respectively (Platt 1994).

The clinical manifestations associated with SCD are the result of chronic anaemia and the blockage of small blood vessels (Belcher 2014; Kuypers 1998). The rigid sickled cells adhere to the lining of blood vessels. The resultant vaso-occlusion blocks oxygen delivery to tissues and organs, leading to inflammation (De Franceschi 2011; Vinchi 2013). Over time, repetitive vaso-occlusion damages blood vessels leading to vasculopathy (Kato 2006; Pegelow 1997). The resulting pathophysiology is the basis for clinical manifestations including, but not limited to, vaso-occlusive pain episodes (VOE), acute chest syndrome, acute splenic sequestration, and stroke (Kato 2007). The vasculopathy of SCD is further associated with organ damage including early loss of splenic function, resulting in an increased risk of infection (Ramakrishnan 2010). Additionally, the dense, dehydrated red blood cells are easily destroyed, leading to haemolytic anaemia. This chronic anaemia results in increased fatigue, pulmonary hypertension, and leg ulcerations (Higgs 1982; Nolan 2005).

There are multiple therapies, used singly or in combination, to manage and treat SCD and its co-morbidities. Prophylactic measures to prevent pneumococcal infections include daily penicillin and early immunization in children. Blood transfusion therapy is highly effective for many acute, life-threatening complications of SCD including stroke and acute chest syndrome. Chronic blood transfusion carries the risk of iron

overload, infections, and transfusion reactions (Wood 2004). Hydroxyurea, a chemotherapeutic drug, is the only disease-modifying medication and has been shown to reduce the risk of many common SCD-related complications including vaso-occlusive pain episodes, acute chest syndrome, and stroke (Wong 2014). However, international availability and accessibility are limited and the long-term effects are not well known (Meremikwu 2011). Bone marrow transplant is currently the only potential curative therapy for patients with SCD (Bolanos-Meade 2012; Hoppe 2001), but the significant risks and the low proportion of eligible patients mean this treatment is rarely used.

In the management of sickle cell anaemia, daily folic acid supplementation of 1 mg/day is a common treatment due to a presumed folate deficiency in the setting of haemolytic anaemia and rapid red blood cell turnover (Falletta 1995). Folate, a water-soluble B vitamin, plays a role in cell division and erythropoiesis. However, there is limited evidence for the efficacy of folate supplementation to reduce anaemia, and a recent systematic review concluded that "while it is possible that folic acid supplementation may increase serum folate levels, the effect of supplementation on anaemia and any symptoms of anaemia remains unclear" (Dixit 2016).

Description of the intervention

The B vitamins include eight water-soluble vitamins that play inter-related roles in cellular functioning: thiamine; riboflavin; niacin; pantothenic acid; vitamin B6; folate; and vitamin B12.

Thiamine (B_1) is essential for the metabolism of carbohydrates and the synthesis of branched-chained amino acids. Food sources of thiamine include meat, fish, and whole grains. The U.S. Recommended Dietary Allowance (RDA) for thiamine is 1.2 and 1.1 mg/day for men and women, respectively. Dietary supplements containing thiamine mononitrate and thiamine hydrochloride (stable and water soluble forms) typically provide about 1.5 mg of thiamine per dose (IOM 1998).

Riboflavin (B_2) plays a major role in energy metabolism. The dietary intake of riboflavin, from food sources including eggs, green leafy vegetables, and organ meats (kidney, heart, liver) (Murray 2000) is highly bioavailable (Zempleni 1996). In the U.S., the RDA for adults is 1.3 and 1.1 mg/day for men and women, respectively. Dietary supplements, containing either the free form or riboflavin 5'-phosphate, typically provide 1.7 mg per dose (IOM 1998).

Niacin (B_3) is involved in carbohydrate metabolism, and DNA replication and repair (IOM 1998; Kim 1994; Lautier 1993). Niacin is found in many foods including meat, fish, poultry, and bread products. Free niacin, e.g., found in beans and liver, is highly bioavailable (IOM 1998), but in cereal grains, niacin is only 30% bioavailable (Carpenter 1985; Carter 1982). The Food and Nutrition Board (FNB) developed niacin equivalents (NE) to reflect the fact that niacin requirements are also met by the conversion of tryptophan (1 mg NE = 1 mg niacin = 60 mg of tryptophan). In the U.S., the RDA for niacin in adults is 16 and 14 mg/day NEs for men and women, respectively (IOM 1998).

Pantothenic acid (B_5) plays a role in carbohydrate metabolism as well as cholesterol and lipid synthesis (IOM 1998; Tahiliani 1991). It is found in all plant and animal foods, and the limited available studies estimated that approximately 50% of food-bound pantothenic acid is bioavailable (Tarr 1981). Recommended intakes of pantothenic acid are based on an adequate intake (AI), which specifies 5 mg/day for adults (IOM 1998).

Vitamin B6 contributes to the metabolism of amino acids, lipids, one-carbon units, and carbohydrates. Liver, whole grain cereals, and soy-based products are rich sources of B6. Dephosphorylated forms of B6 are absorbed by passive diffusion in the small intestine (Hamm 1979). In a mixed diet, vitamin B6 is approximately 75% bioavailable (Gregory 1997; Tarr 1981). In the U.S., the RDA for adults is 1.3 mg/day (IOM 1998).

Biotin (B_7) is involved in glucose, fatty acid, and amino acid metabolism (Dakshinamurti 1994). It exists as free or protein-bound forms in food. There is very limited information on biotin bioavailability and its content in foods. The AI for biotin, which is 30 µg/day for U.S. adults, is based on limited evidence (IOM 1998).

Folate (B_9) plays a role in the synthesis of nucleic and amino acids (Bailey 2004; Ulrich 2008). Sources of folate include dark green leafy vegetables, meat, and fish. Bioavailability from food folates is approximately 50% (IOM 1998; Pfeiffer 1997). In supplements and fortified foods, folate is in the oxidized and stabile form of folic acid. Without food, approximately 100% supplemented folic acid is bioavailable. When consumed with food, bioavailability decreases to 85% (Carmel 2005; Pfeiffer 1997).

The FNB developed dietary folate equivalents (DFE) to reflect the fact that the bioavailability of folic acid is higher than folate (1 μg DFEs = 1 μg food folate = 0.5 μg folic acid without food = 0.6 μg folic acid with food). In the USA, the RDA for adults is 400 μg/day for men and women. Dietary supplements of folic acid typically contain 400 μg per dose (IOM 1998).

Vitamin B12 is required for proper cell division and DNA synthesis. Major food sources of vitamin B12 are of animal-origin. Data on the bioavailability of B12 from food are limited; however, studies show that the fractional absorption of B12 decreases as oral dose increases (Chanarin 1979; Adams 1971). From an oral dose of 1 μg, only 50% is absorbed (Adams 1971). The RDA for B12 takes this assumed absorption into account, and in the USA, the RDA for adults is 2.4 μg/day (IOM 1998).

How the intervention might work

In individuals with SCD, the sickled blood cells have a shorter lifespan (10 to 20 days) compared to normal red blood cells (90 to 120 days) (West 1992). This rapid cell turnover leads to a decrease in the red blood cell count and increased erythropoiesis. The metabolic demands of increased red blood cell formation require a concomitant increase in protein and energy metabolism (Hibbert 2006).

B vitamins involved in the production of red blood cells include B6, Folate, and B12. Vitamin B6 is needed for the synthesis of heme, the iron containing component of haemoglobin. An indicator of vitamin B6 deficiency is small, microcytic red blood cells due to a decrease in haemoglobin synthesis (IOM 1998). Both folate and vitamin

B12 are required for DNA synthesis and cell division, which is vital for proper cell growth and replication. Low folate and vitamin B12 intake are associated with megaloblastic anaemia, which is characterized by abnormally large red blood cells (IOM 1998; Lindenbaum 1988).

Several B vitamins also play a role in energy metabolism. Deficiencies in thiamine, riboflavin, niacin, pantothenic acid, vitamin B6, and biotin are associated with conditions (i.e. fatigue, muscle wasting) related to impairments in pathways involved in energy production including carbohydrate, amino acid, and lipid metabolism (IOM 1998).

Based on these data, it has been proposed in the literature that the concentration of B-vitamins required to meet the increased metabolic demand of red blood cell formation might impose a higher nutritional requirement for B-vitamins in people with SCD compared to the general population. Since it is not always possible for people to achieve these levels of vitamin B intake from diet alone, food-fortification and oral supplementation are commonly used strategies to improve the intake status of target populations. Food fortification refers to the process of adding nutrients to foods that are commonly eaten with the goal of meeting a physiological requirement at a population level, whereas oral supplementation refers to the provision of micronutrients in the form of tablets and powders at dosages that exceed the RDA to meet individual-level requirements. Medical foods are foods designed to meet nutritional needs of clinical patients with distinct nutritional requirements that cannot be met by diet alone and delivered at the individual level. It is proposed that food

fortification and oral supplementation with B vitamins may improve SCD-related health outcomes.

Why it is important to do this review

Folate supplementation for SCD is based on the hypothesis that an increased rate of red blood cell degradation leads to a depletion of folate stores in patients with haemolytic anaemia. However, a recent systematic review of randomised trials of folic acid supplementation indicated that it is unclear whether or not folic acid supplementation in SCD reduces the risk of anaemia (Dixit 2016). Furthermore, a study evaluating the optimal dosages of B-vitamins involved in RBC production (B6, Folate, and B12) in paediatric SCD patients found evidence to support required supplementation at doses that are 2.5 to 10 times greater than the U.S. RDA (Van der Dijs 2002).

This review is needed because the prior review focused on folate alone, and given metabolic pathways supporting red blood cell production require multiple B vitamins there is a need for a broader review to study all B vitamins. Furthermore, this review will include non-randomised studies of the intervention (NRSI) because current clinical practice guidelines consider folate supplementation as routine and this leads naturally to a lack of RCT evidence for folate and/or other B-vitamin supplementation on patient-centred outcomes in SCD. The consideration of NRSIs in this review will leverage cohort and other study designs to allow a fuller exploration of dose-response and of long-term benefits and risks of the intervention.

This review will consider the evidence from randomised, quasi-randomised, and non-randomised studies of intervention (supplementation or fortification (or both)) with B vitamins to understand the benefits and risks of vitamin B supplementation in SCD. These data are needed to develop guidelines for nutritional adequacy of B vitamins in people with SCD that will contribute to minimizing SCD-associated morbidity. The information from this review may also inform the design of future randomised trials.

Objectives

To assess the effectiveness of intervention with B-vitamin supplementation in children and adults with SCD, compared to control group participants who receive no intervention, an existing policy, or another type of intervention.

Methods

Criteria for considering studies for this review

Types of studies

In this review, we plan to assess the effectiveness of B vitamin supplementation in children and adults with SCD, compared to control group participants who receive no intervention, an existing policy, or another type of intervention. We anticipate that we will not be able to achieve this goal by including only randomised studies, therefore we propose to include the following study designs.**Randomised controlled trials**

- Randomized controlled trials (RCTs), with randomisation at individual level.

Non-randomised studies of interventions (NRSIs)

- Non-randomised controlled trials (where allocation is done using methods other than randomised or quasi-randomised).
- Controlled before-and-after studies.
- Interrupted time series (ITS) with at least three measurement points both before and after the intervention.
- Cohort studies, where a group is followed over time to assess the association of the intervention (for example, supplementation with B vitamins or consumption of foods fortified with B vitamins) with a subsequent outcome, including prospective (recruits participants and follows into the future) and retrospective (identifies participants from past records and follows forward in time).
- Quasi-randomized trials (where allocation of treatment is not made by using a random sequence).
- Case-Control studies that are nested within a prospective study

We will include randomised controlled trials and non-randomised studies of interventions, as described above (EPOC 2015). The randomised controlled trials and non-randomised studies of interventions will not be pooled, and we plan to conduct separate meta-analyses for the two categories of study design.

Types of participants

We will include individuals with SCD (laboratory confirmed) of all ages, genders, and SCD genotypes including sickle cell anaemia (HbSS), haemoglobin sickle cell disease (HbSC), Sβ-thalassemia (HbSB+,HbSB^{o}), and other rare genetic variants.

Types of interventions

Inclusion criteria

Studies will be included if the intervention is oral supplementation of B vitamins, either singly or in combination, at any dose and for any duration. For the purpose of this review, oral supplementation refers to the delivery of B vitamins alone or with other micronutrients either as a tablet, capsule, liquid or powder. We will also include studies of food fortification with B vitamins.

We plan to make the following comparisons:

1. Supplementation with B vitamins versus placebo (where placebo refers to diet without fortification, as defined by the trial authors);
2. Fortification of diet with B vitamins versus placebo;
3. Supplementation with B vitamins versus diet fortification with B vitamins;
4. Supplementation with B vitamins and diet fortification versus diet fortification only.

We will include studies with a co-intervention so long as the intervention and comparison groups have the same co-intervention.

Exclusion criteria

We will exclude studies:

5. assessing the intervention of vitamin B12 by intramuscular or subcutaneous injections. Diseased populations with a severe vitamin B12 deficiency are most likely to receive this intervention, which normally needs to be administered by a healthcare professional (Lane 2002).
6. that primarily study patients who have received a blood transfusion within three months before the start of the study. The lifespan of a red blood cell

is typically 60 to 90 days, therefore, excluding based on this criterion will prevent confounding of haematological biomarker measurements red blood cell folate measurements (Franco 2009, McDonald 1978).

Types of outcome measures

Primary outcomes

7. Haematological indices*
   a) Haemoglobin concentration (g/L)
   b) Haematocrit (g/dL)
   c) Mean corpuscular volume (femtoliters)
8. SCD-related morbidities* (as defined by the authors), including but not limited to:
   a) vaso-occlusive pain episodes (presence, duration, frequency, intensity, or severity)
   b) acute chest syndrome
   c) central nervous system complications (including ischemic and hemorrhagic stroke)
   d) infection
9. Measures of plasma and serum B-vitamin concentration* (as defined by the authors)

*These outcomes will be included in 'Summary of Findings' tables

Secondary outcomes

10. Quality of life (as measured by validated scales, e.g. Health-related Quality of Life Assessment (HRQL) Scale)

11. Adverse and serious adverse effects (SAEs) of the intervention (as defined by the authors), including:
    a) SAEs (death, life threatening, requiring hospitalizations, resulting in significant disability, congenital anomaly, requiring intervention to prevent permanent impairment)
    b) Adverse effects related to gastrointestinal effects

Search methods for identification of studies

There will be no restrictions regarding language or publication status.

Electronic searches

The Cochrane Cystic Fibrosis and Genetic Disorders Group's Information Specialist will conduct a systematic search of the Group's Haemoglobinopathies Trials Register for relevant RCTs and NRSIs.

The Haemoglobinopathies Trials Register is compiled from electronic searches of the Cochrane Central Register of Controlled Trials (CENTRAL) (updated with each new issue of the Cochrane Library) and weekly searches of MEDLINE. Unpublished work is identified by searching the abstract books of five major conferences: the European Haematology Association conference; the American Society of Hematology conference; the British Society for Haematology Annual Scientific Meeting; the Caribbean Health Research Council Meetings; and the National Sickle Cell Disease Program Annual Meeting. For full details of all searching activities for the register, please see the relevant section of the Cochrane Cystic Fibrosis and Genetic Disorders Group Module.

We will search the following databases (with no language or date restrictions) for relevant trials and NRSIs:

- PubMed (www. ncbi.nlm.nih.gov/pubmed); 1946 onwards);
- Embase Ovid (1974 onwards);
- CINAHL EBSCO (Cumulative Index to Nursing and Allied Health Literature; 1982 onwards);
- CAB Abstracts via Web of Science (1910 onwards).

We have devised a draft search strategy for PubMed which is displayed in Appendix 1. This will be used as the basis for search strategies for the other databases listed.

We will also search the following trials registries:

- US National Institutes of Health Ongoing Trials Register Clinicaltrials.gov (www.clinicaltrials.gov);
- World Health Organization International Clinical Trials Registry Platform (www.who.int/trialsearch);
- ISRCTN Registry (www.isrctn.com).

Searching other resources

We will search the reference lists of included articles and reviews for additional relevant studies. We will also contact authors of included studies as well as known research and nutritional SCD experts in the field to identify additional studies including unpublished and ongoing trials.

Data collection and analysis

Selection of studies

The review authors (EBA and RD) will independently assess the titles and abstracts of all articles that are identified from the literature searches to identify eligible reports in accordance with the specified inclusion criteria. If we cannot determine eligibility for inclusion from the titles or abstracts, we will retrieve and review the full-text articles. Two review authors (EBA and RD) will retrieve full text reports of trials that are eligible, and these two authors will independently examine them for compliance with the eligibility criteria. If necessary, review authors will correspond with investigators to obtain further information to determine study eligibility.

We will present the excluded studies with reasons for exclusion in the 'Characteristics of excluded studies' table in RevMan (RevMan 2014).

We will not be blinded to information about articles such as the journal of publication, author names, institution, or the study results.

Where there are disagreements about the inclusion of a study, review authors will involve a third review author (PAC) to discuss and reach a consensus.

Data extraction and management

Two review authors (MSF and CDK) will independently extract the data for primary and secondary outcomes in a customized, piloted data collection form developed using the Cochrane Cystic Fibrosis & Genetic Disorders Group's data extraction template. Two authors (MSF, EBA, RD) will enter relevant data into the Review Manager (RevMan) software (RevMan 2014), and two authors will check for errors (EBA and RD). The review authors will resolve any issues and concerns in data extraction by discussion and if necessary, review with a third review author (PAC). Data will be

extracted from both full-text versions and abstract. In instances where there is more than one report linked to a single study, we will use one data extraction form per study. If the information in the published article is not clear, we will contact the study authors for further details.

We will extract the following information:

12. Source: study ID (created by review author), report ID (created by review author), review author ID (created by review author), citation (journal or conference, year of publication, etc), contact details)
13. Method: year of the trial; duration of trial; type of randomisation; allocation; concealment method; blinding; trial area; and sampling method; For NRSI study design: assessment of confounding variables, methods used to control for confounding variables, comparability of groups on confounding variables.
14. Participants: number of participants in control and intervention groups; age; sex; similarity of groups at baseline; dates of most recent blood transfusions, and loss to follow up with reasons.
15. Interventions: interventions (dose, route and duration); comparison intervention (dose, route and duration); and co-medication (dose, route and duration)
16. Outcomes: primary and secondary outcomes as mentioned above; any other outcomes assessed by trial authors; outcome definition (diagnostic method, name of scale, definition of threshold); units of measurement (if relevant); for scales, upper and lower limits, and whether a high or low score is

favourable, times of assessment; length of follow -; baseline assessment of outcome variables.

17. Results: number of participants allocated to each intervention group, and, for each outcome of interest: sample size; missing participants; summary data for each intervention group (mean and standard deviation (SD) for continuous data, 2 x 2 table for dichotomous data, etc.); multiple estimated effects (unadjusted and adjusted, if available).
18. Notes: key conclusions of study authors, references to other relevant studies, published or unpublished data; title; authors; contact address; language of publication; year of publication; and funding sources, if any.

Assessment of risk of bias in included studies

Two authors (MSF and EBA) will independently assess the risk of bias of included studies.

RCTs

For randomised controlled trials, we will assess seven domains for each trial. In each domain, we will assign a judgment of 'low', 'high', or 'unclear' risk of bias according to criteria described in chapter 8 of the Cochrane Handbook for Systematic Reviews of Interventions (Higgins 2011). The seven domains are described below.

1. Random Sequence generation: low risk of bias (if the investigators included random component in sequence generation process such as using random number table, computer generated random number, coin tossing, shuffling cards or envelops, etc.); high risk of bias (if the investigators included a non-random component in sequence generation process such as the use of date of birth, date of admission, hospital or clinic

record number, etc.); or unclear risk of bias (If there is no sufficient information about sequence generation process to judge whether high or low risk).

2. Allocation concealment: low risk of bias (If the investigators used the methods such as central allocation, sequentially numbered drug containers of identical appearance, sequentially numbered opaque or sealed envelopes so that participants and investigator enrolling could not foresee the assignment); high risk of bias (If the investigators used an open random allocation schedule, non-opaque envelopes and any other quasi-randomised methods such as alternation or rotation, case record number, etc. so that participants and investigator enrolling could possibly foresee the assignment); or unclear risk of bias (if method of concealment is not described or there is no sufficient information to judge whether high or low risk).

3. Blinding of participants and personnel: low risk of bias (if blinding was done to the study participants or personnel and the method of blinding is described); high risk of bias (if no blinding or incomplete blinding was done to study participants and personnel); or unclear risk of bias (if there is no sufficient information to judge whether high or low risk).

4. Blinding of outcome assessment: low risk of bias (if blinding was done to outcome assessors and method of blinding was described); high risk of bias (if blinding was not done to outcome assessors or if blinding was done, but likely it was broken); or unclear risk of bias (if there is no sufficient information to judge whether high or low risk).

5. Incomplete outcome data: low risk of bias (no attrition, the number of dropouts or withdrawals was balanced in intervention groups with similar reasons); high risk of

bias (imbalance of dropouts or withdrawals in intervention groups, the number and reasons of dropouts or withdrawals was not described); or unclear risk of bias: If there is no sufficient information of attrition or exclusions to judge whether high or low risk.

6. Selective outcome reporting: low risk of bias (all the study's pre-specified outcomes of interest were reported); high risk of bias (not all the study's pre-specified outcomes of interest were reported, use of not pre-specified measurement for assessing outcomes, incomplete reporting of outcome data); or unclear risk of bias (if there is no sufficient information to judge whether high or low risk).

7. Other sources of bias: low risk of bias (the study appears to be free of other source of bias); high risk of bias (presence of other source of bias); or unclear risk of bias (if there is no sufficient information to judge whether high or low risk).

NRSIs

For observational study designs, we will use the validated Risk of Bias in Non-Randomized Studies-of Interventions (ROBINS-I) tool to assess risk of bias for non-randomised studies and observational studies (Sterne 2016). In each domain, we will assign a judgement of "low risk of bias", "moderate risk of bias", "serious risk of bias", "critical risk of bias", and "no information" (Sterne 2016).

We intend to use the methods described by the Effective Practice and Organisation of Care (EPOC) group for evaluating risk of bias in interrupted time series (ITS) studies (EPOC 2010).

Thus, risk of bias assessment includes eight domains for observational studies, with an additional domain for ITS (item 9).

1. Bias due to confounding: low risk of bias (no confounding expected); moderate risk of bias (confounding expected and controlled for and reliability and validity of important domains are sufficient such that residual confounding is not expected); serious risk of bias (confounding not controlled for or unmeasured and reliability and validity of important domains are low enough such that serious residual confounding is expected); critical risk of bias (confounding not controlled for or measured); no information (no mention of expected confounding)

2. Bias in selecting participants into study: low risk of bias (eligible participants included and start of follow up and start of intervention coincides for each participant); moderate risk of bias (appropriate adjustments were made for selection bias or the start of follow up and start of intervention do not coincide for all participants); serious risk of bias (authors do not make appropriate adjustments for moderately strong selection bias and start of follow up and start of intervention do not coincide for all participants); critical risk of bias (authors do not make appropriate adjustments for very strong selection bias due to missing follow up time and the rate ratio is not constant over time); no information (no information on participant selection or on whether the start of follow up and start of the intervention coincide)

3. Bias in classification of interventions: low risk of bias (intervention and status of intervention are well defined); moderate risk of bias (intervention status is well defined and some aspects of intervention status assignment were determined retrospectively); serious risk of bias (intervention status is not well defined and major aspects of intervention status assignment were determined retrospectively); critical

risk of bias (extremely high amount of misclassification of intervention status); no information (no information on intervention and status of intervention)

4. Bias due to deviations from intended intervention: low risk of bias (deviations from intended intervention reflect usual practice and are unlikely to impact outcome and co-interventions were balanced); moderate risk of bias (intended intervention deviates from usual practice but is unlikely to impact the outcome, deviations from the intended intervention and an imbalance of co-interventions across groups are not expected to significantly impact the outcome due to the use of appropriate analyses); serious risk of bias (intended intervention deviates from usual practice and is likely to impact the outcome, deviations from the intended intervention and an imbalance of co-interventions across groups are expected to significantly impact the outcome due to the absence of appropriate analyses); critical risk of bias (intended intervention deviates significantly from usual practice and is likely to impact the outcome, significant deviations from the intended intervention and an imbalance of co-interventions across groups are expected to significantly impact the outcome due to the absence of appropriate analyses); no information (no information on deviations from intended intervention)

5. Bias due to missing data: low risk of bias (data is reasonably complete or missing data is balanced between intervention groups or analyses of missing data is able to remove risk of bias); moderate risk of bias (missing data differs slightly across intervention groups and the analyses is unlikely to remove risk of bias); serious risk of bias (missing data differs significantly across intervention groups and the analyses is unable to remove risk of bias); critical risk of bias (there are significant differences

between interventions in participants with missing data which were not addressed through analyses); no information (no information about missing data)

6. Bias in measurement of outcomes: low risk of bias (methods of outcome measurement are comparable across intervention groups, outcome measure is not influenced by participant or outcome assessor knowledge of the intervention received, and error in outcome measurement is not related to intervention status); moderate risk of bias (methods of outcome measurement are comparable across intervention groups, outcome measure is not significantly influenced by participant knowledge of the intervention received, and error in outcome measurement is minimally related to intervention status); serious risk of bias (methods of outcome measurement are not comparable across intervention groups, outcome measure is significantly influenced by participant and outcome assessor knowledge of the intervention received, and error in outcome measurement is related to intervention status); critical risk of bias (methods of outcome measurement are significantly incomparable across intervention groups); no information (no information about methods of outcome assessment)

7. Bias in selection of the reported result: low risk of bias (reported results correspond to all intended outcomes, analyses, and sub-cohorts); moderate risk of bias (outcome measurements and analyses are clearly defined, there is no indication of both the selection of the reporting from multiple analyses, and the selection of cohorts or subgroups for analyses and reporting is based on the results); serious risk of bias (outcome measurements are not consistently defined, there is indication of both the selection of the reporting from multiple analyses and the selection of cohorts or subgroups for analyses and reporting that is based on the results); critical risk of bias

(there is indication of selective reporting of results that are substantially different from the unreported results); no information (not enough information on selection of reported result)

8. Other sources of bias: baseline nutrient status in longitudinal studies (low risk of bias, models adjust for starting nutrient status; moderate risk of bias, models adjust but measurement of starting nutrient status has low accuracy and/or precision; high risk of bias, models do not consider baseline nutrient status and only include measurements of nutrient status after supplementation).

9. Additional items for ITS

- Was the intervention independent of other changes?: low risk (there are compelling arguments that the intervention occurred independently of other changes over time and the outcome was not influenced by other confounding variables/historic events during the study period);high risk (it is reported, or there are grounds to suspect, that the intervention was not independent of other changes over the time period of the study).
- Was the shape of the intervention pre-specified?: low risk (the point of analysis is the point of intervention or a rational explanation for the shape of the intervention effect was provided); high risk (it clear that the above conditions were not met).
- Was the intervention unlikely to affect data collection?: low risk (it is reported that the intervention itself was unlikely to affect data collection);high risk (the intervention itself was likely to affect data collection).
- Was knowledge of the allocated interventions adequately prevented during the study?: low risk (the authors state explicitly that the primary outcome variables

were assessed blindly, or or the outcomes are objective); high risk (the outcomes were not assessed blindly).

- Were incomplete outcome data adequately addressed?: low risk (missing outcomes measures were unlikely to bias data); high risk (missing data likely to bias results).
- Was the study free from selective outcome reporting?: low risk (there is no evidence that outcomes were selectively reported); high risk (some important outcomes are subsequently omitted from the results).
- Was the the study free from other risks of bias?: low risk (there is no other evidence of other risk of bias).

The two review authors will not be blinded to the names of study authors, institutions, journals and results of included studies. If there are concerns or issues, they will be resolved by discussion or with the opinion of a third review author (PAC). Results will be recorded in the relevant Characteristics of Included Studies tables in RevMan (RevMan 2014) and summarized in a 'Risk of bias' table or graph.

Measures of treatment effect

Dichotomous data

For dichotomous data (i.e. SCD related morbidities, adverse effects of supplementation), the review authors will present results using the risk ratio (RR) with 95% confidence intervals(CI).

Count data

For count data (i.e. vaso-occlusive pain episodes, acute chest syndrome, infection), we will present results using the RR with 95% CIs.

Continuous data

For continuous outcomes (i.e. hematocrit, haemoglobin, mean corpuscular volume, measures of plasma and serum B vitamin status), we will present the results using the mean difference and the corresponding 95% CIs for outcomes using the same scale between studies. We will standardize the results to a uniform scale. To do this, we will standardize the mean difference and the 95% CI if the same outcome is measured in a variety of ways (i.e. different scale).

For continuous variables in NRSIs, we will extract and report the absolute change adjusting for baseline differences from a statistical analysis (including regression models, mixed models or hierarchical models). If the relative change adjusted for baseline differences in outcome measures is available (i.e. absolute post-intervention difference between intervention and control groups, as relative change is reported (post-intervention difference / post-intervention level in control group), these data will be extracted and reported (EPOC 2017).

For ITS studies that meet the previously described criteria of analysis and from which we can extract relevant information, we will determine the effect sizes by standardizing the data by dividing the level (or time slope) and standard error (SE) by the standard deviation (SD) of the pre-intervention slope. Where appropriate, the number needed to treat to benefit (NNTB) and the number needed to treat to harm (NNTH) will be reported with 95% CIs. We will present the data in tables or a narrative report if the available data cannot be reported in any of the formats mentioned above.

For the included NRSIs, confounding will be considered and evaluated, and we will make the required adjustment for the change of outcomes (EPOC 2017).

Unit of analysis issues

Since we plan to include cluster randomised studies, non-randomised studies, or multiple observations for the same outcome, we anticipate that issues related to unit of analysis will arise. If any of these study designs are included in our review, we will treat these according to Chapter 16 of the Cochrane Handbook for Systematic Reviews of Interventions (Higgins 2011).

Randomised trials

If we encounter studies in which participants are randomized more than once, we will contact the authors to obtain data on outcomes associated with the initial randomization. When appropriate, we will include relevant subgroups for analysis for studies that have multiple treatment groups. If possible, we will also combine groups to create a single pair-wise comparison. If not, the most appropriate pair of interventions will be selected, and others excluded (Higgins 2011d).

Results that are adjusted for clustering will be extracted whereas results that are not adjusted will be re-analysed, if possible, to take clustering into account or presented in a table. If we include both individually-randomised and cluster-randomised trials and if there is little heterogeneity between trial designs, we will pool the results. If the analyses for clustered randomised trials have been conducted at a different level to that of allocation and do not account for the cluster design, we will calculate the trial's effective sample sizes to account for the effect of clustering in data. If available, we will use the intra-cluster correlation coefficient (ICC) derived from the trial or another source (including other, similar trials). We will then calculate the design effect using the formula provided in the Cochrane Handbook for Systematic Reviews of

Interventions (Higgins 2011). We will conduct sensitivity analysis to determine the effect of variations in ICC.

Cross-over trials

For cross-over trials, we plan to include a paired analysis from the two study periods. If there is a carryover effect or where second-period data are not available, we will collect the mean of difference of both the measurement on experimental intervention and control intervention of each participant and its standard error (SE). Following this, the review authors will meta-analyse effect sizes and SEs with this data using the generic inverse method in RevMan (RevMan 2014). When combining results from cross-over trials in a meta-analysis, we plan to use the methods recommended by Elbourne (Elbourne 2002).

NRSIs

NRSIs may include data measured over multiple time points. Since these data usually have a correlated structure, we plan to analyse the data based on pre-defined time points (as with RCTs), adjusting for confounding. We will follow EPOC recommendations for unit of analysis issues that may arise with the inclusion of ITS studies (EPOC 2017). We will use the preferred method of a statistical comparison of time trends before and after the intervention to analyse ITS studies. For longitudinal cohort studies of binary outcomes, we will meta-analyse the effect size as appropriate (hazard ratio in the time-to-event Cox proportional hazards regression model, cumulative risk in logistic regression model). In longitudinal cohort studies of continuous outcomes with multiple observations per individual, we will meta-analyse the effect size using estimates that convey the association of exposure with average

level of the outcome, and the association of the exposure with the slope of change in the outcome.

Dealing with missing data

If we identify studies which have been published only in abstract form or presented at meetings or conferences, we will contact the investigator for full reports and data as needed. If there are missing or unclear data in relation to attrition rates with no reported reason for dropout, we will request further information from the study authors. In order to allow an intention-to-treat analysis, we will collect data on the number of participants with each outcome event by allocated treatment group (regardless of compliance and whether participants were later excluded from treatment or found to be ineligible).

Assessment of heterogeneity

The review authors plan to assess the heterogeneity through visual examination of the overlap of confidence intervals presented in the forest plots. We will use the Chi^2 test and a P value less than 0.10 to determine statistically significant heterogeneity. The authors will also use the I^2 statistic to quantify any heterogeneity across the studies. We will categorize and interpret the I^2 statistic using the following classification (Deeks 2011; Higgins 2003):

- 0% to 40%: might not be important level of heterogeneity;
- 30% to 60%: may represent moderate heterogeneity;
- 50% to 90%: may represent substantial heterogeneity;
- 75% to 100%: considerable heterogeneity.

We anticipate that heterogeneity may be a concern in non-randomised controlled trials as well as other NRSI study designs. Where there is evidence of heterogeneity, we will summarize findings and present them using a forest plot, but will not present the pooled estimate. Data from controlled and observational studies will be analysed separately.

Assessment of reporting biases

In order to identify selective outcome reporting, the review authors will compare outcomes described in the trial protocol (if available) with those reported in the publication. If the protocol is not available, information about outcomes may be found in the trial registry databases. The review authors will also compare outcomes listed in the 'Methods' section of the final paper with those presented in the 'Results' section. If 10 or more trials are included in the meta-analysis, we plan to use a funnel plot to assess publication bias. If the funnel plot is asymmetrical, we will explore the causes.

Data synthesis

We will pool the results and perform a meta-analysis when there is no clinical variation between studies, provided that studies use similar methods and measure the same outcome in similar populations.

Separate meta-analyses will be conducted for controlled trials. We will not pool data together from any of the other study designs, thus summary statistics for RCTs will be computed separately from summary statistics for NRSIs.

Data analysis will be carried out using RevMan (RevMan 2014). Since we anticipate that heterogeneity will be present between studies, we will use a random-effects model to analyse data. We will use the inverse variance method for continuous variables. For

dichotomous variables, we will use the method proposed by Mantel and Haenzel (Higgins 2011).

We will use the generic inverse variance method in RevMan to perform meta-analysis for non-randomised controlled trials and observational studies in which results have been adjusted for confounding factors. If both adjusted and non-adjusted estimates are provided, we will carry out sensitivity analysis using unadjusted figures to determine the possible impact on the estimate of the treatment effect.

A fixed-effect model will be used if we do not find any statistically significant heterogeneity between the studies.

Subgroup analysis and investigation of heterogeneity

If the authors are able to include sufficient studies (at least 10) in a meta-analysis and identify at least 50% heterogeneity, we plan to undertake the following subgroup analyses:

19. Vitamin B supplementation complex combinations
    a) Single B vitamin
    b) Single B vitamin + other B vitamins
    c) Any combination of B vitamins + iron
20. Different types of SCD
    a) Severe genotypes (HbSS and HbSβº) versus Mild genotypes (HbSC, HbSβ^{+}and rare variants)
21. Demographic status
    a) Children (under 18 years of age) versus adults (18 years of age and older)

b) Males versus females

22. Baseline nutritional status: If there is evidence for heterogeneity in the meta-analysed effect estimates, then meta-regression analyses will be used to understand whether effect estimates vary by average baseline B nutriture status to assess whether background nutriture contributes to heterogeneity in the effect

5. Inclusion of hydroxyurea in treatment/standard of care

Sensitivity analysis

We will conduct a sensitivity analysis to assess the robustness of the findings. If we are able to include 10 or more studies in a meta-analysis, we plan to perform a sensitivity analysis and examine the effect of excluding studies that are classified as having high risk of bias for random sequence generation, allocation concealment, and blinding. A meta-analysis will be performed on all the studies and then including only studies that do not have high risk of bias. We plan to repeat the meta-analyses using a random-effects model and then a fixed-effects model to assess the robustness of the findings based on the analysis method. We will report sensitivity analyses data in summary tables.

Summary of findings table

We will create 'Summary of findings' tables for each comparison listed above using the following outcome measures.

23. Haemoglobin concentration
24. Mean corpuscular volume
25. B vitamin concentration

26. SCD related morbidities - pain episodes
27. SCD related morbidities - acute chest syndrome
28. SCD related morbidities - CNS complications
29. SCD related morbidities - infection

Separate summary of findings tables will be created for randomised and non-randomised studies. We will use the Grades of Recommendations, Assessment, Development and Evaluation (GRADE) approach (Balshem 2011). The tables will include a list of primary outcomes for each comparison, estimates of relative effects, number of participants, and studies contributing data for these outcomes. Limiting the assessment to the trials included, we will use the GRADE approach to assess within-study risk of bias, heterogeneity, precision of effect estimates, directness of evidence, and risk of publication bias. The quality of the included trials will be expressed as one of four levels (high, moderate, low, or very low). We will use pro GRADE software (GRADEpro 2008).

Other references

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Contributions of authors

Roles and Responsibilities

TASK	WHO WILL UNDERTAKE TASK?
Protocol stage: draft the protocol	PJS, PAC, RD, LDV, EBA, CDK
Review stage: select which trials to include (2 + arbiter)	EBA, RD + PAC
Review stage: extract data from trials (2 people)	MSF, CDK
Review stage: enter data into RevMan	MSF, EBA, RD
Review stage: carry out analysis	PAC, RD, EBA
Review stage: interpret the analysis	PJS, PAC, LDV, MSF, EBA, CDK
Review stage: draft the final review	PJS, PAC, LDV, MSF, EBA, CDK
Update stage: update the review	PJS, PAC, LDV, MSF, EBA, CDK

Additional references

Adams 1971

Adams JF, Ross SK, Mervyn RL, Boddy K, King P. Absorption of cyanocobalamin, coenzyme B12, methylcobalamin, and hydroxocobalamin at different dose levels. Scandinavian Journal Gastroenterology 1971;6:249-52.

Bailey 2004

Bailey LB. Folate and vitamin B12 recommended intakes and status in the United States. Nutrition Reviews 2004;62(6):14-20.

Balshem 2011

Balshem H, Helfand M, Schünemann HJ, Oxman AD, Kunz R, Brozek J, et al. GRADE guidelines 3: rating the quality of evidence. Journal of Clinical Epidemiology 2011;64(4):401-6.

Belcher 2014

Belcher JD, Chen C, Nguyen J, Milbauer L, Abdulla F, Alayash AI, et al. Heme triggers TLR4 signalling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. Blood 2014;123(3):377-90.

Bolanos-Meade 2012

Bolanos-Meade J, Fuchs EJ, Luznik L, Lanzkron SM, Gamper CJ, Jones RJ, et al. HLA-haploidentical bone marrow transplantation with posttransplant cyclophosphamide expands the donor pool for patients with sickle cell disease. Blood 2012;120:4285-91.

Carmel 2005

Carmel R. Folic acid. In: Shils M, Shike M, Ross A, Caballero B, Cousins R, editor(s). Modern Nutrition in Health and Disease. 10th edition. Lippincott: Williams & Wilkins, 2005:470-81.

Carpenter 1985

Carpenter KJ, Lewin WJ. A reexamination of the composition of diets associated with pellagra. Journal of Nutrition 1985;115(5):545-52.

Carter 1982

Carter EG, Carpenter KJ. The bioavailability for humans of bound niacin from wheat bran. American Journal of Clinical Nutrition 1982;36:855-861.

Chanarin 1979

Chanarin I. The Megaloblastic Anaemias. 2nd edition. Oxford: Blackwell Scientific, 1979.

Dakshinamurti 1994

Dakshinamurti K. Biotin. In: Shils ME, Olson JA, Shike M, editor(s). Modern Nutrition in Health and Disease. Philadelphia: Lea & Febiger, 1994:426-431.

De Franceschi 2011

De Franceschi L, Cappellini MD, Olivieri O. Thrombosis and sickle cell disease. Seminars in Thrombosis and Hemostasis 2011;37(3):226-36.

Deeks 2011

Deeks JJ, Higgins JPT, Altman DG on behalf of the Cochrane Statistical Methods Group. Chapter 9: Analysing data and undertaking meta-analysis. In: Higgins JPT, Green S (editor(s). Cochrane Handbook for Systematic Reviews of Interventions

Version 5.1.0 (updated March 2011). The Cochrane Collaboration, 2011. Available from handbook.cochrane.org.

Dixit 2016

Dixit R, Nettem S, Madan SS, Soe HHK, Abas ABL, Vance LD, et al. Folate supplementation in people with sickle cell disease. Cochrane Database of Systematic Reviews 2016;2:1-32. [DOI: 10.1002/14651858.CD011130.pub2]

EPOC 2010

EPOC Group. Cochrane Effective Practice and Organisation of Care Review Group. Data collection checklist. http://epoc.cochrane.org/sites/epoc.cochrane.org/files/public/uploads/datacollectionchecklist.pdf (accessed 06 July 2017).

EPOC 2015

Effective Practice and Organisation of Care (EPOC). EPOC Resources for review authors. http://epoc.cochrane.org/epoc-specific-resources-review-authors (accessed 20 June 2016).

Falletta 1995

Falletta JM, Woods GM, Verter JI, Buchanan GR, Pegelow CH, Iyer RV, et al. Discontinuing penicillin prophylaxis in children with sickle cell anaemia. Journal of Pediatrics 1995;127:685-90.

Franco 2009

Franco RS. The measurement and importance of red cell survival. Am J Hematol 2009;84(2):109-114.

GRADEpro 2008

GRADEpro [Computer program]. Version 3.2. Hamilton (ON): GRADE Working Group, McMaster University, 2008. [Other: Computer program]]

Gregory 1997

Gregory JF 3rd. Bioavailability of vitamin B-6. European Journal of Clinical Nutrition 1997;51 Suppl 1:S43-8.

Hamm 1979

Hamm MW, Mehansho H, Henderson LM. Transport and metabolism of pyridoxamine and pyridoxamine phosphate in the small intestine of the rat. Journal of Nutrition 1979;109:1552-9.

Hibbert 2006

Hibbert JM, Creary MS, Gee BE, Buchanan ID, Quarshie A, Hsu LL. Erythropoiesis and myocardial energy requirements contribute to the hypermetabolism of childhood sickle cell anaemia. Journal of Pediatric Gastroenterology and Nutrition 2006;43(5):680-7.

Higgins 2003

Higgins JPT, Thompson SG, Deeks JJ, Altman DG. Measuring inconsistency in meta-analyses. BMJ 2003;327(7414):557-60.

Higgins 2011

Higgins JPT, Green S, editor(s). Cochrane Handbook for Systematic Reviews of Interventions Version 5.1.0 (updated March 2011). The Cochrane Collaboration, 2011. Available from handbook.cochrane.org.

Higgs 1982

Higgs DR, Aldridge BE, Lamb J, Clegg JB, Weatherall DJ, Hayes RJ, et al. The interaction of alpha thalassaemia and homozygous sickle-cell disease. New England Journal of Medicine 1982;306:1441-46.

Hoppe 2001

Hoppe CC, Walters MC. Bone marrow transplantation in sickle cell anaemia. Current Opinion in Oncology 2001;13(2):85-90.

IOM 1998

Institute of Medicine (US). Standing Committee on the Scientific Evaluation of Dietary Reference Intakes and its Panel on Folate, Other B Vitamins, and Choline. Washington, D.C.: National Academies Press (US), 1998.

Kato 2006

Kato GJ, Hsieh M, Machado R, Taylor J 6th, Little J, Butman JA, et al. Cerebrovascular disease associated with sickle cell pulmonary hypertension. American Journal of Hematology 2006;81:503-10.

Kato 2007

Kato GJ, Gladwon MT, Steinberg M. Deconstructing sickle cell disease: Reappraisal of the role of haemolysis in the development of clinical subphenotypes. Blood Reviews 2007;21(1):37-47.

Kim 1994

Kim H, Jacobson EL, Jacobson MK. NAD glycohydrolases: A possible function in calcium homeostasis. Molecular and Cellular Biochemistry 1994;138:237-43.

Kuypers 1998

Kuypers FA, Yuan J, Lewis RA, Snyder LM, Kiefer CR, Bunyaratvej A, et al. Membrane phospholipid asymmetry in human thalassaemia. Blood 1998;91(8):3044-51.

Lane 2002

Lane LA, Rojas-Fernandez C. Treatment of vitamin b12-deficiency anaemia: oral versus parenteral therapy. Ann Pharmacother 2002;36(7-8):1268-1272.

Lautier 1993

Lautier D, Lagueux J, Thibodeau J, Menard L, Poirier GG. Molecular and biochemical features of poly (ADP-ribose) metabolism. Molecular and Cellular Biochemistry 1993;122:171-93.

Lindenbaum 1988

Lindenbaum J, Healton EB, Savage DG, Brust JC, Garrett TJ, Podell ER, et al. Neuropsychiatric disorders caused by cobalamin deficiency in the absence of anaemia or macrocytosis. New England Journal of Medicine 1988;318:1720-8.

McDonald 1978

McDonald MJ, Shapiro R, Bleichman M, Solway J, Bunn HF. Glycosylated minor components of human adult haemoglobin. Purification, identification, and partial structural analysis. J Biol Chem 1978;253(7):2327-2332.

Meremikwu 2011

Meremikwu MM, Okomo U. Sickle cell disease. BMJ Clinical Evidence 2011;Feb 14:2402.

Modell 2008

Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. Bulletin of the World Health Organisation 2008;86:480-7.

Murray 2000

Murray RK, Granner DK, Mayes PA, Rodwell VW. Harper's Biochemistry. 25th edition. Dubuque: Lange/McGraw Hill, 2000.

Nolan 2005

Nolan VG, Wyszynski DF, Farrer LA, Steinberg MH. Hemolysis- associated priapism in sickle cell disease. Blood 2005;106:3264-7.

Pegelow 1997

Pegelow CH, Colangelo L, Steinberg M, Wright EC, Smith J, Phillips G, et al. Natural history of blood pressure in sickle cell disease: risks for stroke and death associated with relative hypertension in sickle cell anaemia. American Journal of Medicine 1997;102:171-7.

Pfeiffer 1997

Pfeiffer CM, Rogers LM, Bailey LB, Gregory JF 3rd. Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual-label stable-isotope protocol. American Journal of Clinical Nutrition 1997;66:1388-97.

Piel 2013

Piel FB, Patil AP, Howes RE, Nyangiri OA, Gething PW, Dewi M, et al. Global epidemiology of sickle haemoglobin in neonates: a contemporary geostatistical model-based map and population estimates. Lancet 2013;381:142-51.

Platt 1994

Platt OS, Brambilla DJ, Rosse WF, Milner PF, Castro O, Steinberg MH, et al. Mortality in sickle cell disease. Life expectancy and risk factors for early death. New England Journal of Medicine 1994;330(23):1639-44.

Ramakrishnan 2010

Ramakrishnan M, Moisi JC, Klugman K, Iglesias JMF, Grant LR, Mpoudi-Etame M, et al. Increased risk of invasive bacterial infection in African people with sickle-cell disease: a systematic review and meta-analysis. Lancet Infectious Diseases 2010;10(5):329-37.

RevMan 2014

Review Manager (RevMan) [Computer program]. Version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, 2014.

Sterne 2016

Sterne JA, Hernán MA, Reeves BC, Savović J, Berkman ND, Viswanathan M, Henry D, Altman DG, Ansari MT, Boutron I, Carpenter JR, Chan AW, Churchill R, Deeks JJ, Hróbjartsson A, Kirkham J, Jüni P, Loke YK, Pigott TD, Ramsay CR, Regidor D, Rothstein HR, Sandhu L, Santaguida PL, Schünemann HJ, Shea B, Shrier I, Tugwell P, Turner L, Valentine JC, Waddington H, Waters E, Wells GA, Whiting PF, Higgins

JP. ROBINS-I: a tool for assessing risk of bias in non-randomised studies of interventions. BMJ Oct 2016;355(i4919):1-7.

Tahiliani 1991

Tahiliani AG, Beinlich CJ. Pantothenic acid in health and disease. Vitamins and Hormones 1991;46:165-228.

Tarr 1981

Tarr JB, Tamura T, Stokstad EL. Availability of vitamin B6 and pantothenate in an average American diet in man. American Journal of Clinical Nutrition 1981;34:1328-37.

Ulrich 2008

Ulrich CM, Reed MC, Nijhout HF. Modeling folate, one-carbon metabolism, and DNA methylation. Nutrition Reviews 2008;66(Suppl 1):S27-30.

Van der Dijs 2002

van der Dijs FP, Fokkema MR, Dijck-Brouwer DA, Niessink B, van der Wal TI, Schnog JJ, Duis AJ, Muskiet FD, Muskiet FA. Optimisation of folic acid, vitamin B(12), and Vitamin B(6) supplements in paediatric patients with sickle cell disease. American Journal of Hematology 2002;69(4):239-46.

Vinchi 2013

Vinchi F, De Franceschi L, Ghigo A, Townes T, Cimino J, Silengo L, et al. Hemopexin therapy improves cardiovascular function by preventing heme-induced endothelial toxicity in mouse models of haemolytic diseases. Circulation 2013;127(12):1317-29.

West 1992

West MS, Wethers D, Smith J, Steinberg M. Laboratory profile of sickle cell disease: a cross-sectional analysis. The Cooperative Study of Sickle Cell Disease. Journal of Clinical Epidemiology 1992;42(8):893.

Wong 2014

Wong TE, Brandow AM, Lim W, Lottenberg R. Update on the use of hydroxyurea therapy in sickle cell disease. Blood 2014;124(26):3850-7.

Wood 2004

Wood JC, Tyszka JM, Carson S, Nelson MD, Coates TD. Myocardial iron loading in transfusion-dependent thalassaemia and sickle cell disease. Blood 2004;103:1934-6.

Zempleni 1996

Zempleni J, Galloway JR, McCormick DB. Pharmacokinetics of orally and intravenously administered riboflavin in healthy humans. American Journal of Clinical Nutrition 1996;63:54-66.

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Sources of support

Internal sources

- No sources of support provided

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External sources

- National Institute for Health Research, UK

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Appendices

1 Pubmed (1946 onwards) Search Strategy

	Search	Items Found
1	Vitamin B Complex[MeSH] OR Vitamin B deficiency[MeSH]	
2	B Vitamin*[tiab] OR Vitamin B1[tiab] OR Vitamin B2[tiab] OR Vitamin B3[tiab] OR Vitamin B5[tiab] OR Vitamin B7[tiab] OR Vitamin B9[tiab] OR B group vitamin*[tiab]	
3	Thiamine[MeSH] OR Thiamine deficiency[MeSH]	
4	Thiamin*[tiab] OR Aneurin[tiab]	
5	Riboflavin[MeSH] OR Riboflavin deficiency[MeSH]	

6	Vitamin G[tiab] OR Riboflavin[tiab]
7	Niacin[MeSH]
8	Niacin[tiab] OR Nicotinic[tiab] OR Induracin[tiab] OR 3 Pyridinecarboxylic Acid[tiab] OR Nicotinamide[tiab] OR Pyridine-3-carboxylic acid[tiab] OR Nicamin[tiab] OR Nicotinate[tiab] OR Niacinamide[tiab]
9	Vitamin B6[MeSH] OR Vitamin B6 deficiency[MeSH]
10	B6 Vitamin[tiab] OR B6 Vitamin[tiab] OR Pyridoxine[tiab] OR Pyridoxal[tiab] OR Pyridoxamine[tiab]

11	Folic Acid[MeSH] OR Folic Acid Deficiency[MeSH]
12	Folic acid[tiab] OR Folate[tiab] OR Metafolin[tiab] OR Levomefolate[tiab] OR Folvite[tiab] OR Folacin[tiab] OR 5-methyltetrahydrofolate[tiab] OR 5-methylTHF[tiab] OR 5-MTHF[tiab] OR L-5-MTHF[tiab] OR Tetrahydrofolate*[tiab] OR Leucovorin[tiab] OR Pteroylpolyglutamic acid[tiab]
13	Vitamin B12 [MeSH] OR Vitamin B12 deficiency[MeSH]
14	Cyanocobalamin[tiab] OR Cobalamin[tiab] OR

	Hydroxycobalamin[tiab] OR Eritron[tiab]
15	Biotin[MeSH]
16	Vitamin H[tiab] OR Biotin*[tiab]
17	Pantothenic Acid[MeSH]
18	Pantothenate[tiab] OR pantothenic[tiab]
19	#1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10 OR #11 OR #12 OR #13 OR #14 OR #15 OR #16 OR #17 OR #18
20	anaemia, sickle cell[MeSH] OR sickle cell[tiab] OR haemoglobin S*[tiab] OR haemoglobin S*[tiab] OR HbS disease[tiab]
21	randomised controlled trials[MeSH] OR random

	allocation[MeSH] OR
	double-blind
	method[MeSH] OR
	single-blind
	method[MeSH] OR
	clinical trial*[tiab] OR
	mask*[tiab] OR
	blind*[tiab] OR
	blind*[tiab] OR
	random*[tiab] OR
	randomised controlled
	trial[pt] OR controlled
	clinical trial[pt] OR
	clinical trial[pt]
22	cohort studies[MeSH] OR
	case-control
	studies[MeSH] OR
	comparative study[pt] OR
	risk factors[MeSH] OR
	cohort[tiab] OR
	compared[tiab] OR
	groups[tiab] OR case

	control[tiab] OR multivariate[tiab]
23	#21 OR #22
24	#19 AND #20 AND #23
25	animals[MeSH] NOT humans[MeSH]
26	#24 NOT #25

2 EMBASE (1974 onwards) Search Strategy

	Search	Items Found
1	exp sickle cell anaemia/	
2	sickle cell aneemia.mp.	
3	sickle cell anaemia.mp.	
4	sickle cell disease: tw	
5	1 OR 2 OR 3 OR 4	
6	exp vitamin B complex/	
7	exp vitamin B deficiency/	
8	exp thiamine/	
9	exp thiamine deficiency/	
10	exp riboflavin/	
11	exp riboflavin deficiency/	
12	exp niacin/	

13	exp vitamin B6/
14	exp vitamin B6 deficiency/
15	exp folic acid/
16	exp folic acid deficiency/
17	exp vitamin b12/
18	exp vitamin b12 deficiency/
19	exp biotin/
20	exp pantothenic acid/
21	B vitamin*:tw
22	B group vitamins:tw
23	Vitamin B1:tw
24	Vitamin B2:tw
25	Vitamin B3:tw
26	Vitamin B5:tw
27	Vitamin B7:tw
28	Vitamin B9:tw
29	Vitamin B12:tw
30	Thiamin*:tw
31	Aneurin:tw

32	Vitamin G:tw
33	Niacin:tw
34	Nicotinic:tw
35	Induracin:tw
36	3 Pyridinecarboxylic Acid:tw
37	Nicotinamide:tw
38	Pyridine-3-carboxylic acid:tw
39	Nicamin:tw
40	Nicotinate:tw
41	Niacinamide:tw
42	B6 Vitamin:tw
43	B 6 Vitamin:tw
44	Pyridoxine:tw
45	Pyridoxal:tw
46	Pyridoxamine:tw
47	Folic acid:tw
48	Folate:tw
49	Metafolin:tw
50	Levomefolate:tw

51	Folvite:tw
52	Folacin:tw
53	5-methyltetrahydrofolate:tw
54	5-methylTHF:tw
55	5-MTHF:tw
56	L-5-MTHF:tw
57	Tetrahydrofolate*:tw
58	Leucovorin:tw
59	Pteroylpolyglutamic acid:tw
60	Cyanocobalamin:tw
61	Cobalamin:tw
62	Hydroxycobalamin:tw
63	Eritron:tw
64	Vitamin H:tw
65	Biotin*:tw
66	Pantothenate:tw
67	pantothenic:tw
68	OR/6-67
69	controlled clinical trial:tw

70	randomised:tw
71	placebo:tw
72	double blind:tw
73	randomly:tw
74	trial:tw
75	clinical trials:tw
76	OR/69-75
77	exp cohort studies/
78	exp case-control studies/
79	exp risk factors/
80	comparative study:pt
81	cohort:tw
82	compared:tw
83	groups:tw
84	case control:tw
85	multivariate:tw
86	OR/77-85
87	76 OR 86
88	5 AND 68 AND 87

3 CINAHL (1982 onwards) Search Strategy

	Search	Items Found

1	("Vitamin B Complex" OR "B Vitamin*" OR "B group vitamin*" OR "Vitamin B1" OR "Vitamin B2" OR "Vitamin B3" OR "Vitamin B5" OR "Vitamin B7" OR "Vitamin B9")
2	(Thiamin* OR Aneurin)
3	(Riboflavin OR Vitamin G)
4	(Niacin OR Nicotinic OR Induracin OR "3 Pyridinecarboxylic Acid" OR Nicotinamide OR "Pyridine-3-carboxylic acid" OR Nicamin OR Nicotinate OR Niacinamide)
5	("Vitamin B6" OR "B6 Vitamin" OR Pyridoxine

	OR Pyridoxal OR Pyridoxamine)
6	(“Folic acid” OR Folate OR Metafolin OR Levomefolate OR Folvite OR Folacin OR “5-methyltetrahydrofolate” OR “5-methylTHF” OR “5-MTHF” OR “L-5-MTHF” OR Tetrahydrofolate* OR Leucovorin OR “Pteroylpolyglutamic acid”)
7	(“Vitamin B12” OR Cyanocobalamin OR Cobalamin OR Hydroxycobalamin OR Eritron)
8	(Biotin OR “Vitamin H”)
9	(“Pantothenic Acid” OR Pantothenate)

10	#1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9
11	(“sickle cell” OR “haemoglobin S dis*” OR “HbS dis*”)
12	(“randomised controlled trial” OR “controlled clinical trial” OR randomised OR placebo OR “drug therapy” OR randomly OR trial OR groups)
13	(“cohort studies” OR “case-control studies” OR “comparative study” OR risk factors” OR “cohort” OR compared OR groups OR “case control” OR multivariate)
14	#12 OR #13
15	#10 AND #11 AND #14

4 CAB Abstracts (1910 onwards) Search Strategy

Search	Items Found
TS= ((“sickle cell” OR “haemoglobin S disease” OR “haemoglobin S disorder” OR “HbS disease”) AND (“Vitamin B Complex” OR “B Vitamins” OR “B group vitamin*” OR “Vitamin B1” OR “Vitamin B2” OR “Vitamin B3” OR “Vitamin B5” OR “Vitamin B7” OR “Vitamin B9” OR “Thiamin” OR “Thiamine” OR “Aneurin” OR “Riboflavin” OR “Vitamin G” OR “Niacin” OR “Nicotinic” OR “Induracin” OR “3	

Pyridinecarboxylic Acid”

OR “Nicotinamide” OR

“Pyridine-3-carboxylic

acid” OR “Nicamin” OR

“Nicotinate” OR

“Niacinamide” OR

“Vitamin B6” OR “B6

Vitamin” OR

“Pyridoxine” OR

“Pyridoxal” OR

“Pyridoxamine” OR

“Folic acid” OR “Folate”

OR “Metafolin” OR

“Levomefolate” OR

“Folvite” OR “Folacin”

OR “5-

methyltetrahydrofolate”

OR “5-methylTHF” OR

“5-MTHF” OR “L-5-

MTHF” OR

“Tetrahydrofolate” OR

“Tetrahydrofolates” OR

"Leucovorin" OR "Pteroylpolyglutamic acid" OR "Vitamin B12" OR "Cyanocobalamin" OR "Cobalamin" OR "Hydroxycobalamin" OR "Eritron" OR "Biotin" OR "Vitamin H" OR "Pantothenic Acid" OR "Pantothenate") AND ("randomised controlled trial" OR "controlled clinical trial" OR "randomised" OR "placebo" OR "drug therapy" OR "randomly" OR "trial" OR "groups"))

5 ClinicalTrials.gov Search Strategy

	Search	**Items Found**
1	("sickle cell" OR "haemoglobin S" OR "HbS disease")	

2	(“vitamin B OR thiamine OR “vitamin B6” OR folate OR “vitamin B12” OR niacin OR “pantothenic acid” OR riboflavin OR Biotin)	
3	#1 AND #2	

6 ICTRP Search Strategy

	Search	**Items Found**
1	(“sickle cell” OR “haemoglobin S” OR “HbS disease”)	
2	(“vitamin B” OR thiamine OR “vitamin B6” OR folate OR “vitamin B12” OR niacin OR “pantothenic acid” OR riboflavin OR Biotin)	
3	#1 AND #2	

7 ISRCTN Search Strategy

	Search	**Items Found**

1	("sickle cell" OR "haemoglobin S" OR "HbS disease")
2	("Vitamin B Complex" OR "B Vitamins" OR "B group vitamin*" OR "Vitamin B1" OR "Vitamin B2" OR "Vitamin B3" OR "Vitamin B5" OR "Vitamin B7" OR "Vitamin B9")
3	(Thiamin OR Thiamine OR Aneurin)
4	(Riboflavin OR "Vitamin G")
5	(Niacin OR Nicotinic OR Induracin OR "3 Pyridinecarboxylic Acid" OR Nicotinamide OR "Pyridine-3-carboxylic acid" OR Nicamin OR

	Nicotinate OR Niacinamide)
6	(“Vitamin B6” OR “B6 Vitamin” OR Pyridoxine OR Pyridoxal OR Pyridoxamine)
7	(“Folic acid” OR Folate OR Metafolin OR Levomefolate OR Folvite OR Folacin OR “5-methyltetrahydrofolate” OR “5-methylTHF OR “5-MTHF” OR “L-5-MTHF” OR Tetrahydrofolate OR Tetrahydrofolates OR Leucovorin OR “Pteroylpolyglutamic acid”)
8	(“Vitamin B12” OR Cyanocobalamin OR Cobalamin OR

	Hydroxycobalamin OR Eritron)
9	(Biotin OR “Vitamin H”)
10	(“Pantothenic Acid” OR Pantothenate)
11	#2 OR #3 OR #4 OR #5 OR #6 OR #7 OR #8 OR #9 OR #10
12	#1 AND #11