Secular trends in folate and vitamin B_{12} status in over half a million Norwegian adults between 2000 and 2019

Siri Hundstad Quamme

Supervisors:

Thomas Olsen

Kathrine Jørgensen Vinknes

Kjetil Retterstøl

Master Thesis

Department of Nutrition, Faculty of Medicine

UNIVERSITY OF OSLO

November 2023

Abstract

Background: Folate (vitamin B_9) and vitamin B_{12} (B12) are essential nutrients for maintaining normal haematological and neurological function as well as preventing birth defects. There are indications that Norwegian women of childbearing age have low folate status, and there is continuous debate on whether to initiate mandatory folic acid foritfication. Further, in line with the Nordic Nutrition Recommendations 2023, the proportion of individuals eating plant-based is increasing, thereby reducing the consumption of the main dietary sources of B12. Thus, there is interest in assessing recent trends in folate and B12 status in Norwegian adults.

Methods: Data on serum folate, B12, plasma total homocysteine (tHcy) and plasma methylmalonic acid (MMA) analysed by Fürst Medical Laboraties were obtained. Over one million blood samples from 675,189 subjects aged 18 to 50 years who attended their general physician between 2000 and 2019 were analysed. Adjusted menas (estimated marginal means) and changes in metabolite concentrations between 2000-2004 and 2015-2019 were calculated using linear mixed models controlling for estimated glomerular filtration rate (eGFR), sex and age group, with random terms accounting for variation within subjects and within the laboratory methods of quantification.

Results: Women accounted for 52.9% of the sample and the geometric mean (geometric SD) age was 34.8 (1.32) years. The adjusted mean (95% CI) serum folate concentration decreased from 14.0 (13.0, 15.1) to 13.2 (12.3, 14.2) nmol/L between the two time periods, and serum B12 increased from 300 (294, 306) to 326 (319, 333) pmol/L. Plasma tHcy decreased from 11.3 (10, 12.8) to 10.1 (8.9, 11.4) µmol/L, while plasma MMA concentrations were relatively stable around 0.16 µmol/L. Folate decreased most dramatically in men (15%), with a corresponding 10% increase in tHcy. Women experienced a 5% decrease in folate concentrations and a 10% decrease in tHcy. In 2015-2019, 45.7% of all subjects had elevated plasma tHcy. Over 80% of women of reproductive age had folate concentrations below the World Health Organization (WHO) recommendation of 25.5 nmol/L, and over 20% were deficient (<10 nmol/L). Serum B12 concentrations were satisfactory, using serum B12 <221 pmol/L as a cutoff for low status.

Conclusion: Overall, folate status decreased and B12 status increased in Norwegian adults attending their general physician between 2000 and 2019. Folate status was concerning in both men and women, and plasma tHcy elevated in a significant proportion of the population.

Acknowledgements

This master's thesis was completed at the Department of Nutrition, Faculty of Medicine, University of Oslo.

Writing this thesis has been all but a straightforward process, but I am very happy to have learnt so much about my favourite vitamins, research, data science, and myself. I would very much like to thank my supervisors Thomas Olsen and Kathrine Vinksnes for excellent advice and encouragement. I am equally thankful for your flexibility, support and understanding, allowing me to follow my own timeline. You have given me time when I needed it, and been available when I needed help. You also presented a poster with the preliminary results at the HCY2023 conference in Cambridge, which was very motivating. Thankyou for showing me how exciting researching can be. I want to express my gratitude to Kjetil Retterstøl and the Atherosclerosis and Lipidology research group, including me in scientific discussions and giving weekly glimpses into your interesting work.

I would also like to thank my family and friends for your continuous support throughout this process. Your wise words and warm hugs rekindled my energy when I needed it most. Thankyou to my sisters Elin and Mari, my dad Geir and stepmom Heidi for great laughs and reminding me who I am. Three special thanks go to my mother Lena, my best friend Nora, and my dog Zofia for guiding me through the roughest patches and always being there for me. I don't know how I would have finished my thesis without you.

Mandal, November 14th 2023

Siri Hundstad Quamme

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

B12	Vitamin B_{12}
CVD	Cardiovascular disease
eGFR	Estimated glomerular filtration rate
GP	General practitioner
HoloTC	Holotranscobalamin
MMA	Methylmalonic acid
MTHFR	Methylenetrahydrofolate reductase
MTR	Methionine synthase
NHANES	National Health and Nutrition Examination Survey
NNR	Nordic Nutrition Recommendations
NTD	Neural tube defect
RBC	Red blood cell
RI	Recommended intake
tHcy	Total homocysteine
THF	Tetrahydrofolate
WHO	World Health Organization

1 Introduction

Vitamin B_9 (folate) and vitamin B_{12} (B12) are essential nutrients for maintaining normal haematological and neurological functions as well as preventing neural tube defects (NTDs) (1). They participate in a complex interplay linked by one-carbon metabolism, where the concentration of one vitamin may influence the function of the other (2). Groups at risk of low folate and B12 status include women of reproductive age and individuals consuming few animal products. In Norway, the number of individuals eating plant-based is increasing, and there are indications that women of reproductive age have low folate status (3, 4). Thus, there is interest in assessing recent time trends in folate and B12 status among Norwegian adults.

In this section, the dietary sources and intake recommendations of folate and B12 are introduced, followed by a brief description of the physiology, deficiency pathology, and metabolic interplay of the vitamins. The biochemical assessment of folate and B12 status is covered, alongside an overview of the current knowledge on status in Norway. Lastly, the rationale and objectives of the present study are provided.

1.1 Dietary sources of folate and vitamin B_{12}

Folate and B12 are vitamins, meaning they are not produced by the human body, and regular consumption is necessary to meet the body's requirement (5, 6). The main dietary sources of folate include legumes, dark green leafy vegetables and specific fruits, and fruit products such as orange juice (7). Other sources include walnuts, squash and pumpkin seeds as well as plant based products made from legumes. Folic acid-fortified foods constitute an important source in countries with national fortification policies (8).

Vitamin B_{12} is produced by microbes in soil and in the intestine of animals (5). Its synthesis requires cobalt, which is usually supplied in fortified feed (9). The foods contributing most to B12 intake are thus animal products such as meat, fish, dairy, and eggs (7). As B12 is mainly stored in the liver, liver contains especially high B12 levels. Other B12 sources include seaweed (especially Nori), tempeh (fermented soybeans), and mushrooms. However, these should not be relied upon as solitary sources of B12 as the content varies. Spirulina, a blue-green algae, is often marketed as a B vitamin source, but contains inactive B12 analogues (10).

1.2 Recommended intake of folate and vitamin B_{12}

The current Norwegian Dietary Guidelines are based on the a 2011 report from the National Council for Nutrition, and the 2012 Nordic Nutrition Recommendations (NNR) (11, 12). Specific recommendations on micronutrient intake are adapted from the NNR and the Scientific Committee on Food, the European Food Safety Authority. The NNR recommended intakes (RIs) of folate and B12 from 2012 (12) and 2023 (1) are shown in Table 1.

Table 1: Recommended daily intakes of folate and vitamin B_{12} from the Nordic Nutrition Recommendations 2012 and the Nordic Nutrition Recommendations 2023.

NNR 2012			NNR 2023		
Age months/ years	**Folate (µg)**	**Vitamin B_{12} (µg)**	**Age months/ years**	**Folate (µg)**	**Vitamin B_{12} (µg)**
< 6 m	-	-	< 6 m	64	0.4
6-11 m	50	0.5	7-11 m	86	1.2
12-23 m	60	0.6	1-3 y	120	1.5
2-5 y	80	0.8	4-6 y	130	1.5
6-9 y	130	1.3	7-10 y	190	2.5
Females			**Females**		
10-13	200	2.0	11-14	280	3.5
14-17	300	2.0	15-17	310	4.0
18-30	400*	2.0	18-24	330*	4.0
31-60	300*	2.0	25-50	330*	4.0
61-74	300	2.0	51-70	330	4.0
>= 75	300	2.0	>= 70	330	4.0
Pregnant	500	2.0	**Pregnant**	600	4.5
Lactating	500	2.6	**Lactating**	490	5.0
Males			**Males**		
10-13	200	2.0	11-14	250	3.0
14-17	300	2.0	15-17	320	4.0
18-30	300	2.0	18-24	330	4.0
31-60	300	2.0	25-50	330	4.0
61-74	300	2.0	51-70	330	4.0
>= 75	300	2.0	>= 70	330	4.0

*Does not include supplementation recommended to women of reproductive age.

1.2.1 Recommended intake of folate

The NNR folate RIs are based on the intake necessary to maintain normal serum and red blood cell (RBC) folate concentrations (1). Folic acid supplementation in pregnant women before 12 weeks of gestation has been shown to greatly reduce the incidence of NTDs in their children (13). As not all pregnancies are planned, 400 µg folic acid has been recommended to all Norwegian women of reproductive age since 1998 (14). The folate concentration in human milk is relatively constant (15). To ensure that the mother maintains a healthy folate status, it is recommended for supplementation to be continued during lactation. No adverse effects of naturally occurring dietary folate have been observed, but high doses of folic acid may have negative cosequences (16). Thus, a safe upper limit has been set at 1000 µg/d.

1.2.2 Recommended intake of vitamin B_{12}

The NNR B12 RIs were until 2023 based on the daily dose of B12 injections needed to normalize haematological status in patients with pernicious anaemia, an autoimmune disease described in *section 1.4.4*, but are now based on the daily dose needed to keep B12, methylmalonic acid (MMA), holotranscobalamin (holoTC) and plasma total homocysteine (tHcy) within reference ranges (17). Due to high body stores of B12, only a slightly higher intake has been recommended for pregnant women. Lactating women lose B12 in breast milk and are recommended to further increase their intake (15). Those consuming a plant-based diet are strongly advised to supplement B12 to prevent deficiency (18). Since high doses of B12 have not been observed to yield negative health consequences, no upper limit has been set (1).

1.3 Physiology of folate

1.3.1 Structure

The folates, or vitamin B_9, encompass a group of water-soluble vitamins, the tetrahydrofolates (THF), which are central in one-carbon metabolism (19). Structurally, the folates consist of a pterin unit, a para-aminobenzoyl group, and up to several glutamate residues. The different oxidation levels of folate, which exert unique functions, are illustrated in **Figure 1**.

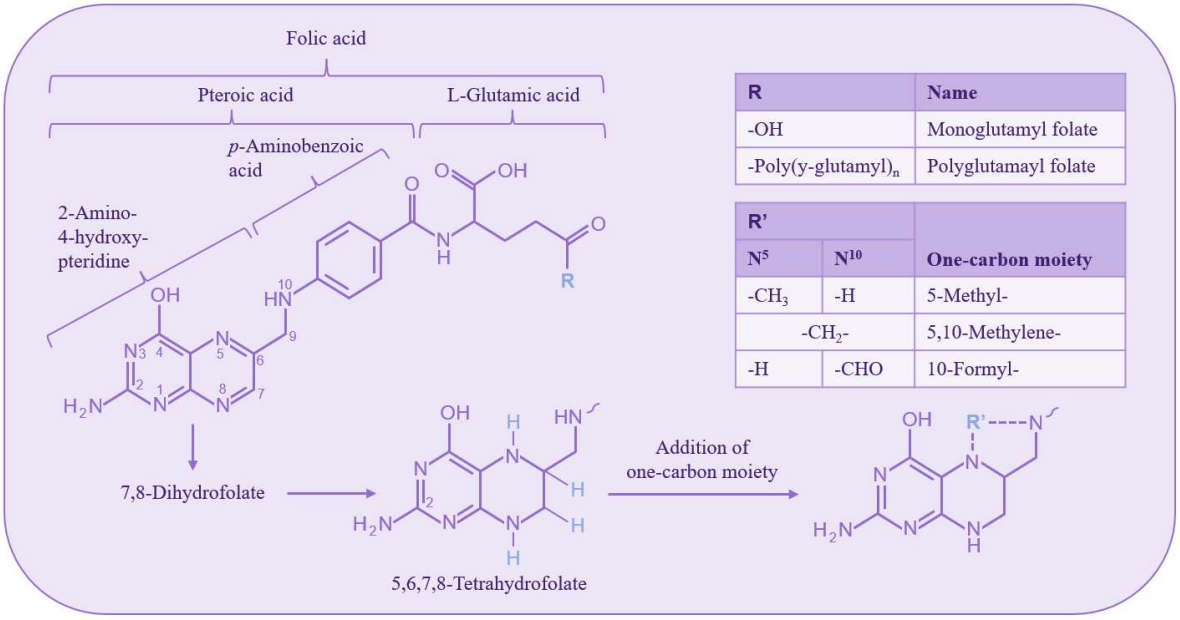


Figure 1: Chemical structure of folates. Adapted from Blom *et al* (20).

1.3.2 Digestion and absorption

Dietary folate is a polyglutamate with 2-9 glutamate residues attached (21). The bioavailability is highly dependent on the number of glutamate residues. Only the monoglutamate form of THF can be absorbed in the intestine, therefore hydrolyzation is carried out in the gastrointestinal tract by the glutamate carboxypeptidase II enzyme (22). THF monoglutamate is then

transported across the intestinal border by the proton-coupled folate transporter (PFCT) (23). In serum, THF monoglutamate circulates freely. In cells, glutamates are re-added, locking the THF polyglutamate's position inside the cells (19). Up to 40 % of cellular folate is located in the mitochondria. Thus, intracellular and liver folate are the most important body stores. Folic acid is used in folate supplements and fortified products, owing to its chemical stability (16). Folic acid is a pro-vitamin absorbed by PCFT and must be reduced to become biologically active. Once fully reduced to THF, it is identical to folate from natural food.

1.3.3 Functions

In the cell, folate participates in a complex system of one-carbon transfers that produce methionine, purines, and thymidylate (dTMP). These reactions are described in **Table 2**.

Table 2: Folate functions.

Function	Folate form	Metabolic reaction	Importance
Methionine synthesis	5-methyl THF	Methionine is synthesized from homocysteine by the enzyme methionine synthase (MTR), using vitamin B12 and 5-methyl THF as cofactors. 5-methyl THF is the carbon donor.	Methionine is needed to produce SAM, the body's main carbon donor.
Purine synthesis	10-formyl THF	Purine synthesis requires an aldehyde group donated by 10-formyl THF. 10-formyl THF is formed in mitochondria and in cytosol through THF coupling with formate. The main formate source is the hydroxymethyl group of serine.	The purines AMP and GMP are needed to form RNA.
Thymidylate synthesis	5,10-methylene THF	dTMP is synthesised in the cytosol and nucleus from dUMP. In this reaction 5,10-methylene THF is the cofactor of thymidylate synthase.	dTMP is required for DNA synthesis.

THF, tetrahydrofolate; SAM, S-adenosyl methionine; AMP, adenosyl monophosphate; GMP, guanosine monophosphate; dTMP, deoxy-thymidylate monophosphate; dUMP, deoxy-uridine monophosphate.

1.3.4 Regulation of folate-mediated one-carbon metabolism

One-carbon metabolism requires a complex interplay between folate and other nutrients (24). Folate intake varies over time and clearance is rapid, making supply to the cells far from constant (25). Thus, regulatory mechanisms are required to keep one-carbon metabolism operating in a stable manner. The most important is the control of methylene-tetrahydrofolate reductase (MTHFR) activity through S-adenosyl-methionine (SAM). MTHFR catalyses the irreversible reduction of 5,10-methylene THF to 5-methyl THF, a cofactor for producing methionine from homocysteine. Homocysteine is an amino acid that serves as an intermediate in methionine metabolism. SAM is an allosteric inhibitor of MTHFR, thus 5-methyl THF is only synthesized when SAM concentrations are low. The mechanism balances the supply of folate available for methionine synthesis against the supply for nucleotide synthesis. In cases of low B12 status, this may lead to what is termed the "folate trap", described in *section 1.5.1*.

1.3.5 Folate deficiency and related diseases

Stages of developing folate deficiency

Folate deficiency usually develops over a few months since the body stores can last approximately 2-3 months (16). The first signs are haematological, with reduced plasma and RBC folate, and increased tHcy concentrations (26). Over time, low folate hinders normal RNA and DNA production necessary for cell division, leading to the development of megaloblastic cells in rapidly replicating tissues. Different cutoffs for serum folate have been used to determine deficiency as the path from low folate status to anaemia is unclear. Concentrations > 7 nmol/L greatly reduce the risk of megaloblastic anaemia, but deficiency is also likely < 10 nmol/L (27).

Neural Tube Defects

Neural tube defects are a group of conditions that arise when the neural tube does not close properly during the first weeks of pregnancy (20). Resulting conditions include spina bifida and anencephaly, the first leading to lifelong paralysis and the latter incongruent with life. The mother's folate status and genetic profile seem to be the strongly correlated with NTD risk. Folic acid fortification has been shown to reduce the incidence of NTDs by up to 70%, and supplementation for women of reproductive age is instrumental in NTD prevention (28). The debate on whether to introduce mandatory folic acid fortification in Norway is not settled and the benefits are being weighed against potential harms, such as increased risk of some cancers.

Folate and cancer

Low folate status has been associated with several cancer diseases, including breast, prostate, leukemia, lymphoma, and colorectal cancer (16). A proposed mechanism is excess uracil in DNA leading to DNA breaks, due to a lack of thymidylate (29). Researchers have also proposed a potential role of folic acid from fortified foods or supplements to act as an antagonist of natural folate, thereby increasing cancer risk (30). Moreover, hypotheses have been raised regarding elevated folate concentrations accelerating cancer growth, considering the role of folate in DNA synthesis (31). High folate intake and serum concentrations have been associated with increased risk of prostate cancer (32, 33). However, a large meta-analysis from 2013 (n = 49,621) found no increase or decrease in overall cancer incidence during the first 5 years of folic acid treatment (34). Recent studies have found that adequate folate status is protective against breast cancer (35), colorectal cancer (36) and childhood acute lymphoblastic leukaemia (37). Furthermore, better folate status has been linked to reduced toxicity associated with anti-folate chemotherapy (38). Although there may be a U-shaped association between folate status and cancer risk, the exact relationship between folate and cancer remains unclear.

Folate and cardiovascular disease

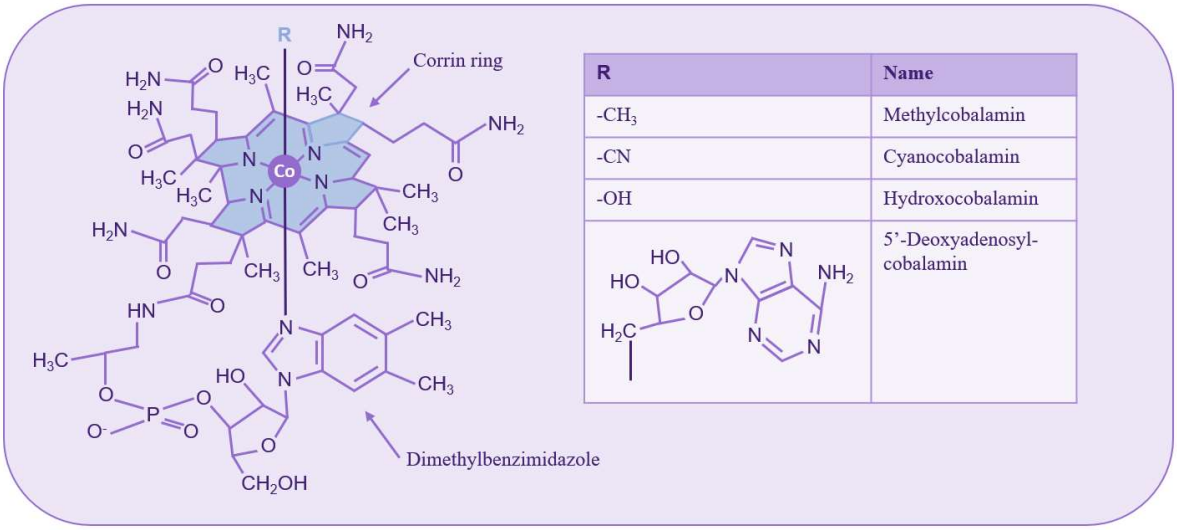
Since folate acts as a coenzyme in the remethylation of homocysteine to methionine, folate deficiency can cause accumulation of homocysteine. Elevated plasma tHcy has been associated with hypertension and cardiovascular disease (CVD) in observational studies (16). Three recent studies have found beneficial effects of folic acid supplementation, alone or in combination with other B-vitamins on endothelial function, mortality risk, CVD risk, and carotid intima-media thickness in specific groups (39-41).

A clearer association has been established between folic acid supplementation and stroke risk; a 2016 meta-analysis showed a 10% reduction in stroke risk and 4% reduction in overall CVD risk, most notably in those with lower folate status (42). There are indications that folic acid supplementation influences cardiovascular risk in some patients. Research is needed to establish which groups may benefit and whate dosage is appropriate. It remains unclear whether higher folate status is protective against CVD, or simply indirectly associated via homocysteine.

1.4 Physiology of vitamin B_{12}

1.4.1 Structure

Vitamin B_{12} (cobalamin) is a water-soluble compound characterized by a planar corrin ring containing a central cobalt atom connected to a ribose-3-phosphate-dimethylbenzimidazole structure below the planar surface (5). The vitamin takes on different properties by which ligand is covalently bound to the cobalt atom (43). The ligand separates supplemental (cyano- and hydroxocobalamin) and active forms of B12. Different B12 variants are shown in **Figure 2**.



R	Name
-CH_3	Methylcobalamin
-CN	Cyanocobalamin
-OH	Hydroxocobalamin
	5'-Deoxyadenosyl-cobalamin

Figure 2: Chemical structure of vitamin B_{12}. Adapted from Kozyraki (43) and Lippincott Biochemistry, Figure 28.6 (44).

1.4.2 Digestion and absorption

Dietary B12 is released from protein by protein-cleaving enzymes in the stomach, before it is bound to haptocorrin (45). In the upper small intestine, B12 is released from haptocorrin and bound to instrinsic factor (IF), which originates from the parietal cells of the gastric wall. The IF-B12 complex is absorbed through receptor-mediated endocytosis. This process is limited in capacity at approximately 1,5-2,0 µg per meal, necessitating frequent consumption. Once inside the enterocyte lysosome, IF is degraded and B12 released to the cytosol. Next, B12 may be processed to an active cofactor, or sent into the portal vein. Circulating B12 binds to transcobalamin I or transcobalamin II. The complex is called holotranscobalamin (holoTC), or active B12. The enterohepatic circulation conserves large amounts of B12, and the majority of the body stores are located in the liver (2).

1.4.3 Function

Vitamin B_{12} has two known roles in the human body, executed by the two different active forms of the vitamin, methyl-B12 and adenosyl-B12. The functions of B12 are outlined in **Table 3**.

Table 3: Vitamin B_{12} functions.

Function	B12 form	Metabolic reaction	Importance
Methionine synthesis	Methyl-B12	Methionine is synthesised from homocysteine by methionine synthase (MTR), using methyl-B12 and 5-methyl THF as cofactors.	Methionine is needed to produce SAM, the main carbon donor in the body.
Cofactor for methyl-malonyl CoA mutase	Adenosyl-B12	Adenosyl-B12 is a cofactor for methylmalonyl CoA mutase in the mitochondrion, converting methylmalonyl CoA to succinyl CoA.	Methylmalonyl CoA mutase is a key enzyme in the metabolism of odd-chain fatty acids and ketogenic amino acids.

THF – tetrahydrofolate; SAM – S-adenosyl methonine; CoA – coenzyme A

1.4.4 Vitamin B_{12} deficiency and related diseases

Pernicious anaemia

The most well-known cause of vitamin B12 deficiency, other than low intake, is inseparably linked to the discovery of the vitamin itself (46). Researchers discovered B12 in a study where pernicious anaemia was treated and death prevented by giving the patients lightly cooked liver. Pernicious anaemia was later recognized as an autoimmune disorder attacking the parietal cells of the stomach that produce IF, which is necessary for B12 absorption (26).

Stages of developing vitamin B_{12} deficiency

Due to effective storage and conservation of B12, deficiency may take years to develop. The first signs of B12 insufficiency are increased plasma MMA, then decreased serum holoTC and B12 (47). Plasma MMA accumulates from methylmalonyl-CoA when not enough B12 is present for methylmalonyl-CoA mutase to produce succinyl-CoA. Later, plasma tHcy is raised, followed by a reduction of RBC B12 and hypersegmentation of neutrophil nuceli. Over time, megaloblastic anaemia develops. Patients with prolonged deficiency may report infertility and foetal loss, fatigue, neuropathy, memory loss, and depression. Dementia and psychosis have been reported in the most severe cases (48). Symptoms may not present in an orderly manner, and anaemia first becomes evident when the deficiency is relatively pronounced. Hence, clinicians should not rely on anaemia symptoms alone to suspect B12 deficiency (49). The cutoffs defining B12 status are debated (2). However, serum B12 < 148 pmol/L is commonly used as a cutoff for deficiency, and 148-221 pmol/L is considered low B12 status (50).

Neurological outcomes

Severe B12 deficiency causes demyelination of neurons in the central and peripheral nervous system (51). The nerve damage may cause neuropathy, reduced cognition or even dementia (47). Studies have found an association between B12 deficiency and depression, but the exact relationship remains unclear. Few studies have investigated the effect of B12 treatment on neurological outcomes, cognitive function and dementia, and the results are contradictory (47, 52). More research is needed to examine the effect of B12 supplementation on neurological outcomes, alone or in combination with other B-vitamins. Evidence is emerging showing that simultaneous high folic acid status and low B12 status is detrimental to cognitive health in the elderly (30), as demonstrated in the US NHANES cohort of 1999-2002 (53). Thus, researchers should consider folate status while investigating B12 and cognitive health.

1.5 Interactions between folate and vitamin B_{12}

Folate and B12 participate in a fine-tuned balance, regulated through SAM, to meet the cell's need for one-carbon units and nucleotides (6). Their roles are interconnected, as they both are cofactors for MTR. Consequently, changes in the concentration of one vitamin may affect the function of the other. An outline of folate and B12 metabolism is presented in **Figure 3**.

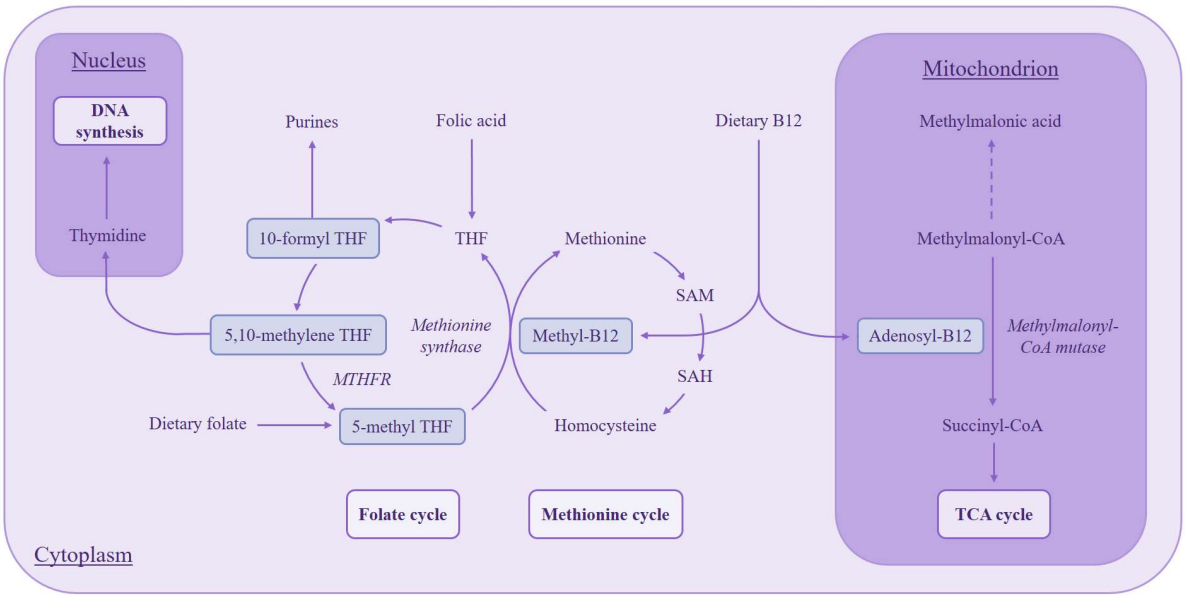


Figure 3: Folate and vitamin B_{12} metabolism. Active forms of the vitamins are marked in blue. THF, tetrahydrofolate; MTHFR, methylenetetrahydrofolate reductase; B12, vitamin B_{12}; SAM, S-adenosyl methionine; SAH, S-adenosyl homocysteine; CoA, Coenzyme A; TCA cycle, tricarboxylic acid cycle. Adapted from Green *et al* (2).

1.5.1 The folate trap

In B12 deficiency, the close regulation of folate-mediated one-carbon metabolism is disrupted (54). Vitamin B_{12} depletion reduces SAM synthesis so that MTHFR is not inhibited, and folate is continuously converted to 5-methyl THF. Because the reaction is irreversible, 5-methyl THF accumulates. This has been termed "The folate trap". Consequently, less folate in the form of 5,10-methylene THF becomes available for nucleotide synthesis, This may ultimately lead to megaloblastic anaemia and neurological symptoms, as seen in severe B12 deficiency.

1.5.2 Folate-masked vitamin B_{12}-deficiency

High folate status may mask the megaloblastic anaemia seen in B12 deficiency by overriding the folate trap with excess folate, allowing both methionine and purine synthesis (30). If B12 deficiency is allowed to progress undetected over time, the risk of neurological damage increases. High folate status may aggravate the risk of clinical manifestations in individuals with low B12 status (2, 30). This is particularly a concern in folate repleted populations.

1.6 Groups at risk of folate and B12 deficiency

Certain groups are at higher risk of folate or B12 deficiency. These are shown in **Table 4**, along with group-specific recommendations. The most common causes of deficiency are inadequate intake compared to requirement, malabsorptive conditions, alcohol abuse, and genetic polymorphisms. Malabsorptive conditions include celiac disease, inflammatory bowel disease, tropical sprue, and surgery to the stomach or intestine. Other vulnerable groups include patients taking select medical drugs that increase folate requirement, such as antiepileptic drugs (55), metformin (diabetes type II), sulfasalazine (IBD), methotrexate (cancer and rheumatoid arthritis) and triamterene (diuretic) (1, 56).

Another issue to be aware of, though of smaller magnitude, is that in recent years, Nordic countries have reported more frequent hospitalizations of long-term nitrous oxide ("laughing gas") users (57, 58). The gas oxidizes and thereby inactivates B12, which can lead to severe neurological symptoms.

Table 4: Groups at risk of folate and vitamin B_{12} deficiency.

Group	Cause of elevated risk	Group-specific recommendations
Women of reproductive age, pregnant and lactating women	Increased requirement of folate to support the foetal growth and prevent NTDs. Increased requirement of folate and B12 to account for loss in breast milk.	Supplement 400 µg folic acid daily.* Serum folate >25 nmol/L.**
Elderly	Impaired B12 absorption i.e. due to atrophic gastritis, reducing HCl and IF production (59).	No specific recommendation.
Vegetarians and vegans	Inadequate B12 intake.	Supplement B12.***
People with alcohol addiction	Decreased absorption in the intestine and uptake in the liver. Lower dietary intake.	No specific recommendation.
People with certain genetic polymorphisms	Mutations in glutamate carboxypeptidase II (GCPII), the transport protein reduced folate carrier (RFC), or the metabolic enzymes methylenetetrahydrofolate reductase (MTHFR) and methionine synthase (MTR).	Serum folate >15 ***

B12, vitamin B_{12}; NTDs, Neural Tube Defects; IF, intrinsic factor. *Norwegian National Nutrition Council (60). **World Health Organization (61). ***Nordic Nutrition Recommendations 2023 (1).

1.7 Assessing folate and vitamin B_{12} status

1.7.1 Biomarkers of folate status

Finding a precise biomarker for folate status is challenging due to different folate vitamers being present in the cells, and the complex interactions of folate with other nutrients such as the vitamins B12, B2 and B6 (62). The fact that age, renal function, and genetic polymorphisms also influence folate status, poses another challenge. Thus far, the most widely used biomarkers have been serum folate and RBC folate, as well as plasma tHcy. Plasma tHcy is negatively correlated with folate since folate catalyses the re-methylation of homocysteine to methionine. RBC folate is an indicator of long-term folate status, reflecting folate concentrations over the past 120 days, the age of an average RBC. Serum folate reflects recent folate intake but may indicate folate status if measured repeatedly over a longer period of time.

1.7.2 Biomarkers of vitamin B_{12} status

The most recognized biomarkers of vitamin B12 status include serum or plasma total B12, serum holoTC, plasma MMA, and plasma tHcy (63). Serum or plasma total B12 is most commonly measured (64). It reflects intake over time and liver stores, and the analysis is both available and affordable. Nevertheless, it lacks sensitivity and specificity, as do MMA and tHcy. A model combining these four markers, termed cB_{12}, has been suggested (65):

$$cB_{12} = \log_{10}\left[\frac{holoTC \cdot B_{12}}{MMA \cdot tHcy}\right] - (age\ factor)$$

Studies have already shown a promising ability of the model to detect neurologic conditions related to B12 deficiency, in samples where one marker alone did not (66, 67). However, most laboratories do not measure holoTC. Thus, methods for estimating cB_{12} using only three ($3cB_{12}$) or even two ($2cB_{12}$) markers have also been proposed (68).

1.7.3 Shared biomarkers

Plasma tHcy has been shown to correlate closely with folate status and, to a lesser degree, with B12 status (63, 69). It is thus often used as a functional biomarker of folate status. In folate repleted populations, however, B12 status is the main determinant of tHcy concentration (70). As mentioned above, the individual biomarkers of folate and B12 status lack sensitivity and specificity. Therefore, the biomarkers should preferably be evaluated together (62, 63).

1.8 Folate and vitamin B_{12} status in Norway

1.8.1 Intake among Norwegian adults

The NORKOST 3 study from 2010 reported dietary intake among 1,787 Norwegian adults aged 18-70 years (71). Median folate intake did not meet the recommendation in men or women (279 and 231 µg/d, respectively), however B12 intake was adequate (8.9 and 6.0 µg/d, respectively). Bread and vegetables were the most important dietary sources of folate. The main sources of B12 were fish, meat, and milk. Men had a higher consumption of meat and dairy products, which may have contributed to the higher B12 intake. When including supplements, a small increase in folate was seen in both sexes. Women in the South-Eastern and Northern regions had a lower intake than women from other regions. A study on the Indigenous Sami population in Northern Norway, whose diet differs slightly from the non-Sami, found an adequate intake of both folate and B12 in both sexes (72).

1.8.2 Intake among women of reproductive age

In 2005, it was reported that few women start folate supplementation before pregnancy, and that NTD incidence was unchanged, despite the recommendation issued in 1998 (14). A study published in 2019 on nutrient intake in pregnant Norwegian women showed that over half of the women were likely to have an inadequate folate intake (4). In Norwegian lactating women, B12 status and B12 content in human milk during the first 6 months of pregnancy was adequate, according to a study published in 2020 (73).

1.8.3 Status in vegetarians and vegans

The proportion of Norwegians consuming a vegan, vegetarian, pescetarian or flexitarian diet was 27% in 2022, according to market research conducted by the food conglomerate Orkla. A recent study on folate and B12 status in Norwegian vegans (n = 115) and vegetarians (n = 90) found that both groups had adequate concentrations of folate, tHcy, B12 and MMA (74). Approximately half of the vegans and one third of the vegetarians reported using folate supplements. Of all participants, 14% had low B12 concentrations (<221 pmol/L). More vegetarians than vegans had B12 intakes, dietary and supplemental, below the RI. The results indicated that vegans, who have made more drastic dietary changes, were more concerned with taking supplements to meet nutritional requirements than vegetarians. Age was also associated with B12 status, with the youngest age groups showing lower concentrations.

1.9 Rationale

Several institutions routinely conduct studies on the diet of the general Norwegian population, which provide the basis for estimations of folate and B12 intake. NORKOST 3 is the largest national dietary survey in Norway to date and gives an overview of the habitual dietary intake among adults. Nevertheless, the study was conducted over 10 years ago and covered a limited population and did not measure biomarkers to assess B-vitamin status.

Groups at risk of folate and B12 deficiency have been studied more recently, however the participant numbers have been limited. Low folate status is far too common in pregnant women, and there is a lack of data on folate status in women of reproductive age. Several countries have already implemented folic acid fortification to secure adequate status in this group, but in Norway, the jury is still out. There is also concern regarding the B12 status of especially adolescents and women of reproductive age, as a switch towards more plant-based diets is seen and recommended by Norwegian and Nordic authorities, and the RIs for B12 have doubled since the NNR 2012.

Presently, the folate and B12 status of the Norwegian population is unclear. An overview of the current situation and recent trends is necessary to inform policymakers on strategies to secure adequate folate and B12 status for optimal health among Norwegians.

2 Objectives

The overall aim of this master thesis was to uncover the development of folate and B12 status in healthy Norwegian adults attending their general practitioner between 2000 and 2019.

The specific objectives were:

- To assess the trends in serum folate and B12, and plasma tHcy and MMA in Norway from 2000-2019.
- To investigate whether there are differences in folate and B12 status between the sexes, age groups and geographical regions.
- To examine the prevalence of simultaneous adequate folate status and low or deficient B12 status.
- To examine the correlation between biomarkers of folate and B12 status.

3 Materials and Methods

3.1 Design

All residents in Norway are entitled to a personal general practitioner (GP). The aim of this thesis was to examine folate and B12 status among healthy Norwegian adults who attended their GP from 2000-2019. Data on B-vitamins were obtained from Fürst Medical Laboratories (Fürst), which is the largest medical laboratory in Norway, covering all regions. The design was serial cross-sectional, and data was categorized into 5-year intervals.

3.2 Study sample

Our dataset included all patients in the ages 18-50 years whose blood samples were analysed for serum folate, serum B12, plasma tHcy and/or plasma MMA concentrations by Fürst in the period 2000-2019. Only subjects up to 50 years of age were included to reduce the risk of morbidities in the sample group. The Fürst laboratory mainly serves the primary healthcare system, and most samples are collected in the offices of GPs. Thus, the dataset did not include samples from hospitalized patients. However, patients receiving outpatient care or at home hospital care could not be excluded.

To counteract potential skewing of data by patients with health conditions that may affect folate or B12 status, exclusion criteria were defined. According to Statistics Norway, the mean number of visits to the GP during the study period was 2.2 among 20-29 year olds and 2.6 among 30-49 year olds (75). In 2020, around 30 out of 100 Norwegian residents had their blood analysed for folate or B12 (64). Unless there was a special interest in monitoring folate and B12 concentrations due to illness or ongoing treatment, it is unlikely that these vitamins were analysed after each visit. Hence, subjects with more than one test per year were excluded.

Reduced kidney function has been associated with increased plasma tHcy concentrations even in moderate kidney failure, and estimated glomerular filtration rate (eGFR) has been established as a better determinant for tHcy than creatinine (76). Plasma MMA concentrations also increase with impaired renal function (77). Therefore, eGFR was calculated, and subjects with reduced eGFR were excluded from the study. An overview of the exclusion criteria and the purpose behind each criterion are outlined in **Table 5**. A subject flowchart is shown in **Figure 4**.

Table 5: Exclusion criteria.

Exclusion criteria	Purpose of exclusion
> 1 measurement within at least one year or >20 measurements in total	Subjects likely to have a special interest in monitoring B-vitamin and/or functional marker concentrations, possibly related to an elevated risk of deficiency or a health condition.
Missing data on creatinine.	Hinders calculation of eGFR.
Missing data on sex.	Hinders calculation of eGFR.
eGFR < 60 mL/min/1.73m^2 or eGFR ≥ 140 mL/min/1.73m^2	Subjects with reduced renal function and glomerular hyperfiltration (76-79). eGFR was calculated from creatinine, sex and age using the CKD-EPI equation (80).

eGFR, estimated glomerular filtration rate; CKD-EPI, Chronic Kidney Disease Epidemiological Collaboration.

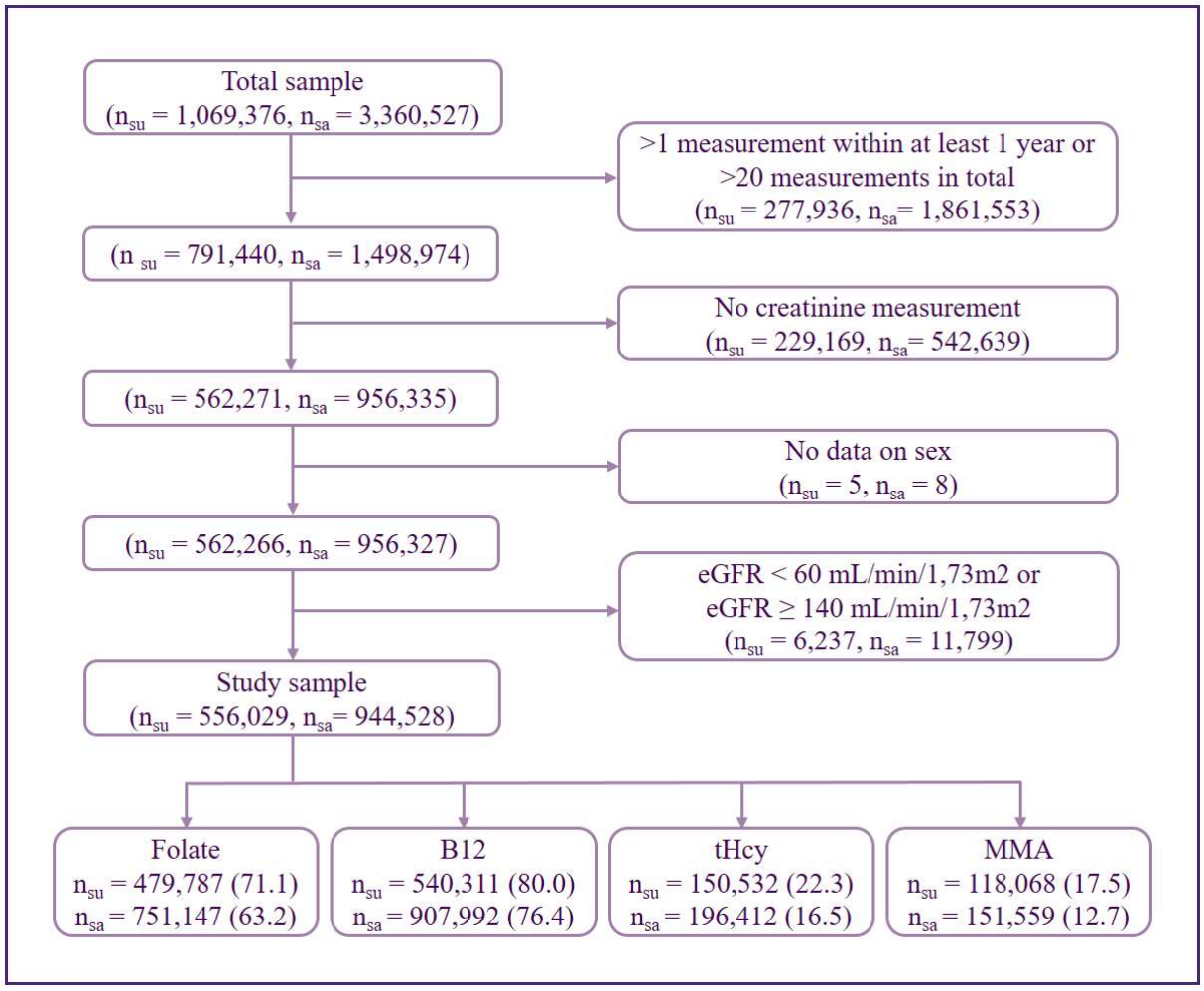


Figure 4: Subject flowchart. n_{su}, number of subjects; n_{sa}, number of samples; B12, serum vitamin B_{12}; tHcy, plasma total homocysteine; MMA, plasma methylmalonic acid. The percentages of subjects and samples having the given analyte mearused are shown in parentheses.

Subjects with one or more B12 measurements >800 pmol/L were excluded from analyses involving B12 because they may have received B12 injections (81). Subjects with tHcy > 50 µmol/L were excluded from tHcy analyses due to likelihood of having a disease or genetic polymorphism causing very high tHcy concentrations (82). Subjects with MMA > 0.50 µmol/L were excluded from MMA analyses because they may have had a genetic polymorphism causing high concentrations, such as a common single nucleotide polymorphism in the 3-hydroxyisobutyryl-CoA hydrolase (HIBCH, rs291466) (83). No subjects were excluded from folate analyses because the methods used by Fürst only measured folate up to 50 nmol/L.

3.3 Biomarkers

The dataset included folate, vitamin B12, tHcy, MMA and creatinine. Folate deficiency was defined using the WHO cutoff of <10 nmol/L in serum concentration (61). In women of reproductive age, serum concentrations above 25.5 nmol/L are recommended to prevent NTDs (84). For B12, the cutoff for deficiency was set at < 148 pmol/L and low B12 status was defined as < 221 pmol/L (8, 50). Elevated concentration of plasma tHcy was defined as > 11 µmol/L (85), while elevated plasma MMA was defined as > 0.27 nmol/L (86). Information on the methods and instruments used by Fürst to analyse each metabolite are shown in **Table 6**.

Table 6: Methods and instruments used in the biochemical analyses of folate, vitamin B_{12}, homocysteine, methylmalonic acid and creatinine.

Biomarker	Method	Instrument	CV
S-Folate	ICL	Siemens Advia Centaur	-
		Siemens Advia Centaur XP (2009)	-
		Siemens Advia Centaur XPT (2014)	7.81
S-B12	ICL	Siemens Advia Centaur	-
		Siemens Advia Centaur XP (2009)	-
		Siemens Advia Centaur XPT (2014)	7.66
P-tHcy	Competing FPIA	Abbott IMx	-
	ICL (2003)	Siemens Advia Centaur (2003)	-
		Siemens Advia Centaur XP (2009)	-
		Siemens Advia Centaur XPT (2014)	6.99
P-MMA	GC-MS (2004)	-	-
	LC-MS/MS (2011)	-	0.27
	UPLC-QqQ-MS/MS (2014)	Waters Aquity (2014)	8.80
S-Creatinine	Colorimetric assay	Roche modular	-
	Kinetic-colorimetric assay (2009)	Siemens Advia 2400 (2014)	1.35
		Siemens Advia Chemistry XPT (2016)	2.83

CV, Coefficient of variation; S, serum; P, plasma; B12, vitamin B_{12}; tHcy, total homocysteine MMA, methylmalonic acid; ICL, chemiluminescence immunoassay; FPIA, fluorescence polarization immunoassay; GC-MS, Gas-chromatography mass-spectrometry; LC-MS/MS, Liquid chromatography tandem mass spectrometry; UPLC-QqQ-MS/MS, Ultra high performance liquid chromatography coupled with triple-quadrupole mass spectrometry. Year started in parentheses.

3.4 Statistics

Descriptive statistics were performed to compare biomarker concentrations over the time period, using the RStudio version 2023.06.0 software for Windows, running R version 4.2.3. Analytes were log-transformed as their distributions were skewed. Geometric means and geometric standard deviations of the biomarker concentrations were computed. Estimated marginal means, hereby referred to as adjusted means, were computed through linear mixed effects regression models with the given analytes as response variables (Model 1). Subject ID, accounting for within-person variation, as well as method or instrument changes were included as random effects. The covariates were eGFR, sex, and age group. The age groups were 18-29 years, 30-39 years and 40-50 years. To reveal the time trends from 2000-2004 to 2015-2019, a variable for the 5-year intervals ("year group") was used. Model 1 was the main model.

To illustrate, Model 1 was as follows:

$$ln(analyte) = year\ group\ +\ sex\ +age\ group +\ eGFR\ + (1|subject\ ID)\ +\ (1|method)$$

Similar models (Model 2-5) were used to test whether time trends were different between women and men, age groups, and geographical regions, using Sattertwhaite's method (87). Subject ID was included as a random term in all models, and method/instrument as a random term in the adjusted models. For subgroup analyses, the subgroup variables (sex, age group, region) were included as interaction terms in both the respective unadjusted models (Model 2) and adjusted models (Models 1, 3 and 4). To test for differences between unadjusted and adjusted models, the anova method was employed. An overview of the models is presented in **Table 7**. A separate analysis on folate status in women was conducted using Model 6. A p-value of < 0.05 was considered significant.

Table 7: Linear mixed effect models for time trend analysis for all subjects and across subgroups.

Model	Subgroup	Interaction term	Covariates	Random terms
Model 1	All subjects	-	eGFR, age group, sex	Subject ID, method
Model 2	All or *	- or *	-	Subject ID
Model 3	By sex	Sex	eGFR, age group	Subject ID, method
Model 4	By age group	Age group	eGFR, sex	Subject ID, method
Model 5	By region	Region	eGFR, age group, sex	Subject ID, method
Model 6	Women	Age group	eGFR	Subject ID, method

eGFR, estimated glomerular filtration rate. *Respective subgroup in the unadjusted analysis.

To illustrate the subgroup analyses with interaction term, the unadjusted model for differences between age groups (based on Model 2) was as follows:

$$ln(analyte) = year\ group + age\ group + (1|subject\ ID) + year\ group * age\ group$$

The adjusted model for differences between age groups (Model 4) was as follows:

$$ln(analyte) = year\ group + age\ group + sex + eGFR + (1|subject\ ID) + (1|method) + year\ group * age\ group$$

3.5 Ethics

The study was part of the research project "Analysing trends in the lipid profile of the Norwegian population after the year 2000". The project, with the extension of covering folate, B12, tHcy and MMA, was approved by the data protection officer at the University of Oslo July 2nd 2020, following data protection impact assessment. The project was approved by the Regional Ethical Committee on September 24th, 2020 (Ref. 14907).

3.6 Use of articifical intelligence

The chatbot GhatGPT, an online service that uses artificial intelligence to answer the user's questions, was used in one instance in October 2023 to improve R code.

3.7 Contribution

The student was responsible for the data cleaning and statistical analysis. The full dataset contained over 4.4 million observations from over 1 million individual subjects, requiring the use of methods suitable for big data. To prepare for analysis, a subset of 40,000 observations was utilized. The student found, developed, and wrote codes that reduced computation time and performance requirement, while accurately performing the desired operations. Codes were then executed on the full data set. An extensive list of the RStudio codes used is provided in the appendix (*Appendix D*).

4 Results

4.1 Subject characteristics

A total of 1,189,050 measurements from 675,189 subjects were included in the final sample. **Table 8** describes the subject characteristics. Women accounted for 53% of the sample and the geometric mean (geometric SD) age was 34.8 (1.32) years. The majority of measurements were from East Norway, which was in accordance with the population size of this region. Among the excluded subjects, there was a higher proportion of women and younger subjects as well as more tHcy and MMA samples. See characteristics of the excluded subjects in *Table S1*, which is found in *Appendix C: Supplemental Material*.

Table 8: Subject characteristics.

Subjects, n	675,189
Sex, n (%)	
Female	356,854 (52.9)
Male	318,335 (47.1)
Age group, n (%)	
18-29 years	247,877 (36.7)
30-39 years	200,684 (29.7)
40-50 years	226,628 (33.6)
Region, n (%)	
East	476,065 (75.9)
South	16,128 (2.57)
West	63,125 (10.1)
Mid	63,891 (10.2)
North	7,707 (1.23)

4.2 Mean metabolite concentrations

B-vitamin biomarker concentrations are outlined in **Table 9**. For all biomarkers, the test volume increased drastically from the first 5-year period to the last. Aside from creatinine, B12 was the most frequently measured biomarker. Fürst started analysing MMA concentrations in 2004; hence, data on MMA in the 2000-2004 interval are only from 2004. Mean metabolite concentrations for each year from 2000 to 2019 are found in *Table S2*.

Table 9: Number of samples and mean metabolite concentrations in 5-year intervals from 2000 through 2019.

	Years				
	2000-2004	**2005-2009**	**2010-2014**	**2015-2019**	**p[4]**
Samples, n (%)[1]					
Folate	45,063 (6)	121,330 (16.2)	241,969 (32.2)	342,787 (45.6)	
B12	86,130 (9.5)	173,068 (19.1)	361,236 (39.8)	531,374 (58.5)	
tHcy	14,623 (7.5)	34,888 (17.8)	65,834 (33.5)	80,742 (41.1)	
MMA	5,726 (3.8)	21,826 (14.4)	52,471 (34.6)	74,995 (49.5)	
Creatinine	97,540 (7)	205,765 (14.8)	430,697 (31.1)	652,198 (47)	
Metabolite concentrations					
Folate, nmol/L (n_{su} = 479,787, n_{sa} = 751,147)					
Geometric mean (SD)	13.9 (1.65)	13.6 (1.65)	14.7 (1.66)	14.1 (1.73)	
Adjusted mean (CI)[2]	14.0 (13.0, 15.1)	14.2 (13.2, 15.3)	15.1 (14.0, 16.3)	13.2 (12.3, 14.2)	<0.001
B12, pmol/L (n_{su} = 540,311, n_{sa} = 907,992)					
Geometric mean (SD)	299 (1.33)	298 (1.33)	300 (1.35)	332 (1.38)	
Adjusted mean (CI)[2]	300 (294, 306)	302 (296, 309)	305 (298, 311)	326 (319, 333)	<0.001
tHcy, µmol/L (n_{su} = 150,532, n_{sa} = 196,412)					
Geometric mean (SD)	10.1 (1.34)	11.5 (1.37)	10.7 (1.40)	10.7 (1.40)	
Adjusted mean (CI)[2]	11.3 (10, 12.8)	13.4 (11.8, 15.2)	11.2 (9.9, 12.6)	10.1 (8.9, 11.4)	<0.001
MMA, µmol/L (n_{su} = 118,068, n_{sa} = 151,559)					
Geometric mean (SD)	0.16 (1.38)	0.15 (1.38)	0.15 (1.43)	0.15 (1.45)	
Adjusted mean (CI)[2]	0.15 (0.15, 0.16)	0.16 (0.15, 0.16)	0.17 (0.16, 0.17)	0.16 (0.16, 0.17)	<0.001[5]
Creatinine, µmol/L (n_{su} = 675,189, n_{sa} = 1,189,050)					
Geometric mean (SD)	86.3 (1.14)	72.8 (1.20)	70.9 (1.20)	67.5 (1.21)	
Adjusted mean (CI)[3]	86.0 (84.4, 87.6)	72.5 (71.2, 73.9)	71.8 (70.5, 73.1)	69.6 (68.4, 70.9)	<0.001

B12; vitamin B_{12}; tHcy, total homocysteine; MMA, methylmalonic acid, SD, standard deviation (as percentage); CI, 95% confidence interval; n_{su} = number of subjects; n_{sa} = number of samples.
[1]Percentage of total measurements within the 5-year period containing the given metabolite.
[2]Estimated marginal means by linear mixed effects regression, controlled for eGFR, sex and age group with method/instrument as a random term (Model 1).
[3]Estimated marginal means by linear mixed effects regression, controlled for sex, age group, and method/instrument as a random term (Model 1, minus eGFR).
[4]P-value derived from linear mixed effects regressions.
[5]Time trend from 2005-2009 to 2015-2019 due to few measurements in 2004.

4.3 Folate status

The distributions of folate concentrations among women and men in 5-year intervals are shown in **Figure 4**. The raincloud plot is a combination of a distribution plot, a scatterplot, which in this case has been modified due to the large sample size, and a line plot showing the average and spread of the distribution. The modified scatter plot consists of points that each represent many observations. The proportion of samples showing folate and B12 deficiency as well as elevated tHcy and MMA are shown in *Table S3*.

The data indicate lower folate status in men than women across the study period. There was an increase in overall folate status from 2000-2004 to 2010-2014, with the geometric mean being highest in 2010-2014. Additionally, the proportion of measurements indicating folate deficiency decreased from 24.5 to 20.7% between 2000-2004 and 2010-2014. However, the proportion increased to 24.6% in 2015-2019. Although the adjusted mean also increased until 2010-2014, it dropped to its lowest concentrations in the period 2015-2019.

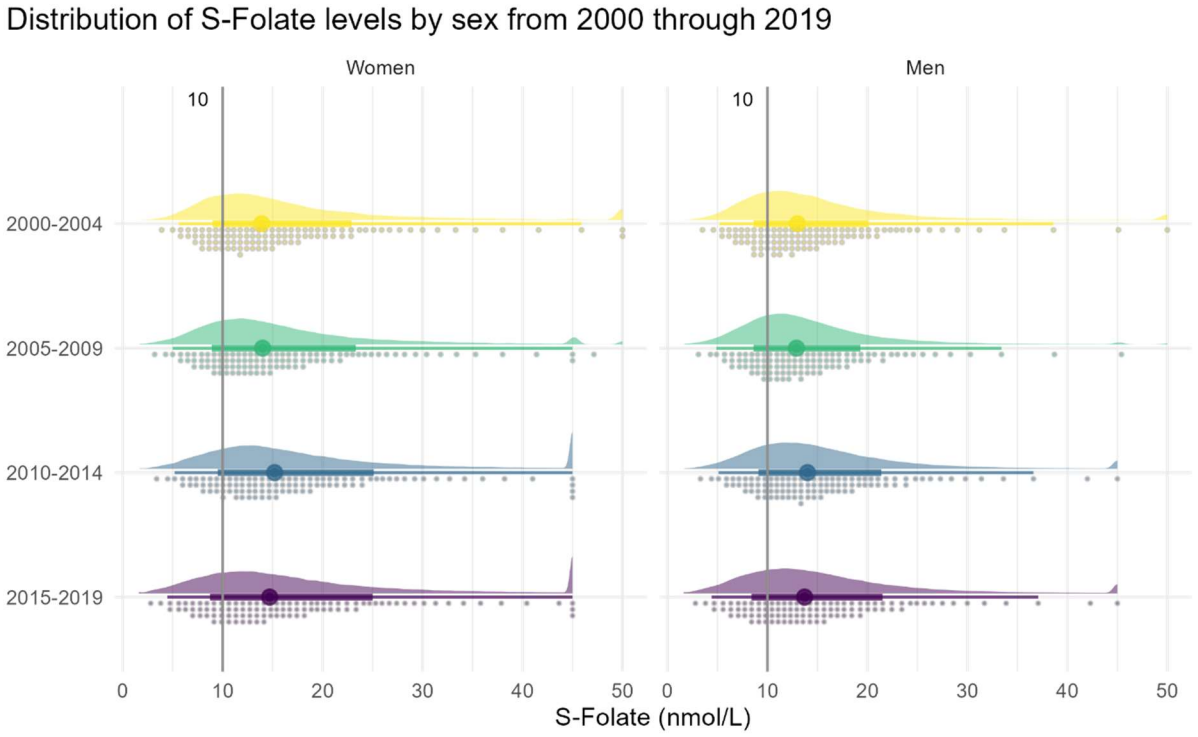


Figure 4: Raincloud plot displaying the distribution of serum folate concentrations (x-axis) in women (n_{su} = 264,699, n_{sa} = 427,969) and men (n_{su} = 215,088, s_{sa} = 323,178) from 2000 through 2019 (y-axis). The cutoff for folate deficiency (10 nmol/L) is shown as a vertical line.

4.3.1 Folate status in women

Figure 5 illustrates folate status among women of different age groups, indicating an increase in serum concentrations, most notably in the 30-39 y age group. Of all women included, 84.4% had folate concentrations below the WHO recommendation of serum folate of >25.5 nmol/L in women of childbearing age. In the youngest age group, 86.7% of the samples showed folate concentrations below the recommendation, and the same was true for 81.9% of 30-39 year old women. The prevalence of folate deficiency among women across all three age groups was 22.1%. In the youngest age group, 25.9% were deficient, whereas 20.8% in the 30-39 y age group were deficient.

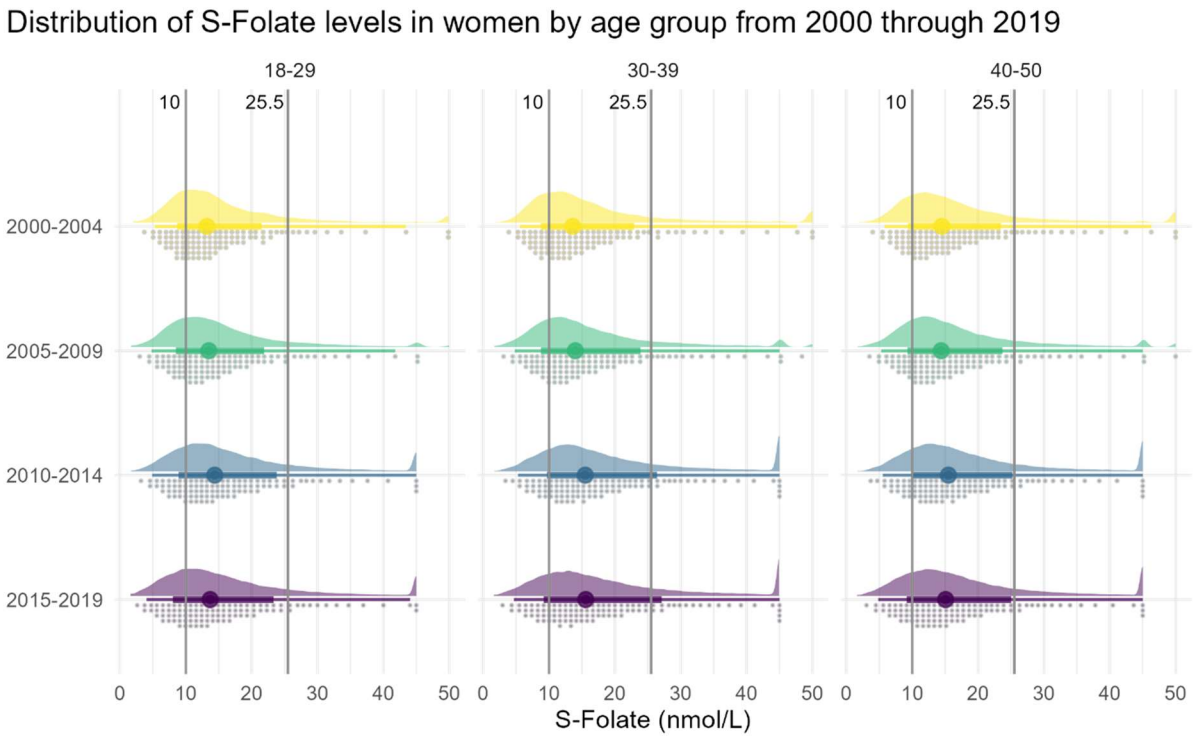


Figure 5: Raincloud plot displaying the distribution of serum folate concentrations (x-axis) in women (n_{su} = 264,699, n_{sa} = 427,969) by age from 2000 through 2019 (y-axis). The cutoff for folate deficiency (<10 nmol/L) and the recommended serum folate concentration for women of reproductive age (>25.5 nmol/L) are shown as vertical lines.

4.4 Vitamin B_{12} status

Vitamin B_{12} status improved during the 20-year period, from 83.3% of samples showing an adequate status in the 2000-2004 period, to 89.9% in 2015-2019. This shift was graphically represented by a rightward flattening of the distribution curve (**Figure 6**). Throughout this period, the proportion of samples showing deficiency was relatively stable ranging between 0.3 and 0.6%. The increase in adjusted mean from 300 pmol/L in the first 5-year-period to 326 pmol/L in the last period was not evenly distributed over time. Instead, there was a marked increase between the periods of 2010-2014 and 2015-2019.

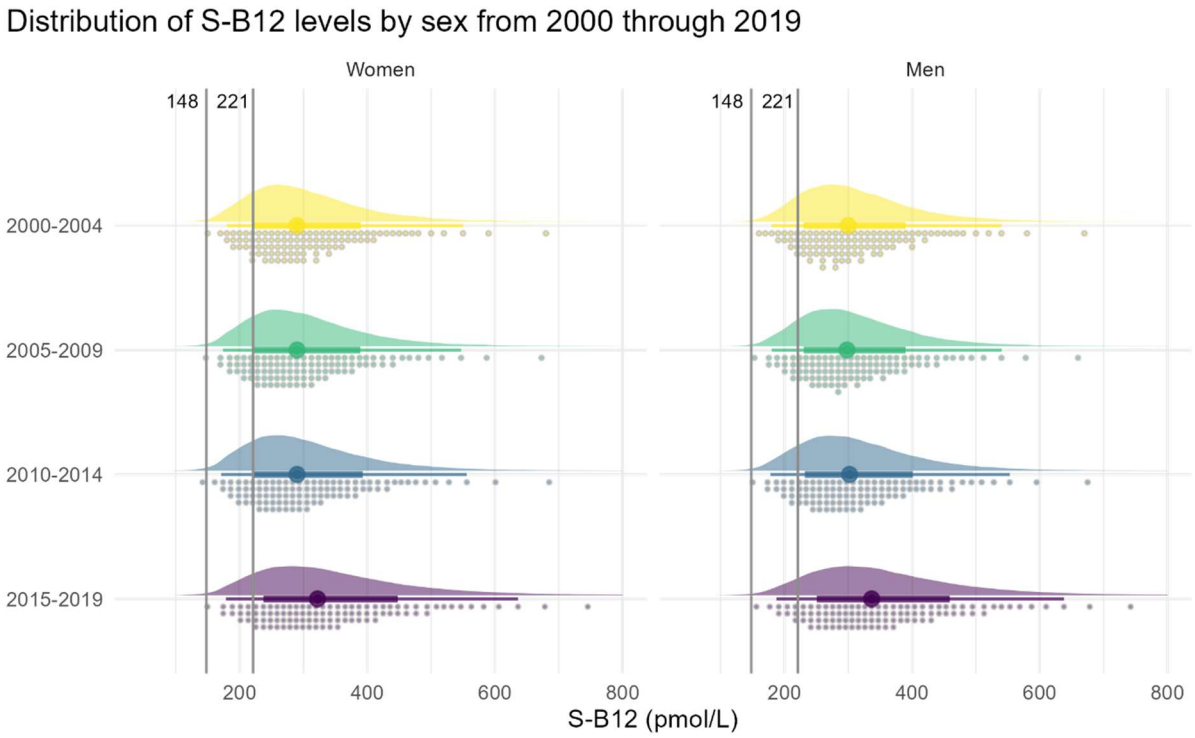


Figure 6: Raincloud plot displaying the distribution of serum vitamin B12 concentrations (x-axis) in women (n_{su} = 263,272, s_{sa} = 455,350) and men (n_{su} = 277,039, s_{sa} = 452,642) from 2000 through 2019 (y-axis). The cutoffs for low B12 status (221 pmol/L) and B12 deficiency (148 pmol/L) are shown as vertical lines.

4.5 Homocysteine status

Plasma tHcy concentrations increased substantially from the first 5-year period (2000-2004) to the next (2005-2009), with 36.6 and 54.4% of measurements showing elevated tHcy, respectively (**Figure 7**). However, in the 2010-2014 period, only 45.6% of the measurements indicated elevated tHcy, and a similar proportion was observed during the last study period. Both the geometric and the adjusted mean increased from the first to the second 5-year period, before decreasing. The adjusted mean decreased to a concentration below what was observed in the first period.

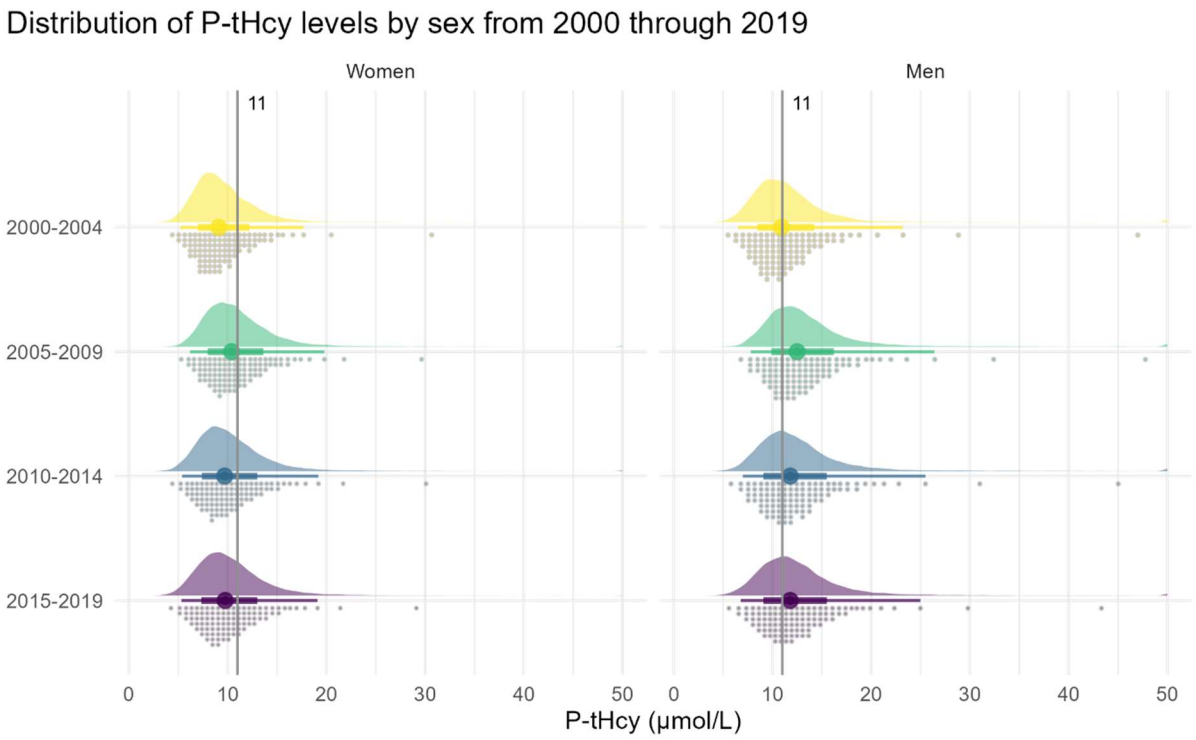


Figure 7: Raincloud plot displaying the distribution of plasma total homocysteine (tHcy) concentrations (x-axis) by women (n_{su} = 84,377, s_{sa} = 111,250) and men (n_{su} = 66,155, s_{sa} =85,162) from 2000 through 2019 (y-axis). The cutoff for elevated tHcy (11 µmol/L) is shown as a vertical line.

4.6 Methylmalonic acid status

Plasma MMA concentrations remained relatively stable over the study period, as seen in **Figure 8**. In 2004, 4.4% of the measurements indicated concentrations above 0.27 µmol/L. The proportion decreased to 5.0% in the subsequent 5-year period. In the next two periods, the prevalence of elevated MMA was 6.4%. The adjusted mean increased from 0.15 µmol/L to 0.17 µmol/L from 2004 to 2010-2014, and was 0.16 µmol/L in the last period.

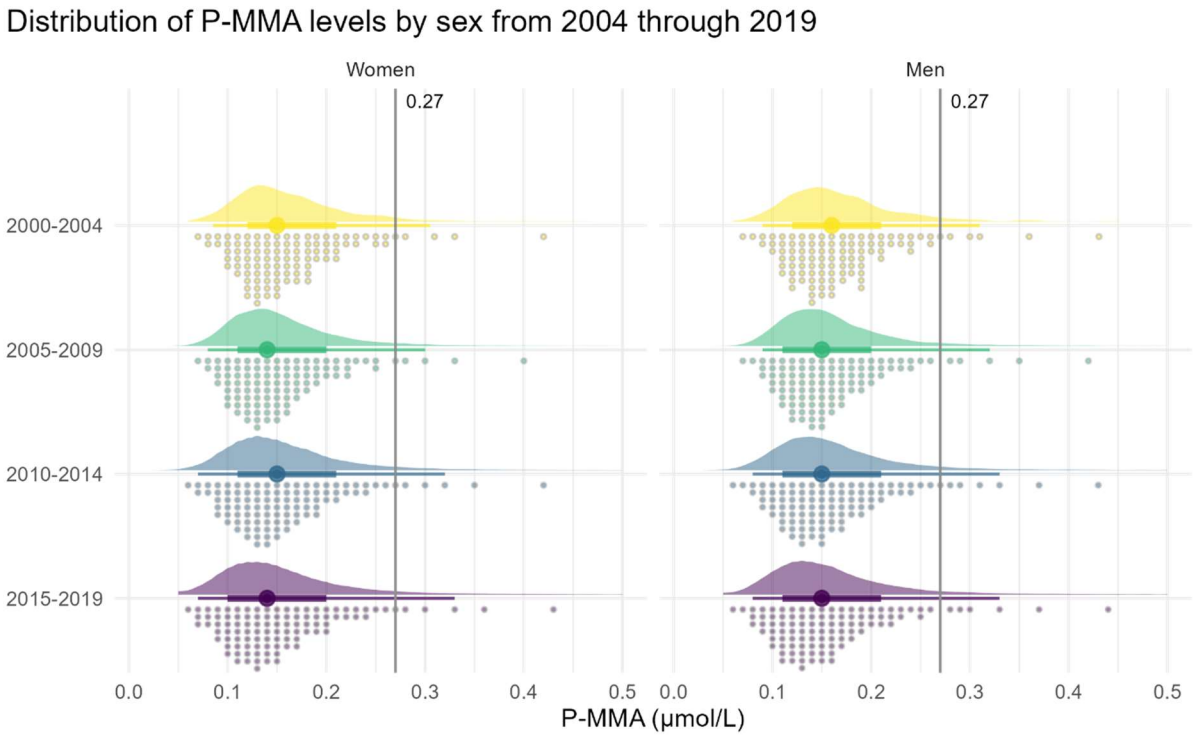


Figure 8: Raincloud plot displaying the distribution of plasma methylmalonic acid (MMA) concentrations (x-axis) by women (n_{su} = 67,844, s_{sa} = 88,389) and men (n_{su} = 50,224, s_{sa} = 63,170) from 2004 through 2019 (y-axis). The cutoff for elevated MMA (0.27 µmol/L) is shown as a vertical line.

4.7 Modelled time trends in folate, vitamin B_{12}, homocysteine and methylmalonic acid concentrations in subgroups from 2000 through 2019

The modelled time trends in percent change in metabolite concentrations for the different subgroups are summarized in **Table 10**. Both the unadjusted and the adjusted models showed significant changes in metabolite concentrations from 2000-2004 to 2015-2019. Model adjustment for eGFR, sex and age group (see Table 7 for specific models) significantly altered the time trends for all metabolites.

Notably, there was a decrease in folate concentrations among men and women, and in the youngest age group. The decrease was most pronounced in men, and was not as strong in East Norway as in the other regions. Plasma tHcy concentrations decreased overall, however there was a substantial difference between the sexes. Specifically, there was a 10.2% decrease among women, whereas men exhibited a 9.5% increase in tHcy concentrations. The strongest decrease was seen in the 30-39 y age group and in East Norway.

A positive trend in B12 concentrations was found in all subgroups, and was somewhat higher in men than in women. Plasma MMA concentrations increased in women and men, the two youngest age groups as well as in East and Mid Norway. In women, both B12 and MMA increased by 9%. In men, both B12 and MMA increased by 13%.

Table 10: Percent changes in metabolite concentrations from 2000-2004 to 2015-2019 (95% confidence interval).

	Folate	B12	tHcy	MMA[1]
All, n	(n_{su} = 479787, n_{sa} = 751,147)	(n_{su} = 540,311, n_{sa} = 907,992)	(n_{su} = 150,532, n_{sa} = 196,412)	(n_{su} = 118,068, n_{sa} = 151,559)
Unadjusted[2]	3.04 (2.53, 3.55)	11.03 (10.78, 11.28)	6.42 (5.82, 7.01)	-4.7 (-5.86, -3.52)
Adjusted[3]	-5.88 (-6.7, -5.04)	9.01 (8.6, 9.43)	-11.12 (-12.03, -10.21)	7.69 (5.19, 9.22)
By sex				
Women, n	(n_{su} = 264,699, n_{sa} = 427,696)	(n_{su} = 263,272, n_{sa} = 455,350)	(n_{su} = 84,377, n_{sa} = 111,250)	(n_{su} = 67,844, n_{sa} = 88,389)
Unadjusted[2]	3.29 (2.64, 3.95)	11.19 (10.84, 11.53)	6.24 (5.47, 7.01)	-4.37 (-5.87, -2.84)
Adjusted[4]	-5.48 (-6.39, -4.56)	9.09 (8.56, 9.62)[c]	-10.17 (-11.18, -9.14)	9.17 (7.3, 11.08)
Men, n	(n_{su} = 215,088, n_{sa} = 323,178)	(n_{su} = 277,039, n_{sa} = [45c2],642)	(n_{su} = 66,155, n_{sa} = 85,162)	(n_{su} = 50,224, n_{sa} = 63,170)
Unadjusted[2]	-5.18 (-7.61, -2.7)[c]	15.32 (13.95, 16.7)	30.09 (26.48, 33.8)[c]	-10.96 (-15.91, -5.72)[b]
Adjusted[4]	-14.96 (-17.45, -12.39)[c]	12.97 (11.4, 14.56)	9.54 (6.13, 13.06)[c]	12.92 (6.27, 19.99)[c]
By age-group				
18-29 y, n	(n_{su} = 172,637, n_{sa} = 228,399)	(n_{su} = 197,740, n_{sa} = 274,828)	(n_{su} = 46,927, n_{sa} = 55,301)	(n_{su} = 39,320, n_{sa} = 46,119)
Unadjusted[2]	0.28 (-0.72, 1.29)	14.24 (13.7, 14.79)	6.36 (5.06, 7.68)	-5.86 (-8.3, -3.36)
Adjusted[5]	-8.38 (-9.51, -7.24)	12.16 (11.48, 12.84)	-10 (-11.31, -8.68)	7.41 (4.55, 10.35)
30-39 y, n	(n_{su} = 142,733, n_{sa} = 225,021)	(n_{su} = 156,254, n_{sa} = 265,172)	(n_{su} = 45,406, n_{sa} = 58,653)	(n_{su} = 35,744, n_{sa} = 45,565)
Unadjusted[2]	10.97 (7.09, 15)[c]	15.36 (13.44, 17.32)[c]	5.96 (1.51, 10.6)[b]	-13.16 (-20.49, -5.17)
Adjusted[5]	1.06 (-2.7, 4.96)[b]	12.71 (10.69, 14.77)[c]	-14.09 (-17.74, -10.28)	11.47 (1.89, 21.96)
40-50 y, n	(n_{su} = 164,417, n_{sa} = 297,727)	(n_{su} = 186,317, n_{sa} = 367,992)	(n_{su} = 58,199, n_{sa} = 82,458)	(n_{su} = 43,004, n_{sa} = 59,875)
Unadjusted[2]	9.8 (6.08, 13.65)[c]	12.52 (10.7, 14.36)[c]	12.63 (8.12, 17.32)[c]	-4.15 (-12.85, 5.41)
Adjusted[5]	-0.82 (-4.4, 2.9)[c]	9.61 (7.7, 11.56)[c]	-12.45 (-16.01, -8.75)[c]	6.37 (-3.3, 17)

Table 10, continued.

	Folate	B12	tHcy	MMA[1]
By region				
East	(n_{su} = 328,889, n_{sa} = 530,194)	(n_{su} = 375,576, ns_a = 657,882)	(n_{su} = 106,007, n_{sa} = 139,415)	(n_{su} = 76,212, n_{sa} = 98,291)
Unadjusted[2]	5.77 (5.2, 6.33)	11.79 (11.52, 12.06)	6.17 (5.52, 6.83)	-4.94 (-6.22, -3.64)
Adjusted[6]	-4.2 (-5.08, -3.31)	8.97 (8.47, 9.47)	-11.35 (-12.3, -10.39)	7.31 (5.67, 8.96)
South	(n_{su} = 11,711, n_{sa} = 16,094)	(n_{su} = 13,537, n_{sa} = 19,180)	(n_{su} = 2,263, n_{sa} = 2,738)	(n_{su} = 1,849, n_{sa} = 2,255)
Unadjusted[2]	-11.6 (-17.99, -4.71)[b]	7.38 (3.47, 11.44)	10.1 (0.34, 20.82)	-6.25 (-77.39, 288.7)
Adjusted[6]	-19.38 (-25.51, -12.74)[c]	4.91 (0.88, 9.1)	-5.56 (-13.81, 3.48)[b]	4.99 (-74.41, 330.71)
West	(n_{su} = 51,200 n_{sa} = 70,854)	(n_{su} = 51,326, n_{sa} = 71,279)	(n_{su} = 18,485, n_{sa} = 22,519)	(n_{su} = 17,044, n_{sa} = 20,725)
Unadjusted[2]	-2.23 (-6.17, 1.88)[a]	7.93 (5.67, 10.24)[c]	7.08 (2.62, 11.73)	-0.34 (-15.98, 18.21)
Adjusted[6]	-11.7 (-15.59, -7.64)[b]	5.4 (2.98, 7.87)[c]	-9.65 (-13.6, -5.52)	-24.3 (-65.62, 66.68)
Mid	(n_{su} = 46,145, n_{sa} = 68,622)	(n_{su} = 52,950, n_{sa} = 82,243)	(n_{su} = 9,240, n_{sa} = 11,658)	(n_{su} = 9,499, n_{sa} = 11,860)
Unadjusted[2]	-5.51 (-16.27, 6.63)[c]	11.36 (4.53, 18.65)	5.86 (-15.72, 32.97)	-4.12 (-19, 13.49)
Adjusted[6]	-14.11 (-24.12, -2.78)[c]	8.73 (1.86, 16.07)	-10.31 (-27.98, 11.71)	11.41 (7.91, 15.03)
North	(n_{su} = 6,131, n_{sa} = 8,327)	(n_{su} = 6,464, n_{sa} = 8,853)	(n_{su} = 2,048, n_{sa} = 2,439	(n_{su} = 654, n_{sa} = 769)
Unadjusted[2]	-9.02 (-21.43, 5.35)[c]	8.61 (1.8, 15.87)	7.89 (-15.18, 37.23)	-6.42 (-30.33, 25.7)
Adjusted[6]	-18.45 (-29.75, -5.32)[c]	6.16 (-0.68, 13.48)	-8.86 (-27.53, 14.63)	6.2 (-20.87, 42.52)

B12 , vitamin B12; tHcy, total homocysteine; MMA, methylmalonic acid; CV, coefficient of variation; n_{su}, number of subjects; n_{sa}, number of samples.

[1]Change in MMA between 2005-2009 and 2015-2019.

[2]Unadjusted (Model 2).

[3]Adjusted for eGFR, sex and age group (Model 1).

[4]Adjusted for eGFR, and age group (Model 3).

[5]Adjusted for eGFR, and sex (Model 4).

[6]Adjusted for eGFR, sex and age group (Model 5).

[a]Significantly different from reference category, $p < 0.05$.

[b]Significantly different from reference category, $p < 0.01$.

[c]Significantly different from reference category, $p < 0.001$.

4.8 Modelled time trend in folate concentrations in women from 2000 through 2019

Overall, there was a 5.5% decrease in folate concentrations among women. A separate analysis of the trend in serum folate concentrations in women of different age groups uncovered a 9.4% and 0.8% decrease in the youngest and oldest age group from 2000-2004 to 2015-2019, respectively. Women in the 30-39 y age group experienced a 1.5% increase in folate concentrations. The time trends were significant.

4.9 Correlation of folate and B12 with functional biomarkers

The number of subjects with adequate and inadequate folate and B12 concentrations as well as functional biomarker concentrations are shown in **Table 11**. A high proportion (67.7%) of subjects with low folate status had elevated tHcy. There was a clear trend towards elevated tHcy in subjects with lower B12 concentrations. Elevated tHcy was seen in 45% of those with normal B12 status, and in 69% of those with B12 deficiency. Among those with low B12 status or deficiency, the proportion with elevated MMA was also higher (36.1%) than in those with adequate B12 status (5.2%). Nevertheless, 75.9% of the individuals with B12 deficiency did not have elevated MMA.

Table 11: B-vitamin status by vitamin and functional biomarker concentrations.

	tHcy[1]			**B12**[2]			
Folate, n (%)	**Total**	**< 11 µmol/L**	**≥ 11 µmol/L**	**Total**	**< 148 pmol/L**	**148-221 pmol/L**	**≥ 221 pmol/L**
< 10 nmol/L	24,769	8,008	16,761	146,470	1,160	24,480	120,830
	(100)	(32.3)	(67.7)	(100)	(0.8)	(16.7)	(82.5)
≥ 10 nmol/L	83,903	49,617	34,286	465,777	2,106	54,289	409,382
	(100)	(59.1)	(40.9)	(100)	(0.5)	(11.7)	(87.9)

	tHcy[3]			**MMA**[4]		
B12, n (%)	**Total**	**< 11 µmol/L**	**≥ 11 µmol/L**	**Total**	**< 0.27 µmol/L**	**≥ 0.27 µmol/L**
< 148 pmol/L	753	237	516	636	483	153
	(100)	(31.5)	(68.5)	(100)	(75.9)	(24.1)
148-221 pmol/L	15,722	6,237	9,485	12,272	10,805	1,467
	(100)	(39.7)	(60.3)	(100)	(88.0)	(12.0)
≥ 221 pmol/L	101,910	55,939	45,971	79,101	75,009	4,092
	(100)	(54.9)	(45.1)	(100)	(94.8)	(5.2)

tHcy, total homocysteine; B12, vitamin B_{12}; MMA, methylmalonic acid. [1]n_{su} = 87,992, n_{sa} = 108,672. [2]n_{su} = 408,623, n_{sa} = 612,247, [3]n_{su} = 95,347, n_{sa} = 118,385, [4]n_{su} = 74,397, n_{sa} = 92,009.

The majority of subjects had adequate B12 and folate status. However, approximately a quarter of those with adequate B12 status had simultanous low folate status. Further, 12.2% of subjects with adequate folate status had low B12 status. Only a small number of samples showed B12 deficiency. Of those with B12 deficiency, 64% had simultaneous adequate folate concentrations. Among those with simultaneous folate and B12 deficiency (n_{su} = 3,794, n_{sa} = 3,991), 76.8% had elevated tHcy.

Pearson's correlation coefficient between serum vitamin and functional biomarker concentrations are shown in **Table 12**. A negative correlation was observed between folate and tHcy concentrations as well as between folate and B12 concentrations. A weaker negative correlation was found between B12 and MMA concentrations. When tHcy and MMA measurements were combined, the negative correlation with B12 was slightly stronger than when the two measurements were considered separately.

Table 12: Partial correlation between metabolites.

	n_{su}	n_{sa}	r	p-value
Folate – tHcy	87,992	108,672	-0.34	< 0.001
Folate – B12	408,623	612,247	-0.34	< 0.001
B12 – tHcy	95,347	118,385	-0.24	< 0.001
B12 – MMA	74,397	92,009	-0.20	< 0.001
B12 – (tHcy*MMA)	51,053	61,540	-0.27	< 0.001

tHcy, total homocysteine; B12, vitamin B_{12}; MMA, methylmalonic acid; n_{su}, number of subjects; n_{sa}, number of samples; r, Pearson's correlation coefficient. Adjusted for eGFR, sex, age group and method/ instrument for the given metabolite analyses.

5 Discussion

Folate and vitamin B_{12} status were analysed in more than 675,000 Norwegian adults from 2000-2019, using serum vitamin and functional biomarker concentrations. Overall, time trend analysis revealed a decrease in serum folate concentrations, and an increase in serum B12 concentrations. Folate decreased the most in men, and correspondingly, plasma tHcy increased in men, but decreased in women. Methylmalonic acid, a marker of B12 status, increased in both women and men, despite increases in serum B12. In 12% of the samples, simultaneous low B12 concentration and adequate folate status was observed. Furthermore, the prevalence of suboptimal folate status (< 25.5 mol/L) among women of reproductive age was high in the 18-29 y and 30-39 y age groups (86.7% and 81.9%, respectively), as was the prevalence of folate deficiency (25.9% and 20.8%). The following sections will discuss methodological and statistical considerations, key findings as well as implications and future research.

5.1 Methodological considerations

5.1.1 Design

This study was of an observational nature, examining serial cross-sectional data over 20 years. The design has been termed serial cross-sectional in some papers, including a large study that monitored universal health coverage in China (88). Observational studies are unable to uncover causal relationships between variables due to the presence of confounding variables (89). However, they can provide insights into the prevalence, incidence, or trends of a health condition in a population (90). Cross-sectional studies are well-suited to provide a "snapshot" of a population at a specific point in time. A strength of the present study is that in combining cross-sectional data from several time periods, trends and associations can be observed.

5.1.2 Subjects and exclusion

The original dataset contained all individuals whose blood samples were analysed for folate, B12, tHcy, or MMA by the Fürst Medical Laboratories during 2000-2019. The study sample was narrowed down with the aim of studying a healthy population. The exclusion criteria were based on common cutoffs that indicate the potential presence of a health condition or a genetic polymorphism related to folate and B12 metabolism. The lack of confirmatory data on such conditions may have led to the wrongful exclusion of the intended subjects. However, the mean number of measurements per subject per year among the excluded was 1.44. On average, Norwegians visited their GP 2.5 times a year in 2010 (75). It is unlikely that blood samples were drawn each visit, especially to examine B vitamin status. In 2020, analyses of folate, B12, tHcy and MMA were ordered in 30%, 37%, 5% and 5% of non-hospitalized Norwegians,

respectively (64). Thus, the higher mean number of samples among the excluded indicates that the criteria were successful in excluding subjects who had their B-vitamin satus examined more frequently.

5.1.3 Generalizability of results

The results from this study may not necessarily be generalizable to all healthy Norwegian adults. Firstly, healthy adults may not visit their GP as frequently as those who have health concerns. Thus, the data may have contained a larger proportion of people with such concerns. This could particularly apply to men, who have been found to visit their GP approximately half as often as women (75). Further, some individuals who regularly visit their GP may be more concerned with staying healthy than the general population, and seek regular check-ups as well as preventative advice. This may in particular pertain to women. Secondly, tHcy and MMA are usually not measured unless there is reason to suspect folate and/or B12 deficiency or there is a need to monitor status. In the present study, tHcy analysis was ordered in 14.5% of cases where folate analysis was ordered, and MMA in 8.1% of cases where B12 analysis was ordered. Thus, results from the tHcy and MMA measurements may have overrepresented individuals with related health concerns.

Thirdly, individuals with B12 > 800 pmol/L, tHcy > 50 µmol/L, and MMA > 0.50 µmol/L were excluded from analyses. No sensitivity analysis was conducted to evaluate whether these exclusion criteria were appropriate. Indiviudals with genetic polymorphisms that result in higher concentrations may thus be underrepresented in this study. One such example is C677T mutations in the MTHFR enzyme causing elevated tHcy, and these polymorphisms are present in approximately 40-50% of northern Europeans (60). Around 10% are TT homocygotes, causing the highest tHcy concentrations. Another example is mutations in the HIBCH gene (rs291466) causing elevated MMA, especially in CC homocygotes, as described in a young, healthy Irish cohort (n = 2,208) (91). Approximately one third of these were CC homocygotes.

Fourthly, the sample was limited to individuals aged 18-50 years. The 18-49 y age group represents approximately 45% of the Norwegian population, whereas the 50-66 y age group represents around 20% of the population (92). National healthcare statistics show that around 40% of GP visits occur in the 18-50 years age group, and around 25% in the 50-66 years age group (93). Including older adults would have increased the generalizability of the results, as the 50-66 y age group constitutes a relative large proportion of the population and the influence of potential health conditions on the results would not necessarily be so large, as indicated by only slightly more frequent GP consultations.

The present study did not include information on pregnancy and lactation, both of which have been shown to affect B-vitamin concentrations. Specifically, pregnancy has been associated with decreased B12, tHcy, and MMA concentrations, whereas lactation has been associated with increased B12 and MMA concentrations (2). Additionally, lactation has been associated with potential lower folate status, as the concentration of folate in human milk is kept relatively constant even when maternal folate status is on the lower end (94). Depending on the proportion of pregnant and lactating women represented in the study population, metabolite concentrations among female participants may have been under- or overestimated.

Although the study population may not accurately have covered the full variability in healthy Norwegian adults, the sample size indicated that some degree of variation remained in the sample after the exclusion. With 675,189 included subjects, the present study covered a relatively large proportion of the Norwegian population (4.48 M and 5.33 M in 2000 and 2019, respectively) (95). Thus, the results provide a comprehensive overview of the recent trends in folate and B12 status and can be generalized to most healthy Norwegian adults aged 18-50 years, albeit in a population setting.

5.1.4 Biomarker analysis

During the study period, several changes in instruments and methods occurred in the Fürst Medical Laboratory (outlined in Table 6). Correlation of measurements within each instrument or method were controlled for in the regression analyses. However, the correlations between different instruments or methods were not controlled for, which may have affected results. Folate and B12 concentrations were measured by ICL throughout the period.

Creatinine was measured using colorimetric assay until 2009 followed by kinetic-colorimetric assay. From the first 5-year period to the last, serum creatinine dropped drastically, from 86.0 to 69.6 µmol/L. A study from 2016 using data from the Nord-Trøndelag Helath Study (HUNT) from 1995-1997 and 2006-2008 found no large difference in the prevalence of chronic kidney disease, however pointed out that better blood pressure control may have improved kidney function in recent years (96). If the large decrease in creatinine, corresponding to increased eGFR, would have affected the present results, it is likely that improved tHcy status would have been seen. It is possible that controlling for eGFR in the adjusted models mitigated this effect.

In 2003, the method of analysing tHcy changed from competing FPIA to ICL, which uses a different detection method (97). The performance of different tHcy assays are generally considered comparable, although ICL is slightly more imprecise than FPIA (98). The CV for

tHcy measured by the Siemens Advia Centaur instrument (6.99%) was higher than the desired threshold of 5% (99), but for a high throughput setting such as at Fürst, the instrument was suitable. Any significant influence of the potentially higher variation in ICL measurements on the results is unlikely due to the large number of samples measured in the later 5-year periods.

Three method changes occurred for the MMA analysis. Studies have found a strong correlation between GC-MS and LC-MS/MS (100), and low inter-assay variability (<10%) between LC-MS/MS and UPLC-QqQ-MS/MS (101). In the present study, MMA concentrations were very similar across the study period in spite of the CV increasing from 0.27% to 8.80% between the two latter methods. There was an increase in the total amount of measurements taken, with a 6-fold increase for B12 and 30-fold increase for MMA from the first to the last 5-year interval. Although the 2004 MMA measurements were excluded from the regression analysis, the overall results may have been distorted by the variation caused by this uneven distribution of samples.

The samples were measured in different Fürst laboratories across Norway, which was not controlled for. Nevertheless, the majority of measurements were conducted in Eastern Norway, corresponding well with the population dispersion. Data on lot changes for instrument calibration were only available from 2015, so adjustement was not possible. This may have influenced results. However, overall, the biomarker analyses were conducted with appropriate methods and the CVs were generally low, indicating high precision of the chemical assays.

5.1.5 Statistical considerations

The data material provided from Fürst allowed for statistical adjustment for eGFR, method and/or instrument changes, sex, and age group. Controlling for renal function, a key determinant of the analytes, and for correlation within instruments or methods, were strengths of the present study. The statistical adjustment changed the time trends to some degree in all metabolites, with the greatest changes observed for folate and tHcy concentrations. Adjustment led to a downward shift in all trends except for MMA, where the opposite was seen. A large difference was seen in tHcy trends when introducing sex as an interaction term and controlling for age group, uncovering a decreasing trend in women and an increasing trend in men. Before adjustment, regression models of MMA showed decreasing concentrations. Adjustement led to increasing concentration in all subgroups except West Norway, however here, the trend was not significant. It is unclear what caused the substantial changes observed when adjusting for covariates, but the inclusion of variables such as instrument changes and eGFR into the models resulted in shifts in trends. There may have been other confounding variables present that were not controlled for in the analysis.

5.2 Discussion of results

5.2.1 Trends in serum folate concentrations

An overall decrease in folate status was observed, with the decrease being most pronounced in men and in the 18-29 years age group. Data from the US National Health and Nutrition Examination Survey (NHANES) in the post-fortification years 1999-2010 (n = 46,873) showed a 17% decrease in overall serum folate concentrations (102). The prevalence of serum folate <10 nmol/L was less than or equal to 1% post-fortification, as compared to 24% prior to fortification (n = 23,359). Our data indicated a similar prevalence of folate deficiency to that observed in the US pre-fortification era with 24.5% in 2000-2004 and 24.6% in 2015-2019. More recent NHANES data showed lower folate concentrations in 2017-2018 (35.0 nmol/L) than in 2007-2016 (38.0 nmol/L) (8), which is consistent with the reduced serum folate concentration observed in Norwegian adults from 2000-2004 to 2015-2019.

Newly published data from a nationally representative sample in the UK showed a decline in folate status from 2008-2015 (103). Among 19-64 year olds, geometric mean (2.5^{th}, 97.5^{th} percentile) serum folate was reduced from 17.6 (6-8, 48.8) nmol/L to 14.0 (5.2, 57.3) nmol/L. Men experienced a stronger decline (4 percentage points) than women (3.1 percentage points). This is consistent with the reduction seen in Norwegian adults, from adjusted mean (CI) 14.2 (13.2, 15.3) nmol/L in 2005-2009 to 13.2 (12.3, 14.2) nmol/L in 2015-2019. The UK data showed an increase in both the prevalence of low folate status (<13 nmol/L) from 29% to 52%, as well as folate deficiency (<7 nmol/L) from 3% to 11%. The increase was most pronounced in men, which corresponds well with the strong decline in folate status in Norwegian men.

The high prevalence of folate deficiency across the study period, around 25%, and the discrepancy between the sexes, may be related to the indications for measuring folate. Folate is genereally not measured unless the patient reports symptoms possibly indicating anaemia, such as fatigue, or is planning pregnancy. Thus, there may be an overrepresentation of individuals with low folate status in the study sample as compared to the general population. This effect may be stronger in men, who seek medical attention less frequently. On the other hand, the study sample may overrepresent women who are health conscious or planning pregnancy.

The trends in folate status did not align with the estimated folate intake reported in a study in 2022 on the development of the Norwegian diet (104). Looking at main folate sources, there was an estimated 68% increase in dry legume consumption and 12% increase in fresh legume consumption, as well as a 46% increase in the consumption of nuts. Overall, there was a 34% increase in vegetable consumption, while fruits and berry consumption increased by 24%. In

contrast, the consumption of broccoli and cabbage, belonging to the folate-rich dark green leafy vegetables, decreased by 1%. From 1999 to 2009, there was a 57% increase in the consumption of imported processed fruits, including orange juice concentrate which is an important folate source, but a 10% decrease was seen from 2009 to 2019.

Although folate intake seems to have increased based on engross data, several factors may explain why serum folate does not reflect this change. The path from food production and import to consumption involves food loss, processing and cooking. Notably, approximately 50% of folate content can be lost during cooking, especially in green vegetables cooked for a prolonged time (105). Between 2005 and 2013, ultra-processed food products accounted for 58.8% of food purchases in Norway, although a slight reduction was seen over this time period (106). These factors related to food management and preparation could potentially impact the folate content in the foods consumed, and thus serum folate concentrations.

Further, engross data estimated a 7.5 % decrease in total energy intake per person per day, from 12.0 MJ to 11.1 MJ over the 20-year period (104). The estimated daily energy intake from the 2011 Norkost 3 study, which used 24h recalls, was 9.4 MJ (10.9 MJ for men, 8.0 MJ for women) (71). This highlights both the discrepancy between engross and intake data and the fact that an overall reduction in food intake over the 20-year period cannot be ruled out. The Norkost 3 study also found that 58% of women and 47% of men used dietary supplements, and that the inclusion of supplements resulted in a small increase in estimated folate intake.

5.2.2 Trends in plasma total homocysteine concentrations

At the same time as serum folate concentrations decreased, tHcy concentrations increased in men (9.5%), but decreased in women (-10.2%). The decrease was most pronounced in the 30-39 y age group (-14.1%). Geometric mean plasma concentration in 2000-2004 was 10.1 µmol/L, which is comparable to the geometric means found in men and women (10.8 and 9.1 µmol/L, respectively), in a paper from the Hordaland Health study in 1998 (n = 11,941) (69). The adjusted mean tHcy concentration in the current study was higher (11.3 (10, 12.8) µmol/L). A study in the US NHANES cohort in 2008 compared tHcy concentrations between the two periods 1991-1994 (n = 7,781) and 1999-2004 (n = 17,891) and found a decrease in women and men of all age groups, as expected due to the introduction of folic acid fortified products (107). From 1991-1994 to 2003-2004, median tHcy concentration dropped from 8.11 (7.90–8.36) µmol/L to 7.88 (7.67–8.07) µmol/L, representing a 2.8% decrease. Mean tHcy concentration in a Danish twin study (n = 1,206) from 2007 ranged from 6.9 µmol/L in women under 30 years to 8.7 µmol/L in men aged 30 years or older (108).

The NHANES study applied equations to adjust for the different methods used in two periods (107). However, in the current study, we were unable to apply these equations due to the use of different methods in NHANES, except for one case, specifically the Abbott IMx immunoassay kit, which was used by Fürst between 2000 and 2003. Measurements on the Abbott AxSYM used in NHANES, to which all other measurements in that study were adjusted, were 6% higher than the measurements obtained using the Abbott IMx instrument. As a result, measurements of tHcy concentrations could be lower in the Norwegian sample than in the US sample during the 2000-2004 period.

On the other hand, the C677T polymorphism of the MTHFR gene may have contributed to the higher concentrations observed in Norway compared to the US. The CT and CC variants are present in approximately 40% and 50% of Northern Europeans, respectively, whereas around 10% have the rare TT variant (60). The polymorphisms lead to a shift in folate distribution in disfavour of 5-methyl THF, thus plasma tHcy is raised (109). Other essential nutrients, such as vitamin B_2 (B2), vitamin B_6 (B6) and choline, also influence tHcy status (110-112). As of November 2023, there are no studies available on B2, B6 or choline status allowing evaluation of their potential influence on tHcy status. Unfortunately, there is a lack of recent data to compare the trend in tHcy concentrations observed in our study, both within Norway and internationally. However, this study revealed a clear decrease in tHcy status in women and an increase in men.

5.2.3 Trends in serum vitamin B_{12} concentrations

Plasma B12 concentrations increased in all subgroups over the 20-year period. The trend was similar across all groups, but a slightly steeper increase was observed among men and in the 30-39 y age group. Recent NHANES data from 2007-2018 (n = 9,297) showed increasing B12 concentrations with age (8). However, when considering individuals who did not use supplements, the highest B12 concentrations were found in the 19-39 y age group, which corresponds well with the present findings. The prevalence of deficiency in the NHANES cohort, defined as serum B12 < 148 pmol/L, was 3.6%, and the adjusted geometric mean serum B12 concentration was 390 (384, 396) pmol/L. In the present study, adjusted mean serum B12 was lower, but increased from 300 pmol/L to 326 pmoL/L between 2000-2004 and 2015-2019.

The NHANES data from 1999-2004 showed a deficiency prevalence of 2.9% using the same cutoff, serum B12 <148 pmol/L, with a higher prevalence in women (86). Thus, the prevalence of deficiency has increased in the US. The current study found a slight increase in the prevalence of deficiency from 2000-2004 (0.34%) to 2015-2019 (0.41%), however, the proportion of low

B12 status (148-221 pmol/L) was reduced from 16% to 10%. The largest increase in B12 concentration was observed in the 40-50 years age group. There is debate regarding the cutoffs used to define B12 deficiency, and this is discussed in *section 5.2.8*.

When comparing trends in B12 status with national dietary intake data, our results are in accordance with the consumption of B12-rich foods (104). Over the past two decades, estimated meat consumption, including meat products and cross-border shopping, increased by 17%, while egg consumption increased by 25%. Total milk consumption decreased by 63% over same time period, but yoghurt consumption increased by 43%, cream and sour cream by 7%, and cheese by 26%. These animal products constitute the main dietary sources of B12 in a standard Western diet and the increased consumption may explain the rise in B12 concentration.

5.2.4 Trends in plasma methylmalonic acid concentrations

Plasma MMA increased in both men and women, and across all age groups in the present study. The overall prevalence of elevated MMA increased from 4.4% to 6.4%. Similarly, a prevalence of 5.9% was found in the NHANES data from 1999-2004 (86). The adjusted geometric mean MMA plasma concentration in the NHANES population from 2007-2018 was 0.150 (0.147-0.153) µmol/L (8). In this study, only a slightly higher range of 0.15-0.17 µmol/L was observed across the 20-year period. In the NHANES data from 2007-2018, MMA status was slightly higher in men (152 µmol/L) than in women (149 µmol/L). In the present study, men experienced a stronger increase in MMA concentrations, as did the 30-39 y age group.

The increase in MMA concentrations was not supported by the observed increase in B12 concentrations. It is possible that this was observed because of the young sample studied. In a study comparing B12 and MMA status in middle-aged and elderly subjects (n = 6,946), MMA increased in the elderly when B12 concentrations dropped below 400 pmol/L (113). Thus, MMA has been deemed more sensitive to low B12 concentrations in the elderly compared to younger adults. Further, common genetic variations such as in the HIBCH gene can alter MMA concentrations to an extent that would influence results (91). It is possible that MMA samples from 2015-2019 included more subjects with such polymorphisms than in 2005-2009. The MMA measurements in the last period also had the highest CV (8.8%) of all analyses. The utility of MMA as a marker of B12 status is further discussed in *section 5.2.8*.

5.2.5 Regional differences in the trends in folate and vitamin B12 status

Regional differences were observed in the reduction in folate status, with the most pronounced decreases occurring in the South (-19.4%) and North (-18.5%). The NORKOST 3 study found a significantly lower intake of folate among women in the South-East and North compared to

Oslo (71). The same was found in men, however the difference was not significant. Apart from a removal of fruit price subsidies in Northern Norway in 2002, it is unclear why this is the case. The less dramatic reduction in folate status in the East (-4.2%) may be linked to a shift towards more minimally processed foods in the capital city Oslo (106). An increase in tHcy was seen in all regions, supporting the decreasing trend in folate, but to a lesser degree in the South.

This study found a lower increase in B12 in the West, but the NORKOST 3 study revealed no significant regional differences in B12 intake in 2010-2011 (71). However, in 2020, the use of prescribed B12 medication was markedly higher in the West (64). Analyses of tHcy and MMA were also ordered more frequently in the West than in other regions. It is possible that the change observed in the West was not as large because local physicians were more concerned with B-vitamin status throughout the study period due to the Hordaland Homocysteine Study that started in 1992 (114).

There were no significant differences in MMA trends across the regions. There were fewer MMA samples than for the other analytes, especially from the North. Fewer samples originated from the Northern region across all analytes. No statistics on GP consultations by region were available that could help explain the differences in the trends observed. However, a 2020 report pointed out that there are regional differences in GP access due to the country's challenging geography with long distances between townships, especially in Northern Norway. This region has a smaller population, and the long distances may result in less frequent GP visits. Further, those who visit their GP may be in a worse health condition as the threshold for seeking medical attention is higher (115). This is supported by a 2020 study which found that fewest B-vitamin related analyses were ordered in Northern Norway compared to the other regions (64).

5.2.6 Combined folate and vitamin B_{12} status

There was a negative correlation between folate and B12 concentration. This may point to the fact that among those with B12 deficiency and low B12 status, there was a higher proportion of subjects with adequate folate status (64% and 69%, respectively) than folate deficiency. Nevertheless, there were more subjects with low B12 status among those with folate deficiency. Among those with adequate folate status, 11.7% had low B12 status and 0.5% had deficiency. Thus, even a non-folic acid fortified sample, the prevalence of simultaneous low B12 status and adequate folate status was over 12%. Unmetabolized folic acid was not studied, but the concentrations thereof were likely low in this non-repleted population.

5.2.7 The cutoffs for folate and vitamin B_{12} deficiency may be too low

There was a negative correlation between folate and tHcy (Pearson's r = -0.34) and 67.7% of those with folate deficiency had elevated tHcy. In those with adequate folate status, 41% had elevated tHcy. There was also a negative correlation between B12 and tHcy (Pearson's r = -0.24), and among those with simultaenous folate and B12 deficiency, 76.8% had elevated tHcy. The overall prevalence of B12 deficiency was low (around 0.5%), yet still, 46% had elevated tHcy in 2015-2019. This could be an indication of inappropriate cutoffs for folate and B12.

For folate deficiency, Fürst uses a cutoff of 5.7 nmol/L and WHO has also recommended 6.8 nmol/L (61). If these cutoffs were used, the prevalence of folate deficiency would have been lower. Nevertheless, the WHO recommendation is based on when tHcy increases. For women of childbearing age, WHO recommends the higher concentration of 25.5 nmol/L to prevent NTDs. Cutoffs for B12 deficiency are also debated, especially since serum B12 responds later to decreasing B12 status than holoTC (116). The NNR 2023 have doubled the RIs for B12 partially based on holoTC measurements (1), and a following adjustment of serum B12 cutoffs may be due. There is no consencus on what cutoff should be used for folate or B12 (62, 63), however the prevalence of deficiency may be larger than indicated by the present results.

5.2.8 Methylmalonic acid and alternative markers of vitamin B_{12} status

The negative correlation between B12 and MMA concentrations was expected, but not particularly strong (Pearson's r = -0.20). Despite the negative correlation, time trends revealed increased B12 and MMA concentrations across subgroups, and 76% of those with B12 deficiency had normal MMA concentrations. A contributing factor may be that, as previously noted, MMA has been deemed a better marker in the elderly than in younger adults, as were studied in the present study (113). Further, HoloTC has been deemed a more sensitive marker of B12 status as it responds quicker to decreasing B12 concentrations (116, 117).

A recent study on a large, mixed population ($n_{subjects}$ = 9,464, $n_{samples}$ = 11,833), found that holoTC performed better than serum B12 and MMA (118). In women <50 years and in men, however, neither holoTC or MMA performed better than serum B12 for the detection of B12 deficiency and subclinical deficiency. This was determined by using the combined marker cB_{12} proposed by Fedosov (68). In the NHANES 1999-2004 sample (n = 12,335), fewer were classified with B12 deficiency when using cB_{12} compared to individual markers (119). Which marker of B12 status is best suited is still unsure, but age seems to be an important factor regarding what biomarker should be used, and evaluating a combination of markers is likely better than single markers (63, 117, 118).

5.3 Future perspectives and applications

5.3.1 Homocysteine and disease risk

Increased plasma tHcy has been associated with over 100 diseases and conditions, most are diseases of the cardiovascular and central nervous system (85). Furthermore, elevated tHcy is associated with increased all-cause mortality. These associations have also been established in the Norwegian population (114). Lowering plasma tHcy through B-vitamin supplementation has been shown to prevent or partially prevent five diseases: NTDs, impaired childhood cognition, cognitive impairment in the elderly, macular degeneration and primary stroke. Researchers have suggested viewing tHcy not only as a biomarker, but also as a guide for the prevention of disease, stating that concentrations over 11 µmol/L may signify the need for intervention (85). The close associations between tHcy and a vast number of health conditions indicate that folate and B12 may play significant roles in the development of many other diseases than merely the classical deficiency diseases.

5.3.2 B-vitamin status and cognitive decline in the elderly

Future research should focus on examining B vitamin status in the Norwegian population aged 50 years and above. With the increasing proportion of elderly, sometimes termed the "silver tsunami", vast healthcare resources are required to meet their needs sustainably. As cognitive decline and Alzheimer's disease affect many individuals and require extensive resources, preventing the development of these conditions can have substantial societal benefits.

Recent research has given strong indications that tHcy lowering through high dose B-vitamin supplementation may reduce brain atrophy in elderly individuals with cognitive decline and Alzheimer's disease (120). Knowing the B-vitamin status of grown adults and the elderly can play a crucial role in understanding and preventing cognitive decline, especially if coupled with national disease and mortality registries. Such registries are readily available in Norway, and their use should be considered by health authorities and researching institutions when funding research for sustainable development.

5.3.3 Should Norway introduce mandatory folic acid fortification?

There is ongoing debate on whether to introduce mandatory folic acid fortification in Norway to ensure healthy folate status, especially in women of reproductive age. Countries such as USA and Canada have experienced success with fortification programmes, resulting in a substantial reduction in the number of children born with NTDs (14). In 2021, the UK announced the introduction of mandatory folic acid fortification in non-whole wheat flour (121).

A study on folate supplement use and birth defects in Norway found an increase in reported supplement use before and during pregnancy between 1999 and 2013, from 9% to 27% (122). During pregnancy only, reported supplement use increased from 19.5% to 48.6%. Nevertheless, the present study found a 9.4% decrease in folate concentrations in women aged 18-29 years. In the UK, researchers found a 3 percentage point reduction in serum folate per year in women aged 19-49 (n = 1,391) from 2008 to 2019 (103). The purpose of the UK study was to examine folate status prior to fortification. The folate concentrations seen in the Norwegian population today, in women and men, are comparable to those seen in the US and the UK pre-fortification.

There are indications that high folic acid intake may promote tumour growth in patients with a pre-existing tumour, mask B12 deficiency, and be detrimental to cognitive health in the elderly (30). Thus, researchers have raised the question of whether supplementing with natural folate forms is preferable, such as 5-methyl THF, as these may not have the same health risks as folic acid (123). Further discussion about the potential role of natural folate in our diets may be due.

The current situation in Norway is concerning, with approximately 83% of women of childbearing age having serum folate below the 25.5 nmol/L threshold that WHO considers safe to prevent NTDs. The NNR 2023 recommends a more plant based diet rich in legumes, one of the main dietary sources of folate (1). This presents an opportunity to promote folate intake among Norwegian women, and men, through consumption of legumes and adherence to the new dietary guidelines. Supplementation of 400 µg before and during pregnancy, as well as while lactating, is still necessary. However, there may be a potential to increase overall serum folate concentrations through the adoption of healthy and environmentally sustainable diets. This could potentially reduce the risk of NTDs while also avoiding consequences of high folic acid intake.

Nevertheless, the risk of missing clinical signs of B12 deficiency before symptoms are overt must be taken into consideration. The results of the present study showed that even in a non-folic acid fortified population, there was a high proportion of subjects with adequate folate status among those with B12 deficiency and with low B12 status. If folic acid fortification was to be introduced, the risk of missing diagnostic signs would be exacerbated. Future research should evaluate the possible benefit of public health interventions to increase folate status, and what type of intervention would be most feasible and effective.

5.3.4 Vitamin B_{12} status in individuals consuming a plant-based diet

A 2022 study on folate and B12 status in Norwegian vegans and vegetarians found adequate status in both groups when supplements were taken into account (74). Vegans were more diligent with supplement use, and the proportion with B12 deficiency was higher in vegetarians. The number of individuals consuming less animal products, the major source of B12 in the diet, is increasing. Notably, in present times, this does not only pertain to vegans or vegetarians, but to a relatively large proportion of the population. Hence, B12 status in the population may fall over the next years. No such trend was observed in this study.

However, firstly, the recommendations to eat predominantly plant based are fairly new. Secondly, the recommended intake of B12 has approximately doubled (1). Thirdly, there is much debate regarding the cutoffs for defining B12 deficiency and low B12 status, the concern being that these are set too low. Consequently, the proportion of individuals with B12 deficiency may rise. Individuals consuming more plant-based diets must be informed about the importance of B12 supplementation, and health professionals must be aware of the issue (18). With the correct information, individuals can be supported to eat in alignment with personal ethical beliefs as well as more healthily and sustainably, without risking severe B12 deficiency.

5.3.5 Future research

Future research on folate and B12 status should:

- Clarify which biomarker, or combination of biomarkers, should be used to determine B12 status in the population as a whole and in different subgroups.
- Map out possible interventions to increase folate status among women of reproductive age and assess both benefits and risks thereof.
- Examine the consequences of subclinical folate and B12 deficiency. This may especially be of interest in men, whose folate status was poor and declining, and who receive less attention in folate research.
- Investigate the intake and status of B2, B6 and choline in the Norwegian population to allow for a better understanding of the roles of these nutrients. Such data would also help evaluating the tHcy concentrations observed.
- Combine vitamin status with dietary intake, including intake from supplements. Studying supplement use may be increasingly important to accurately monitor B12 intake as the Norwegian population consumes less animal products.
- Investigate B-vitamin status in older adult and elderly Norwegians, preferably in combination with national health registries.

6 Conclusion

Secular trends in folate and vitamin B_{12} status in healthy Norwegian adults aged 18-50 years from 2000 through 2019 were studied. Data from over a million samples from over half a million subjects revealed a clear decrease in serum folate and increase in serum B12 concentrations between 2000-2004 and 2015-2019. Folate concentrations decreased most in men, who experienced a corresponding increase in plasma tHcy. This trend aligned with the negative correlation observed between folate and tHcy. In women, folate concentrations decreased less than in men, and tHcy decreased. Across all groups, B12 concentrations increased, and despite a negative correlation observed between the two biomarkers, so did MMA concentrations.

Among women of reproductive age, over 80% had suboptimal folate status according to the WHO recommendation of serum folate > 25 nmol/L, and over 20% were folate deficient (<10 nmol/L). Both the prevalence of deficiency and the trend in folate concentrations were worst among 18-29 year old women. Regional differences were observed, with folate status decreasing less in the East compared to the other regions. Folate status decreased the most, almost 20%, in the South and North. In the West, B12 status increased less than in the other regions. When examining combined B-vitamin status, simultaneous adequate folate status and inadequate B12 status was seen in 12.3% of the samples. However, there are uncertainties regarding what cutoffs should be used to define folate and B12 deficiency. If these are indeed set too low, folate and B12 status may be poorer than indicated in the present study.

Overall, the trends observed in folate status were concerning, particularily in women of reproductive age. The folate concentrations were comparable to those seen in the US and the UK in their respective pre-fortification eras. Elevated tHcy was also seen in almost half of all subjects. The results warrant further investigation into the clinical consequenses of poor folate status, as well as the consideration of possible intervention strategies to improve status. Although B12 status was generally satisfactory, healthcare authorities should ensure that the Norwegian population is informed about the need for supplementation when reducing the consumption of animal products, as has been recommended. Future research should hone in on appropriate biomarkers and cutoffs to define deficiency.

7 References

1. Blomhoff R, Andersen R, Arnesen EK, Christensen JJ, Eneroth H, Erkkola M, et al. Nordic Nutrition Recommendations 2023 : Integrating Environmental Aspects. Copenhagen: Nordisk Ministerråd; 2023 2023. 388 p.
2. Green R, Allen LH, Bjørke-Monsen AL, Brito A, Guéant JL, Miller JW, et al. Vitamin B(12) deficiency. Nat Rev Dis Primers. 2017;3:17040.
3. Stadig flere velger plantebasert [press release]. Orkla 2023.
4. Saunders CM, Rehbinder EM, Carlsen KCL, Gudbrandsgard M, Carlsen KH, Haugen G, et al. Food and nutrient intake and adherence to dietary recommendations during pregnancy: a Nordic mother-child population-based cohort. Food Nutr Res. 2019;63.
5. Carmel R. Chapter 27 Cobalamin (Vitamin B12). In: Ross AC, editor. Modern Nutrition in Health and Disease: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2014.
6. Stover P. Chapter 26 Folic acid. In: Ross AC, editor. Modern Nutrition in Health and Disease: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2014.
7. Norwegian Food Composition Database [Internet]. 2022 [cited 04.25.2023]. Available from: www.matvaretabellen.no.
8. Zhou Y, Wang A, Yeung LF, Qi YP, Pfeiffer CM, Crider KS. Folate and vitamin B12 usual intake and biomarker status by intake source in United States adults aged ≥19 y: NHANES 2007-2018. Am J Clin Nutr. 2023.
9. Additives EPo, Products or Substances used in Animal F. Scientific Opinion on the use of cobalt compounds as additives in animal nutrition. EFSA Journal. 2009;7(12):1383.
10. Watanabe F, Yabuta Y, Tanioka Y, Bito T. Biologically active vitamin B12 compounds in foods for preventing deficiency among vegetarians and elderly subjects. J Agric Food Chem. 2013;61(28):6769-75.
11. Kostråd for å fremme folkehelsen og forebygge kroniske sykdommer : metodologi og vitenskapelig kunnskapsgrunnlag. Oslo: Helsedirektoratet; 2011.
12. Nordic nutrition recommendations 2012 : integrating nutrition and physical activity. 5th ed. ed. Copenhagen: Nordic Council of Ministers; 2014.
13. Czeizel AE, Dudás I, Vereczkey A, Bánhidy F. Folate deficiency and folic acid supplementation: the prevention of neural-tube defects and congenital heart defects. Nutrients. 2013;5(11):4760-75.
14. Staff AC, Løken EB, Holven K, Sygnestveit K, Vollset SE, Smeland S. Effects of public initiatives aimed at reducing neural tube defects with folic acid supplementation. Tidsskr Nor Laegeforen. 2005;125(4):435-7.
15. Ahuja JKC, Casavale KO, Li Y, Hopperton KE, Chakrabarti S, Hines EP, et al. Perspective: Human Milk Composition and Related Data for National Health and Nutrition Monitoring and Related Research. Adv Nutr. 2022;13(6):2098-114.
16. Bailey LB, Stover PJ, McNulty H, Fenech MF, Gregory JF, 3rd, Mills JL, et al. Biomarkers of Nutrition for Development-Folate Review. J Nutr. 2015;145(7):1636s-80s.

17. EFSA Panel on Dietetic Products N, Allergies. Scientific Opinion on Dietary Reference Values for cobalamin (vitamin B12). EFSA Journal. 2015;13(7):4150.

18. Niklewicz A, Smith AD, Smith A, Holzer A, Klein A, McCaddon A, et al. The importance of vitamin B(12) for individuals choosing plant-based diets. Eur J Nutr. 2023;62(3):1551-9.

19. Fox JT, Stover PJ. Folate-mediated one-carbon metabolism. Vitam Horm. 2008;79:1-44.

20. Blom HJ, Shaw GM, den Heijer M, Finnell RH. Neural tube defects and folate: case far from closed. Nat Rev Neurosci. 2006;7(9):724-31.

21. McNulty H, Pentieva K. Folate bioavailability. Proc Nutr Soc. 2004;63(4):529-36.

22. Zhao R, Matherly LH, Goldman ID. Membrane transporters and folate homeostasis: intestinal absorption and transport into systemic compartments and tissues. Expert Rev Mol Med. 2009;11:e4.

23. Zhao R, Goldman ID. The proton-coupled folate transporter: physiological and pharmacological roles. Curr Opin Pharmacol. 2013;13(6):875-80.

24. Lyon P, Strippoli V, Fang B, Cimmino L. B Vitamins and One-Carbon Metabolism: Implications in Human Health and Disease. Nutrients. 2020;12(9).

25. Lin Y, Dueker SR, Follett JR, Fadel JG, Arjomand A, Schneider PD, et al. Quantitation of in vivo human folate metabolism. Am J Clin Nutr. 2004;80(3):680-91.

26. Beck WS. Diagnosis of megaloblastic anemia. Annu Rev Med. 1991;42:311-22.

27. Devalia V, Hamilton MS, Molloy AM. Guidelines for the diagnosis and treatment of cobalamin and folate disorders. Br J Haematol. 2014;166(4):496-513.

28. De-Regil LM, Peña-Rosas JP, Fernández-Gaxiola AC, Rayco-Solon P. Effects and safety of periconceptional oral folate supplementation for preventing birth defects. Cochrane Database Syst Rev. 2015;2015(12):Cd007950.

29. Berger SH, Pittman DL, Wyatt MD. Uracil in DNA: consequences for carcinogenesis and chemotherapy. Biochem Pharmacol. 2008;76(6):697-706.

30. Smith AD, Kim YI, Refsum H. Is folic acid good for everyone? Am J Clin Nutr. 2008;87(3):517-33.

31. Mason JB. Folate, cancer risk, and the Greek god, Proteus: a tale of two chameleons. Nutr Rev. 2009;67(4):206-12.

32. Bo Y, Zhu Y, Tao Y, Li X, Zhai D, Bu Y, et al. Association Between Folate and Health Outcomes: An Umbrella Review of Meta-Analyses. Front Public Health. 2020;8:550753.

33. Collin SM, Metcalfe C, Refsum H, Lewis SJ, Zuccolo L, Smith GD, et al. Circulating folate, vitamin B12, homocysteine, vitamin B12 transport proteins, and risk of prostate cancer: a case-control study, systematic review, and meta-analysis. Cancer Epidemiol Biomarkers Prev. 2010;19(6):1632-42.

34. Vollset SE, Clarke R, Lewington S, Ebbing M, Halsey J, Lonn E, et al. Effects of folic acid supplementation on overall and site-specific cancer incidence during the randomised trials: meta-analyses of data on 50,000 individuals. Lancet. 2013;381(9871):1029-36.

35. Zeng J, Wang K, Ye F, Lei L, Zhou Y, Chen J, et al. Folate intake and the risk of breast cancer: an up-to-date meta-analysis of prospective studies. Eur J Clin Nutr. 2019;73(12):1657-60.

36. Fu H, He J, Li C, Deng Z, Chang H. Folate intake and risk of colorectal cancer: a systematic review and up-to-date meta-analysis of prospective studies. Eur J Cancer Prev. 2023;32(2):103-12.

37. Wan Ismail WR, Abdul Rahman R, Rahman NAA, Atil A, Nawi AM. The Protective Effect of Maternal Folic Acid Supplementation on Childhood Cancer: A Systematic Review and Meta-analysis of Case-control Studies. J Prev Med Public Health. 2019;52(4):205-13.

38. Zwart NRK, Franken MD, Tissing WJE, Lubberman FJE, McKay JA, Kampman E, et al. Folate, folic acid, and chemotherapy-induced toxicities: A systematic literature review. Crit Rev Oncol Hematol. 2023;188:104061.

39. Zamani M, Rezaiian F, Saadati S, Naseri K, Ashtary-Larky D, Yousefi M, et al. The effects of folic acid supplementation on endothelial function in adults: a systematic review and dose-response meta-analysis of randomized controlled trials. Nutr J. 2023;22(1):12.

40. Dhar I, Svingen GF, Bjørnestad E, Ulvik A, Saeed S, Nygård OK. B-vitamin Treatment Modifies the Mortality Risk Associated with Calcium Channel Blockers in Patients with Suspected Stable Angina Pectoris: A Prospective Cohort Study. Am J Clin Nutr. 2023;118(1):77-84.

41. Miao Y, Guo Y, Chen Y, Lin Y, Lu Y, Guo Q. The effect of B-vitamins on the prevention and treatment of cardiovascular diseases: a systematic review and meta-analysis. Nutr Rev. 2023.

42. Li Y, Huang T, Zheng Y, Muka T, Troup J, Hu FB. Folic Acid Supplementation and the Risk of Cardiovascular Diseases: A Meta-Analysis of Randomized Controlled Trials. J Am Heart Assoc. 2016;5(8).

43. Kozyraki R, Cases O. Vitamin B12 absorption: mammalian physiology and acquired and inherited disorders. Biochimie. 2013;95(5):1002-7.

44. Abali EE, Cline SD, Franklin DS, Viselli S, Harvey RA. Biochemistry. Eighth edition Emine E. Abali, Susan D. Cline, David S. Franklin, Susan M. Viselli.; Eight edition. ed. Philadelphia: Lippincott Williams & Wilkins; 2021.

45. Nielsen MJ, Rasmussen MR, Andersen CB, Nexø E, Moestrup SK. Vitamin B12 transport from food to the body's cells--a sophisticated, multistep pathway. Nat Rev Gastroenterol Hepatol. 2012;9(6):345-54.

46. Chanarin I. Historical review: a history of pernicious anaemia. Br J Haematol. 2000;111(2):407-15.

47. Allen LH, Miller JW, de Groot L, Rosenberg IH, Smith AD, Refsum H, et al. Biomarkers of Nutrition for Development (BOND): Vitamin B-12 Review. J Nutr. 2018;148(suppl_4):1995s-2027s.

48. Sahu P, Thippeswamy H, Chaturvedi SK. Neuropsychiatric manifestations in vitamin B12 deficiency. Vitam Horm. 2022;119:457-70.

49. Stabler SP. Clinical practice. Vitamin B12 deficiency. N Engl J Med. 2013;368(2):149-60.

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

51. Metz J. Cobalamin deficiency and the pathogenesis of nervous system disease. Annu Rev Nutr. 1992;12:59-79.

52. Markun S, Gravestock I, Jäger L, Rosemann T, Pichierri G, Burgstaller JM. Effects of Vitamin B12 Supplementation on Cognitive Function, Depressive Symptoms, and Fatigue: A Systematic Review, Meta-Analysis, and Meta-Regression. Nutrients. 2021;13(3).

53. Morris MS, Jacques PF, Rosenberg IH, Selhub J. Folate and vitamin B-12 status in relation to anemia, macrocytosis, and cognitive impairment in older Americans in the age of folic acid fortification. Am J Clin Nutr. 2007;85(1):193-200.

54. Folic acid metabolism in vitamin B12 deficiency. Nutr Rev. 1975;33(4):118-20.

55. Linnebank M, Moskau S, Semmler A, Widman G, Stoffel-Wagner B, Weller M, et al. Antiepileptic drugs interact with folate and vitamin B12 serum levels. Ann Neurol. 2011;69(2):352-9.

56. Shea B, Swinden MV, Tanjong Ghogomu E, Ortiz Z, Katchamart W, Rader T, et al. Folic acid and folinic acid for reducing side effects in patients receiving methotrexate for rheumatoid arthritis. Cochrane Database Syst Rev. 2013;2013(5):Cd000951.

57. Jacobsen D. A gas causing laughter and harm. Tidsskrift for den Norske Lægeforening. 2022.

58. European Monitoring Centre for Drugs and Drug Addiction. Recreational use of nitrous oxide: a growing concern for Europe. Publications Office of the European Union, Luxembourg; 2022.

59. Stabler SP, Lindenbaum J, Allen RH. Vitamin B-12 deficiency in the elderly: current dilemmas. Am J Clin Nutr. 1997;66(4):741-9.

60. Nasjonalt råd for e. Helsemessige gevinster av økt folatinntak : hvordan nå ut til ønsket målgruppe? Oslo: Nasjonalt råd for ernæring; 2004.

61. World Health O. Serum and red blood cell folate concentrations for assessing folate status in populations. Geneva: World Health Organization; 2015 2015. Contract No.: WHO/NMH/NHD/EPG/15.01.

62. Yetley EA, Pfeiffer CM, Phinney KW, Fazili Z, Lacher DA, Bailey RL, et al. Biomarkers of folate status in NHANES: a roundtable summary. Am J Clin Nutr. 2011;94(1):303s-12s.

63. Yetley EA, Pfeiffer CM, Phinney KW, Bailey RL, Blackmore S, Bock JL, et al. Biomarkers of vitamin B-12 status in NHANES: a roundtable summary. Am J Clin Nutr. 2011;94(1):313s-21s.

64. Mouland G, Berg CL, Sharikabad MN, Sommerschild HT. Fylkesforskjeller i utredning og behandling av vitamin B12-mangel. Tidsskrift for den Norske Lægeforening. 2022.

65. Fedosov SN. Metabolic signs of vitamin B(12) deficiency in humans: computational model and its implications for diagnostics. Metabolism. 2010;59(8):1124-38.

66. Brito A, Verdugo R, Hertrampf E, Miller JW, Green R, Fedosov SN, et al. Vitamin B-12 treatment of asymptomatic, deficient, elderly Chileans improves conductivity in

myelinated peripheral nerves, but high serum folate impairs vitamin B-12 status response assessed by the combined indicator of vitamin B-12 status. Am J Clin Nutr. 2016;103(1):250-7.

67. Lildballe DL, Fedosov S, Sherliker P, Hin H, Clarke R, Nexo E. Association of cognitive impairment with combinations of vitamin B_{12}-related parameters. Clin Chem. 2011;57(10):1436-43.

68. Fedosov SN, Brito A, Miller JW, Green R, Allen LH. Combined indicator of vitamin B12 status: modification for missing biomarkers and folate status and recommendations for revised cut-points. Clin Chem Lab Med. 2015;53(8):1215-25.

69. Nygård O, Refsum H, Ueland PM, Vollset SE. Major lifestyle determinants of plasma total homocysteine distribution: the Hordaland Homocysteine Study. Am J Clin Nutr. 1998;67(2):263-70.

70. Green R, Miller JW. Vitamin B12 deficiency is the dominant nutritional cause of hyperhomocysteinemia in a folic acid-fortified population. Clin Chem Lab Med. 2005;43(10):1048-51.

71. Andersen LF, Kigen KM. Norkost 3 – hva spiser nordmenn i 2010? Norsk tidsskrift for ernæring. 2010;8(2):12-3.

72. Petrenya N, Skeie G, Melhus M, Brustad M. No ethnic disparities in nutritional adequacy between the Indigenous Sami and the non-Sami population living in rural Northern Norway—the SAMINOR 2 Clinical Survey. Nutr Res. 2019;64:9-23.

73. Henjum S, Manger M, Hampel D, Brantsæter AL, Shahab-Ferdows S, Bastani NE, et al. Vitamin B12 concentrations in milk from Norwegian women during the six first months of lactation. Eur J Clin Nutr. 2020;74(5):749-56.

74. Henjum S, Groufh-Jacobsen S, Lindsay A, Raael E, Israelsson AM, Shahab-Ferdows S, et al. Adequate vitamin B(12) and folate status of Norwegian vegans and vegetarians. Br J Nutr. 2022;129(12):1-8.

75. Statistics Norway 09535: Konsultasjoner hos fastlegen, etter region, alder, kjønn, statistikkvariabel og år [Online Table]. 2023 [updated 05.23.2023. Available from: https://www.ssb.no/statbank/table/09535/tableViewLayout1/.

76. Arnadottir M, Hultberg B, Nilsson-Ehle P, Thysell H. The effect of reduced glomerular filtration rate on plasma total homocysteine concentration. Scand J Clin Lab Invest. 1996;56(1):41-6.

77. Lindgren A. Elevated serum methylmalonic acid. How much comes from cobalamin deficiency and how much comes from the kidneys? Scand J Clin Lab Invest. 2002;62(1):15-9.

78. Wang A, Yeung LF, Ríos Burrows N, Rose CE, Fazili Z, Pfeiffer CM, et al. Reduced Kidney Function Is Associated with Increasing Red Blood Cell Folate Concentration and Changes in Folate Form Distributions (NHANES 2011-2018). Nutrients. 2022;14(5).

79. Samson ME, Yeung LF, Rose CE, Qi YP, Taylor CA, Crider KS. Vitamin B-12 malabsorption and renal function are critical considerations in studies of folate and vitamin B-12 interactions in cognitive performance: NHANES 2011-2014. Am J Clin Nutr. 2022;116(1):74-85.

80. Levey AS, Stevens LA, Schmid CH, Zhang YL, Castro AF, 3rd, Feldman HI, et al. A new equation to estimate glomerular filtration rate. Ann Intern Med. 2009;150(9):604-12.

81. Butler CC, Vidal-Alaball J, Cannings-John R, McCaddon A, Hood K, Papaioannou A, et al. Oral vitamin B12 versus intramuscular vitamin B12 for vitamin B12 deficiency: a systematic review of randomized controlled trials. Fam Pract. 2006;23(3):279-85.

82. Bjørke-Monsen A-L. What does a high plasma homocysteine level signify? Tidsskr Nor Laegeforen. 2021;141(5).

83. Hannibal L, Lysne V, Bjørke-Monsen AL, Behringer S, Grünert SC, Spiekerkoetter U, et al. Biomarkers and Algorithms for the Diagnosis of Vitamin B12 Deficiency. Front Mol Biosci. 2016;3:27.

84. Chen MY, Rose CE, Qi YP, Williams JL, Yeung LF, Berry RJ, et al. Defining the plasma folate concentration associated with the red blood cell folate concentration threshold for optimal neural tube defects prevention: a population-based, randomized trial of folic acid supplementation. Am J Clin Nutr. 2019;109(5):1452-61.

85. Smith AD, Refsum H. Homocysteine - from disease biomarker to disease prevention. J Intern Med. 2021;290(4):826-54.

86. Bailey RL, Carmel R, Green R, Pfeiffer CM, Cogswell ME, Osterloh JD, et al. Monitoring of vitamin B-12 nutritional status in the United States by using plasma methylmalonic acid and serum vitamin B-12. Am J Clin Nutr. 2011;94(2):552-61.

87. Luke SG. Evaluating significance in linear mixed-effects models in R. Behav Res Methods. 2017;49(4):1494-502.

88. Zhou Y, Li C, Wang M, Xu S, Wang L, Hu J, et al. Universal health coverage in China: a serial national cross-sectional study of surveys from 2003 to 2018. Lancet Public Health. 2022;7(12):e1051-e63.

89. Ranganathan P, Aggarwal R. Study designs: Part 1 - An overview and classification. Perspect Clin Res. 2018;9(4):184-6.

90. Aggarwal R, Ranganathan P. Study designs: Part 2 - Descriptive studies. Perspect Clin Res. 2019;10(1):34-6.

91. Molloy AM, Pangilinan F, Mills JL, Shane B, O'Neill MB, McGaughey DM, et al. A Common Polymorphism in HIBCH Influences Methylmalonic Acid Concentrations in Blood Independently of Cobalamin. Am J Hum Genet. 2016;98(5):869-82.

92. Statistics Norway 07459: Population, by age and year 2023 [Available from: https://www.ssb.no/en/statbank/table/07459/tableViewLayout1/.

93. Statistics Norway 10141: General practitioners consultations, by age, sex and year 2023 [Available from: https://www.ssb.no/en/statbank/table/10141/tableViewLayout1/.

94. O'Connor DL, Green T, Picciano MF. Maternal folate status and lactation. J Mammary Gland Biol Neoplasia. 1997;2(3):279-89.

95. 05803: Population 1 January and population changes during the calendar year, by contents and year [Internet]. 2023 [cited 07.09.2023]. Available from: https://www.ssb.no/en/statbank/table/05803.

96. Hallan SI, Øvrehus MA, Romundstad S, Rifkin D, Langhammer A, Stevens PE, et al. Long-term trends in the prevalence of chronic kidney disease and the influence of cardiovascular risk factors in Norway. Kidney Int. 2016;90(3):665-73.

97. Shipchandler MT, Moore EG. Rapid, fully automated measurement of plasma homocyst(e)ine with the Abbott IMx analyzer. Clin Chem. 1995;41(7):991-4.

98. Demuth K, Ducros V, Michelsohn S, Paul JL. Evaluation of Advia Centaur automated chemiluminescence immunoassay for determining total homocysteine in plasma. Clin Chim Acta. 2004;349(1-2):113-20.

99. Refsum H, Smith AD, Ueland PM, Nexo E, Clarke R, McPartlin J, et al. Facts and recommendations about total homocysteine determinations: an expert opinion. Clin Chem. 2004;50(1):3-32.

100. Obeid R, Geisel J, Herrmann W. Comparison of two methods for measuring methylmalonic acid as a marker for vitamin B12 deficiency. Diagnosis (Berl). 2015;2(1):67-72.

101. Pedersen TL, Keyes WR, Shahab-Ferdows S, Allen LH, Newman JW. Methylmalonic acid quantification in low serum volumes by UPLC-MS/MS. J Chromatogr B Analyt Technol Biomed Life Sci. 2011;879(19):1502-6.

102. Pfeiffer CM, Hughes JP, Lacher DA, Bailey RL, Berry RJ, Zhang M, et al. Estimation of trends in serum and RBC folate in the U.S. population from pre- to postfortification using assay-adjusted data from the NHANES 1988-2010. J Nutr. 2012;142(5):886-93.

103. Jones KS, Collins D, Meadows SR, Koulman A, Page P. National Diet and Nutrition Survey data reveal a decline in folate status in the UK population between 2008 and 2019. Am J Clin Nutr. 2023.

104. Helsedirektoratet. Utviklingen i norsk kosthold [Online document]. Oslo: Helsedirektoratet; 2022 [updated 01.25.2023. Available from: https://www.helsedirektoratet.no/rapporter/utviklingen-i-norsk-kosthold.

105. McKillop DJ, Pentieva K, Daly D, McPartlin JM, Hughes J, Strain JJ, et al. The effect of different cooking methods on folate retention in various foods that are amongst the major contributors to folate intake in the UK diet. Br J Nutr. 2002;88(6):681-8.

106. Solberg SL, Terragni L, Granheim SI. Ultra-processed food purchases in Norway: a quantitative study on a representative sample of food retailers. Public Health Nutrition. 2016;19(11):1990-2001.

107. Pfeiffer CM, Osterloh JD, Kennedy-Stephenson J, Picciano MF, Yetley EA, Rader JI, et al. Trends in circulating concentrations of total homocysteine among US adolescents and adults: findings from the 1991-1994 and 1999-2004 National Health and Nutrition Examination Surveys. Clin Chem. 2008;54(5):801-13.

108. Bathum L, Petersen I, Christiansen L, Konieczna A, Sørensen TI, Kyvik KO. Genetic and environmental influences on plasma homocysteine: results from a Danish twin study. Clin Chem. 2007;53(5):971-9.

109. Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. Biological and clinical implications of the MTHFR C677T polymorphism. Trends Pharmacol Sci. 2001;22(4):195-201.

110. Hustad S, Ueland PM, Vollset SE, Zhang Y, Bjørke-Monsen AL, Schneede J. Riboflavin as a determinant of plasma total homocysteine: effect modification by the

methylenetetrahydrofolate reductase C677T polymorphism. Clin Chem. 2000;46(8 Pt 1):1065-71.

111. Ubbink JB, van der Merwe A, Delport R, Allen RH, Stabler SP, Riezler R, et al. The effect of a subnormal vitamin B-6 status on homocysteine metabolism. J Clin Invest. 1996;98(1):177-84.

112. Ueland PM. Choline and betaine in health and disease. J Inherit Metab Dis. 2011;34(1):3-15.

113. Vogiatzoglou A, Oulhaj A, Smith AD, Nurk E, Drevon CA, Ueland PM, et al. Determinants of plasma methylmalonic acid in a large population: implications for assessment of vitamin B12 status. Clin Chem. 2009;55(12):2198-206.

114. Refsum H, Nurk E, Smith AD, Ueland PM, Gjesdal CG, Bjelland I, et al. The Hordaland Homocysteine Study: a community-based study of homocysteine, its determinants, and associations with disease. J Nutr. 2006;136(6 Suppl):1731s-40s.

115. Saunes IS, Karanikolos M, Sagan A. Norway Health System Review 2020. 2020.

116. Herzlich B, Herbert V. Depletion of serum holotranscobalamin II. An early sign of negative vitamin B12 balance. Lab Invest. 1988;58(3):332-7.

117. Nexo E, Hoffmann-Lücke E. Holotranscobalamin, a marker of vitamin B-12 status: analytical aspects and clinical utility. Am J Clin Nutr. 2011;94(1):359s-65s.

118. Jarquin Campos A, Risch L, Nydegger U, Wiesner J, Vazquez Van Dyck M, Renz H, et al. Diagnostic Accuracy of Holotranscobalamin, Vitamin B12, Methylmalonic Acid, and Homocysteine in Detecting B12 Deficiency in a Large, Mixed Patient Population. Dis Markers. 2020;2020:7468506.

119. Mineva EM, Sternberg MR, Bailey RL, Storandt RJ, Pfeiffer CM. Fewer US adults had low or transitional vitamin B12 status based on the novel combined indicator of vitamin B12 status compared with individual, conventional markers, NHANES 1999-2004. Am J Clin Nutr. 2021;114(3):1070-9.

120. Douaud G, Refsum H, de Jager CA, Jacoby R, Nichols TE, Smith SM, et al. Preventing Alzheimer's disease-related gray matter atrophy by B-vitamin treatment. Proc Natl Acad Sci U S A. 2013;110(23):9523-8.

121. Haggarty P. UK introduces folic acid fortification of flour to prevent neural tube defects. Lancet. 2021;398(10307):1199-201.

122. Gildestad T, Bjørge T, Haaland Ø A, Klungsøyr K, Vollset SE, Øyen N. Maternal use of folic acid and multivitamin supplements and infant risk of birth defects in Norway, 1999-2013. Br J Nutr. 2020;124(3):316-29.

123. Smith AD, Sobczyńska-Malefora A, Green R, Reynolds EH, Refsum H. Mandatory food fortification with folic acid. Lancet Glob Health. 2022;10(10):e1389.

8 Appendices

Appendix A: Ethical Approval

REK
REGIONALE KOMITEER FOR MEDISINSK OG HELSEFAGLIG FORSKNINGSETIKK

Region:	Saksbehandler:	Telefon:	Vår dato:	Vår referanse:
REK sør-øst B	Kristine Lundblad	22845513	24.09.2020	14907
			Deres referanse:	

Kjetil Retterstøl

14907 Trender i lipidprofil i den norske befolkning etter år 2000

Forskningsansvarlig: Universitetet i Oslo

Søker: Kjetil Retterstøl

REKs vurdering

Vi viser til søknad om prosjektendring mottatt 28.08.2020 for ovennevnte forskningsprosjekt (tidligere REK-ref.: 2016/1693). Søknaden er behandlet av leder for REK sørøst B på fullmakt, med hjemmel i helseforskningsloven § 11.

Endringene gjengis i sin helhet:

«3.2.5.1 og 3.6: I tillegg til analysenavnene som er oppført, vil vi også inkludere serum folat, homocystein, vitamin B12, metylmalonsyre og vitamin D i studiepopulasjonen som kovariater.

6.1, 6.7 Personkonsekvensvurdering (DPIA) er gjennomført. Personvernombud ved UiO konkluderer med at risikoen for enkeltpersoners integritet er minimert og svært lav.»

Vedlagt endringsmeldingen er en oppdatert forskningsprotokoll.

Komiteens leder har vurdert søknaden og har ingen forskningsetiske innvendinger mot endringene av prosjektet. Endringene fremstår som kvalitetssikret og forsvarlige.

Vedtak

Godkjent

REK har gjort en forskningsetisk vurdering av endringene i prosjektet, og godkjenner prosjektet slik det nå foreligger, jf. helseforskningsloven § 11.

Vi gjør samtidig oppmerksom på at etter ny personopplysningslov må det også foreligge et behandlingsgrunnlag etter personvernforordningen. Det må forankres i egen institusjon.

Tillatelsen er gitt under forutsetning av at prosjektet gjennomføres slik det er beskrevet i

søknaden, endringssøknad, oppdatert protokoll og de bestemmelser som følger av helseforskningsloven med forskrifter.

Med vennlig hilsen

Ragnhild Emblem
professor, dr. med.
leder REK sørøst B

Kristine Lundblad
Rådgiver

Kopi sendes forskningsansvarlig institusjon og eventuelle medbrukere som er gitt tilgang til prosjektet i REK-portalen.

Klageadgang
Du kan klage på komiteens vedtak, jf. forvaltningsloven § 28 flg. Klagen sendes til REK sør-øst B. Klagefristen er tre uker fra du mottar dette brevet. Dersom vedtaket opprettholdes av REK sør-øst B, sendes klagen videre til Den nasjonale forskningsetiske komité for medisin og helsefag (NEM) for endelig vurdering.

Appendix B: Approval of Data Protection Impact Assessment

UiO : Universitetet i Oslo

Til:

Dato: 2. juli 2020

Godkjennelse av personvernkonsekvensvurdering (DPIA)

Utøver av behandleransvaret ved UiO godkjenner med dette personvernkonsekvensvurderingen for «Trender i lipidprofil i den norske befolkning etter år 2000». Prosjektet oppfyller kravene i personvernlovgivningen, og kan starte slik det er beskrevet i meldeskjemaet.

Med vennlig hilsen,

Are Evju
Utøver av behandleransvaret, UiO

Roger Markgraf-Bye
Personvernombud, UiO

Appendix C: Supplemental Material

Table S1: Characteristics of and number of measurements among the excluded.

Excluded in total, n	
Subjects	394,187
Samples	2,012,668
Sex, n (%)	
Female	275,145 (69.8)
Male	119,030 (30.2)
Age group, n (%)	
18-29 years	166,931 (42.3)
30-39 years	125,488 (31.8)
40-50 years	101,768 (25.8)
Region, n (%)	
East	282,888 (76.8)
South	12,333 (3.35)
West	40,050 (10.9)
Mid	29,802 (8.09)
North	3,399 (0.92)
Subjects by analyte, n (%)	
Folate[1]	318,282 (80.7)
B12[1]	378,136 (95.9)
tHcy[1]	160,739 (40.8)
MMA[1]	145,648 (36.9)
Creatinine[1]	275,367 (69.9)
Samples by analyte, n (%)	
Folate[2]	1,293,678 (64.3)
B12[2]	1,913,683 (95.1)
tHcy[2]	395,082 (19.6)
MMA[2]	331,450 (16.5)
Creatinine[2]	1,414,820 (70.3)

[1]Percentage of total number of subjects.

[2]Percentage of total number of samples.

Table S2, part 1: Number of samples by year, n (%)[1].

Year	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Folate	4,334 (0.6)	7,192 (1)	9,022 (1.2)	10,596 (1.4)	13,919 (1.9)	18,938 (2.5)	19,317 (2.6)	23,815 (3.2)	28,170 (3.8)	31,090 (4.1)
B12	9,633 (0.8)	14,780 (1.3)	17,565 (1.5)	20,514 (1.8)	23,638 (2.1)	28,081 (2.5)	27,787 (2.4)	34,109 (3)	39,839 (3.5)	43,252 (3.8)
tHcy	763 (0.4)	1,723 (0.9)	2,755 (1.4)	4,213 (2.1)	5,169 (2.6)	5,766 (2.9)	5,680 (2.9)	7,010 (3.6)	7,244 (3.7)	9,188 (4.7)
MMA	0 (0)	84 (0.1)	1,025 (0.7)	1,948 (1.3)	2,669 (1.8)	3,287 (2.2)	3,447 (2.3)	4,397 (2.9)	4,539 (3)	6,156 (4.1)
Creatinine	10,217 (0.7)	16,142 (1.2)	19,683 (1.4)	23,696 (1.7)	27,802 (2)	33,433 (2.4)	33,064 (2.4)	40,261 (2.9)	46,862 (3.4)	52,145 (3.8)

Year	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019
Folate	34,120 (4.5)	41,161 (5.5)	48,215 (6.4)	55,722 (7.4)	62,751 (8.4)	63,116 (8.4)	65,963 (8.8)	69,735 (9.3)	69,781 (9.3)	74,192 (9.9)
B12	47,865 (4.2)	59,926 (5.3)	72,262 (6.3)	85,086 (7.5)	96,097 (8.4)	96,952 (8.5)	103,469 (9.1)	108,831 (9.5)	108,051 (9.5)	11,4071 (10)
tHcy	10,152 (5.2)	11,625 (5.9)	13,304 (6.8)	14,561 (7.4)	16,192 (8.2)	15,913 (8.1)	16,318 (8.3)	16,627 (8.5)	15,965 (8.1)	15,919 (8.1)
MMA	6,876 (4.5)	8,267 (5.5)	11,110 (7.3)	12,279 (8.1)	13,939 (9.2)	14,083 (9.3)	14,870 (9.8)	15,196 (10)	15,040 (9.9)	15,806 (10.4)
Creatinine	57,895 (4.2)	71,272 (5.1)	86,203 (6.2)	101,110 (7.3)	114,217 (8.2)	116,183 (8.4)	124,978 (9)	133,003 (9.6)	134,278 (9.7)	143,756 (10.4)

B12; vitamin B_{12}; tHcy, total homocysteine; MMA, methylmalonic acid. [1]Percentage of total measurements for the given biomarker.

Table S2, part 2: Mean metabolite concentrations from 2000 through 2019.

Year	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009
Folate, nmol/L ($n_{subjects}$ = 318,282, $n_{samples}$ = 1,293,678)										
GM (SD)	10.6 (1.58)	11.1 (1.59)	15.3 (1.59)	14.8 (1.63)	15.0 (1.64)	13.4 (1.63)	13.2 (1.61)	14.5 (1.65)	13.9 (1.68)	13.3 (1.66)
AM (CI)[2]	15.9 (14.6, 17.4)	15.73 (14.4, 17.2)	15.56 (14.2, 17.0)	15.4 (14.1, 16.8)	15.2 (14.0, 16.6)	15.1 (13.8, 16.5)	14.9 (13.7, 16.3)	14.7 (13.5, 16.1)	14.6 (13.4, 15.9)	14.4 (13.2, 15.8)
B12, pmol/L ($n_{subjects}$ = 378,136, $n_{samples}$ = 1,913,683)										
GM (SD)	314 (1.34)	314 (1.3)	292 (1.3)	291 (1.34)	289 (1.34)	293 (1.34)	302 (1.34)	298 (1.34)	298 (1.33)	293 (1.33)
AM (CI)[2]	255 (241, 270)	259 (245, 274)	264 (249, 279)	269 (254, 284)	274 (258, 290)	279 (263, 295)	284 (268, 300)	289 (273, 306)	295 (279, 312)	300 (283, 318)
tHcy, µmol/L ($n_{subjects}$ = 160,739, $n_{samples}$ = 395,082)										
GM (SD)	9.4 (1.38)	9.7 (1.39)	9.9 (1.37)	9.6 (1.39)	10.8 (1.38)	11.3 (1.36)	11.1 (1.36)	10.9 (1.36)	11.4 (1.37)	12.5 (1.37)
AM (CI)[2]	13.1 (11.1, 15.5)	12.9 (10.9, 15.2)	12.7 (10.7, 15.0)	12.5 (10.6, 14.7)	12.2 (10.4, 14.4)	12.0 (10.2, 14.2)	11.8 (10, 13.9)	11.6 (9.8, 13.7)	11.4 (9.7, 13.5)	11.2 (9.5, 13.2)
MMA, µmol/L ($n_{subjects}$ = 145,648, $n_{samples}$ = 331,450)										
GM (SD)	-	-	-	-	0.157 (1.38)	0.145 (1.38)	0.148 (1.38)	0.154 (1.38)	0.149 (1.38)	0.151 (1.37)
AM (CI)[2]	-	-	-	-	0.16 (0.16, 0.16)	0.16 (0.16, 0.16)	0.16 (0.16, 0.16)	0.16 (0.16, 0.16)	0.16 (0.16, 0.16)	0.16 (0.16, 0.16)
Creatinine, µmol/L ($n_{subjects}$ = 275,367, $n_{samples}$ = 1,414,820)										
GM (SD)	83.4 (1.14)	85.9 (1.14)	85.8 (1.14)	87.4 (1.14)	87.0 (1.14)	74.8 (1.19)	74.9 (1.19)	72.6 (1.19)	71.6 (1.20)	71.6 (1.20)
AM (CI)[3]	87.6 (85.7, 89.6)	86.3 (84.3, 88.2)	84.9 (83.0, 86.9)	83.6 (81.7, 85.5)	82.3 (80.4, 84.2)	81.0 (79.2, 82.8)	79.7 (77.9, 81.5)	78.5 (76.7, 80.3)	77.2 (75.5, 79.0)	76.0 (74.3, 77.8)

Table S2, part 2, continued: Mean metabolite concentrations from 2000 through 2019.

Year	2010	2011	2012	2013	2014	2015	2016	2017	2018	2019	p[4]
Folate, nmol/L ($n_{subjects}$ = 318,282, $n_{samples}$ = 1,293,678)											
GM (SD)	14.5 (1.65)	13.8 (1.66)	13.8 (1.65)	14.5 (1.66)	16.3(1.64)	16.6 (1.64)	13.3 (1.73)	13.3 (1.79)	13.8 (1.72)	14.0 (1.71)	
AM (CI)[2]	14.3 (13.1, 15.6)	14.1 (12.9, 15.4)	14.0 (12.8, 15.3)	13.8 (12.7, 15.1)	13.7 (12.5, 14.9)	13.5 (12.4, 14.8)	13.4 (12.3, 14.6)	13.2 (12.1, 14.5)	13.1 (12.0, 14.3)	13.0 (11.9, 14.2)	<0.001
B12, pmol/L ($n_{subjects}$ = 378,136, $n_{samples}$ = 1,913,683)											
GM (SD)	286 (1.34)	299 (1.34)	289 (1.37)	304 (1.35)	309 (1.34)	306 (1.35)	323 (1.36)	328 (1.37)	335 (1.38)	363 (1.39)	
AM (CI)[2]	306 (289, 324)	312 (295, 330)	318 (300, 336)	323 (306, 342)	329 (311, 349)	336 (317, 355)	342 (323, 362)	348 (329, 368)	355 (335, 375)	361 (341, 382)	<0.001
tHcy, µmol/L ($n_{subjects}$ = 160,739, $n_{samples}$ = 395,082)											
GM (SD)	10.6 (1.38)	9.8 (1.38)	9.8 (1.39)	11.0 (1.40)	12.1 (1.39)	11.7 (1.39)	11.0 (1.39)	10.3 (1.40)	11.0 (1.38)	9.7 (1.41)	
AM (CI)[2]	11.0 (9.3, 13.0)	10.8 (9.2, 12.8)	10.6 (9.0, 12.5)	10.4 (8.8, 12.3)	10.3 (8.7, 12.1)	10.1 (8.5, 11.9)	9.9 (8.4, 11.7)	9.7 (8.2, 11.5)	9.6 (8.1, 11.3)	9.4 (8.0, 11.1)	<0.001
MMA, µmol/L ($n_{subjects}$ = 145,648, $n_{samples}$ = 331,450)											
GM (SD)	0.157 (1.39)	0.149 (1.47)	0.144 (1.46)	0.151 (1.42)	0.152 (1.41)	0.15 (1.44)	0.147 (1.43)	0.140 (1.44)	0.146 (1.46)	0.150 (1.46)	
AM (CI)[2]	0.16 (0.16, 0.16)	0.16 (0.16, 0.16)	0.16 (0.16, 0.16)	0.16 (0.16, 0.17)	0.16 (0.16, 0.17)	0.16 (0.16, 0.17)	0.16 (0.16, 0.17)	0.16 (0.16, 0.17)	0.16 (0.16, 0.17)	0.16 (0.16, 0.17)	<0.001
Creatinine, µmol/L ($n_{subjects}$ = 275,367, $n_{samples}$ = 1,414,820)											
GM (SD)	69.5 (1.20)	70.1 (1.20)	73.0 (1.20)	71.2 (1.20)	70.13 (1.21)	68.8 (1.22)	69.0 (1.21)	68.9 (1.21)	66.7 (1.21)	64.8 (1.21)	
AM (CI)[3]	74.8 (73.2, 76.6)	73.7 (7.0, 75.4)	72.5 (70.9, 74.2)	71.4 (69.8, 73.0)	70.3 (68.7, 71.9)	69.2 (67.6, 70.7)	68.1 (66.5, 69.6)	67.0 (65.5, 68.5)	66.0 (64.5, 67.5)	64.9 (63.5, 66.4)	<0.001

B12; vitamin B_{12}; tHcy, total homocysteine; MMA, methylmalonic acid, GM, geometric mean; AM, adjusted mean; SD, geometric standard deviation (as percentage); CI, 95% confidence interval. [1]Estimated marginal means by linear mixed effects regression, controlled for eGFR, sex and age group with method/instrument as a random term (Model 1). [2]Estimated marginal means by linear mixed effects regression, controlled for sex, age group, and method/instrument as a random term (Model 1 minus eGFR). [3]P-value derived from linear mixed effects regressions. [4]Time trend from 2005-2009 to 2015-19 due to few measurements in 2004.

Table S3: Status of B vitamins and functional markers in 5-year intervals from 2000-2019.

	Years			
	2000-2004	**2005-2009**	**2010-2014**	**2015-2019**
Folate, n (%) ($n_{subjects}$ = 479,787, $n_{samples}$ = 751,147)				
< 10 nmol/L	11,022 (24.5)	30,038 (24.8)	50,106 (20.7)	84,296 (24.6)
≥ 10 nmol/L	34,041 (75.5)	91,292 (75.2)	191,863 (79.3)	258,491 (75.4)
B12, n (%) ($n_{subjects}$ = 540,311, $n_{samples}$ = 907,992)				
< 148 pmol/L	297 (0.345)	908 (0.525)	2,329 (0.645)	2,180 (0.41)
148-221 pmol/L	14,102 (16.4)	25,489 (14.7)	52,905 (14.6)	51,498 (9.69)
≥ 221 pmol/L	71,731 (83.3)	146,671 (84.7)	306,002 (84.7)	477,696 (89.9)
tHcy, n (%) ($n_{subjects}$ = 150,532, $n_{samples}$ = 196,412)				
< 11 µmol/L	9,273 (63.4)	15,908 (45.6)	35,836 (54.4)	43,831 (54.3)
≥ 11 µmol/L	5,350 (36.6)	18,980 (54.4)	29,998 (45.6)	36,911 (45.7)
MMA, n (%) ($n_{subjects}$ = 118,068, $n_{samples}$ = 151,559)				
< 0.27 µmol/L	5,477 (95.7)	20,743 (95)	49,127 (93.6)	70,219 (93.6)
≥ 0.27 µmol/L	249 (4.35)	1,083 (4.96)	3,344 (6.37)	4,776 (6.37)

B12; vitamin B_{12}; tHcy, total homocysteine; MMA, methylmalonic acid.

Table S4: Percent change in folate concentrations in women by age group between 2000-2004 and 2015-2019, $n_{subjects}$ = 264,699, $n_{samples}$ = 427,969.

	Percent change[1]	**p-value**
18-29 y	-9.4	<0.001
30-39 y	1.5	<0.001
40-50 y	-0.8	<0.001

[1]Derived from linear mixed effects regression with eGFR as covariate, and subject id and method/instrument change as random terms.

Table S5: Suboptimal folate concentrations in women, $n_{subjects}$ = 264,699, $n_{samples}$ = 427,969.

	S-Folate < 10 nmol/L	**S-Folate < 25.5 nmol/L**
All, n (%)	94,644 (22.1)	361,028 (84.4)
By age group, n (%)		
18-29 y	35,958 (25.9)	120,169 (86.7)
30-39 y	26,162 (20.8)	103,095 (81.9)
40-50 y	32,524 (19.9)	137,764 (84.3)

Appendix D: R Codes for Data Cleaning and Statistical Analysis

Preparations

Install and load packages and load data.

```
install.packages(c("tidyverse", "data.table", "writexl", "writexl", "RColorBrewer", "viridis","ggsci", "janitor",
"lme4", "lmerTest", "emmeans", "sjPlot", "broom.mixed", "ppcor"))
library(tidyverse)      library(data.table)     library(writexl)        library(RColorBrewer)
library(viridis)        library(ggsci)          library(janitor)        library(lme4)
library(lmerTest)       library(emmeans)        library(sjPlot)         library(broom.mixed)
library(ppcor)

df<-fread("M:/- SIRIHUN M -/Masteropppgave/Master 7 R/furst-bvitamins-1201.tsv")
```

Remove foreign characters æøå from column names

```
names(df)[5] = "total_rekkefolge"                   names(df)[6] = "rekkefolge_per_aar"
```

Save copy of original data

```
df_original <- df
```

Exclusions

Median samples per person per year

```
df %>% summarise(Median = median(rekkefolge_per_aar))
```

Exclusion and stepwise count of subjects and samples. Add eGFR variable.

```
df %>% distinct(id) %>% count()             #1,069,376
df %>% distinct(sample_id) %>% count() #3,360,527
#------------------------- Subjects with too many tests
df <- df %>% group_by(id) %>%
  filter(!(any(total_rekkefolge > 20) | any(rekkefolge_per_aar > 1))) %>% ungroup()
df %>% distinct(id) %>% count()             #791,440
df %>% distinct(sample_id) %>% count() #1,498,974
#------------------------- Samples with missing creatinine data, or creatinine == 0
df <- df %>% filter(!is.na(kreat)) %>% filter(kreat != 0)
df %>% distinct(id) %>% count()             #682,638
df %>% distinct(sample_id) %>% count() #1,203,755
#------------------------- Samples with missing data on sex
df <- df %>% filter(sex != "NULL")
df %>% distinct(id) %>% count()             #682,635
df %>% distinct(sample_id) %>% count() #1,203,750
#------------------------- Subjects with impaired renal function, determined by eGFR
#Add eGFR variable:
df <- df %>% mutate(egfr = case_when(
sex =="Kvinne" & kreat <= 62 ~ signif(144*(kreat/(0.7*88.42))^(-0.329)*0.993^age, 3),
sex =="Kvinne" & kreat > 62 ~ signif(144*(kreat/(0.7*88.42))^(-1.209)*0.993^age, 3),
sex == "Mann" & kreat <= 80 ~ signif(141*(kreat/(0.9*88.42))^(-0.411)*0.993^age, 3),
sex == "Mann" & kreat > 80  ~ signif(141*(kreat/(0.9*88.42))^(-1.209)*0.993^age, 3)))

#Exclusion:
df <- df %>% group_by(id) %>%
  filter(!(any(egfr < 60) | any(egfr >= 140))) %>% ungroup()
df %>% distinct(id) %>% count()             #675,189
df %>% distinct(sample_id) %>% count() #1,189,050
```

Add variables

Add “county” and region variable

```
df <- df %>% mutate(county=case_when(zip<1300 ~"Oslo", zip>=1300& zip <2100 ~"Viken",
   zip >=2150 & zip <2200    ~ "Viken",              zip == 2713                ~ "Viken" ,
   zip >=2715 & zip <=2717   ~ "Viken",              zip >=2720 & zip <=2743    ~ "Viken",
   zip >=3000 & zip <=3066   ~ "Viken",              zip == 3075                ~ "Viken",
   zip >=3300 & zip <=3521   ~ "Viken",              zip >=3523 & zip <=3526    ~ "Viken",
   zip >= 3529 & zip <=3588  ~ "Viken",              zip == 3593 & zip == 3595 ~ "Viken",
   zip >= 3601 & zip <= 3648 ~ "Viken",              zip >=2100 & zip <2150     ~ "Innlandet",
   zip >=2200 & zip <=2712   ~ "Innlandet",          zip == 2714                ~ "Innlandet",
   zip == 2718               ~ "Innlandet”,          zip >=2750 & zip <=2985    ~ "Innlandet",
   zip == 3522               ~ "Innlandet",          zip == 3527                ~ "Innlandet",
   zip == 3528               ~ "Innlandet",          zip == 3590                ~ "Innlandet",
   zip >= 3650 & zip <=3999  ~ "Innlandet",          zip >=3070 & zip <=3074    ~ "Vestfold & Telemark",
   zip >=3076 & zip <=3297   ~ "Vestfold & Telemark",
   zip >4000 & zip <=4399    ~ "Rogaland",           zip >=4460 & zip <=4465    ~ "Rogaland",
   zip >5500 & zip <=5549    ~ "Rogaland",           zip >=5560 & zip <=5589    ~ "Rogaland",
   zip == 5595               ~ "Rogaland",           zip >=4400 & zip <=4450    ~ "Agder",
   zip >=4473 & zip <=4994   ~ "Agder",              zip >=5003 & zip <=5499    ~ "Vestland",
   zip >=5550 & zip <=5559   ~ "Vestland",           zip >=5590 & zip <=5594    ~ "Vestland",
   zip >=5596 & zip <=5995   ~ "Vestland",           zip >=6700 & zip <=6760    ~ "Vestland",
   zip >= 6770 & zip <=6997  ~ "Vestland",
   zip >=6000 & zip <=6656   ~ "Moere & Romsdal",  zip >=6664 & zip <=6674    ~ "Moere & Romsdal",
   zip >=6689 & zip <=6699   ~ "Moere & Romsdal",  zip == 6761                ~ "Moere & Romsdal",
   zip >=6657 & zip <=6659   ~ "Troendelag",         zip >=6680 & zip <=6688    ~ "Troendelag",
   zip >7000 & zip <=7977    ~ "Troendelag",         zip >7985 & zip <=7995     ~ "Troendelag",
   zip >=7979 & zip <=7983   ~ "Nordland",           zip >=8000 & zip <=8408    ~ "Nordland",
   zip >=8410 & zip <=8985   ~ "Nordland",           zip == 8409                ~ "Troms & Finnmark",
   zip >9000 & zip <=9169    ~ "Troms & Finnmark",  zip >=9180 & zip <=9991   ~"Troms & Finnmark",
   zip == 9171               ~ "Svalbard"))
```

Change county variable to factor

```
df$county <- factor(df$county, ordered = TRUE, levels = c("Oslo", "Viken", "Innlandet",
                              "Vestfold og Telemark", "Rogaland", "Agder", "Vestland", "Moere og Romsdal",
                                    "Troendelag", "Nordland","Troms og Finnmark", "Svalbard"))
```

Gather counties to regions

```
df <- df %>% mutate(region = case_when(county == "Oslo" ~ "East", county == "Viken"  ~ "East",       county
== "Innlandet"   ~ "East", county == "Vestfold og Telemark" ~ "East",  county == "Agder"  ~ "South", county
== "Rogaland"  ~ "West",    county == "Vestland" ~ "West", county == "Moere og Romsdal"  ~ "West",    county
== "Troendelag"  ~ "Mid", county == "Nordland"  ~ "North", county == "Troms og Finnmark" ~ "North",
county == "Svalbard"  ~ "North"))
```

Add variables age group and years (5-year intervals)

```
df <- df %>% mutate(agegr = case_when(
   age <= 29 ~ "18-29",              age >= 30 & age <= 39 ~ "30-39",            age >= 40 ~ "40-50"),
 yeargr = case_when(
   year <= 2004 ~ "2000-2004",                    year >= 2005 & year <= 2009 ~ "2005-2009",
   year >= 2010 & year <= 2014 ~ "2010-2014",     year >= 2015 ~ "2015-2019"))
```

Add variables for the methods/instruments used by Fürst

```
df <- df %>% mutate(method_B12 = case_when(
   year <  2009                    ~ "Siemens ADVIA Centaur",
   year >= 2009 & year < 2014      ~ "Siemens ADVIA Centaur XP",
   year >= 2014                    ~ "Siemens ADVIA Centaur XPT"),
 method_FA = case_when(
   year <  2009                    ~ "Siemens ADVIA Centaur",
   year >= 2009 & year < 2014      ~ "Siemens ADVIA Centaur XP",
   year >= 2014                    ~ "Siemens ADVIA Centaur XPT"),
 method_tHcy = case_when(
   year <  2003                    ~ "IMx FPIA",
   year >= 2003 & year < 2009      ~ "Siemens ADVIA Centaur",
   year >= 2009 & year < 2014      ~ "Siemens ADVIA Centaur XP",
   year >= 2014                    ~ "Siemens ADVIA Centaur XPT"),
 method_MMA = case_when(
   year <  2004                    ~ "No analysis",
   year >= 2004 & year < 2011      ~ "CG-MS",
   year >= 2011 & year < 2014      ~ "LC-MSMS",
   year >= 2014                    ~ "UPLC-triple quadropol MS"),
 method_kreat = case_when(
   year <  2009                    ~ "Roche Modular",
   year >= 2009 & year <2016       ~ "Siemens ADVIA 2400",
   year >= 2016                    ~ "Siemens ADVIA Chemistry XPT"))
```

Modified dataframes and count

Create dataframes that exclude observations outside cutoff levels for the biomarkers. The counts give the n_su and n_sa for Table 8 and Table 9.

```
df_FA <- df %>% group_by(id) %>% filter(!is.na(FA) & FA != 0) %>% ungroup()

#Count n_su and n_sa
df_FA %>% distinct(id) %>% count() #479787
df_FA %>% distinct(sample_id) %>% count() #751147
#Count subjects by sex
df_FA_su <- df_FA %>%  group_by(id) %>% filter(row_number() == 1) %>% ungroup()
fct_count(df_FA_su$sex) #f 264699, m 215088
#Count samples by sex
df_FA %>% group_by(sex) %>% distinct(sample_id) %>% count() %>% ungroup()
#f 427969, m 323178
```

B12 too high, could be undergoing treatment

```
#Count subjects with B12 > 800
counted_subjects <- df %>% filter(B12 > 800 | B12 == 0) %>% distinct(id)

#Subtract subjects with B12 > 800 from df to get df_B12.
df_B12 <- anti_join(df, counted_subjects, by = "id")
df_B12 <- df_B12 %>% filter(!is.na(B12))

#Counted the same way as df_FA.
```

Hcy too high, could have genetic polymorphism.

```
#Count subjects with tHcy > 50
counted_subjects <- df %>% filter(tHcy > 50 | tHcy == 0) %>% distinct(id)

#Subtract subjects with tHcy > 50 from df to get df_tHcy.
df_tHcy <- anti_join(df, counted_subjects, by = "id")
df_tHcy <- df_tHcy %>%  filter(!is.na(tHcy))

#Counted the same way as df_FA.
```

MMA too high, could have genetic polymorphism. According to Fürst, MMA was not measured before 2004, but some observations dated back to 2001. Removed these.

```
#Count subjects with MMA > 0.50
counted_subjects <- df %>% filter(MMA > 0.5 | MMA == 0) %>% distinct(id)

#Subtract subjects with MMA > 0.50 from df to get df_MMA.
df_MMA <- anti_join(df, counted_subjects, by = "id")
df_MMA <- df_MMA %>%  filter(!is.na(MMA)) %>%  filter(year >= 2004)

#Counted the same way as df_FA.
```

Count n in total dataframe by sex. Total n and n for creatinine analyses.

```
df_su <- df %>%  group_by(id) %>% filter(row_number() == 1) %>% ungroup()
fct_count(df_su$sex) #f 356854, m 318335

df %>% group_by(sex) %>% distinct(sample_id) %>% count() %>% ungroup() #f 653203, m 535847
```

Check for duplicates

```
df %>% duplicated() %>% nrow() #NULL
#Check that the separately noted samples results do not contain several registrations of the same results (Using B12 bc. most observations)
df_B12 %>% group_by(sample_id) %>% filter(!is.na(B12)) %>% count() %>% ungroup() #907992
df_B12 %>% filter(!is.na(B12)) %>% distinct(sample_id) %>% count() #907992
df_B12 %>% nrow() #907992
```

Same result for each count - no duplicate registrations.

Compute CV

Define CV variables

```
df_cv <- df %>% mutate(CV_kreat = case_when(dato1 < "2014-01-01" ~ 0,
dato1 >= "2014-01-01" & dato1 < "2014-05-26" ~ 3.500,
dato1 >= "2014-05-26" & dato1 < "2014-09-22" ~ 3.504,
dato1 >= "2014-09-22" & dato1 < "2015-04-07" ~ 3.506,
dato1 >= "2015-04-07" & dato1 < "2015-05-08" ~ 3.500,
dato1 >= "2015-05-08" & dato1 < "2015-09-10" ~ 3.499,
dato1 >= "2015-09-10" & dato1 < "2016-04-02" ~ 3.506,
dato1 >= "2016-04-02" & dato1 < "2016-09-15" ~ 3.500,
dato1 >= "2016-09-15" & dato1 < "2018-01-22" ~ 4.100,
dato1 >= "2018-01-22" & dato1 < "2018-04-23" ~ 4.100,
dato1 >= "2018-04-23" & dato1 < "2018-07-02" ~ 1.400,
dato1 >= "2018-07-02" & dato1 < "2019-12-02" ~ 1.400, dato1 >= "2019-12-02"    ~ 1.400)) %>%
  mutate(CV_FA = case_when(dato1 < "2014-01-01" ~    0,
```

```
dato1 >= "2014-01-01" & dato1 < "2014-03-03" ~ 8.200,
dato1 >= "2014-03-03" & dato1 < "2015-06-15" ~ 8.200,
dato1 >= "2015-06-15" & dato1 < "2016-01-25" ~ 8.274,
dato1 >= "2016-01-25" & dato1 < "2016-05-03" ~ 8.300,
dato1 >= "2016-05-03" & dato1 < "2016-07-11" ~ 8.300,
dato1 >= "2016-07-11" & dato1 < "2016-09-15" ~ 8.300,
dato1 >= "2016-09-15" & dato1 < "2017-01-23" ~ 8.600,
dato1 >= "2017-01-23" & dato1 < "2017-07-03" ~ 7.200,
dato1 >= "2017-07-03" & dato1 < "2017-11-21" ~ 8.000,
dato1 >= "2017-11-21" & dato1 < "2017-11-21" ~ 7.412,
dato1 >= "2017-11-21" & dato1 < "2018-01-22" ~ 8.000,
dato1 >= "2018-01-22" & dato1 < "2018-05-03" ~ 7.300,
dato1 >= "2018-05-03" & dato1 < "2018-09-03" ~ 7.300,
dato1 >= "2018-09-03" & dato1 < "2019-12-02" ~ 7.300, dato1 >= "2019-12-02"      ~  6.100))  %>%
  mutate(CV_B12 = case_when(dato1 < "2014-01-01" ~ 0,
dato1 >= "2014-01-01" & dato1 < "2014-11-05" ~ 7.200,
dato1 >= "2014-11-05" & dato1 < "2015-06-15" ~ 7.382,
dato1 >= "2015-06-15" & dato1 < "2016-05-03" ~ 7.997,
dato1 >= "2016-05-03" & dato1 < "2016-09-15" ~ 8.000,
dato1 >= "2016-09-15" & dato1 < "2017-01-23" ~ 7.700,
dato1 >= "2017-01-23" & dato1 < "2018-01-22" ~ 7.000,
dato1 >= "2018-01-22" & dato1 < "2018-05-03" ~ 7.600,
dato1 >= "2018-05-03" & dato1 < "2019-02-01" ~ 7.600,
dato1 >= "2019-02-01" & dato1 < "2019-12-02" ~ 8.600,
dato1 >= "2019-12-02" & dato1 < "2019-12-02" ~ 7.500, dato1 >= "2019-12-02" ~    7.500 )) %>%
  mutate(CV_tHcy = case_when(dato1 < "2014-01-01" ~ 0,
dato1 >= "2014-01-01" & dato1 < "2014-09-03" ~ 6.000,
dato1 >= "2014-09-03" & dato1 < "2014-09-16" ~ 5.991,
dato1 >= "2014-09-16" & dato1 < "2015-06-15" ~ 6.000,
dato1 >= "2015-06-15" & dato1 < "2016-05-03" ~ 6.481,
dato1 >= "2016-05-03" & dato1 < "2016-09-15" ~ 6.500,
dato1 >= "2016-09-15" & dato1 < "2016-12-12" ~ 6.500,
dato1 >= "2016-12-12" & dato1 < "2018-01-22" ~ 8.200,
dato1 >= "2018-01-22" & dato1 < "2018-05-02" ~ 7.300,
dato1 >= "2018-05-02" & dato1 < "2019-12-02" ~ 7.300, dato1 >= "2019-12-02"  ~    7.100)) %>%
  mutate(CV_MMA = case_when(dato1 < "2013-11-28" ~ 0,
dato1 >= "2013-11-28" & dato1 < "2015-04-15" ~ 8.800,
dato1 >= "2015-04-15" & dato1 < "2015-11-11" ~ 8.800,
dato1 >= "2015-11-11" & dato1 < "2017-11-06" ~ 8.795,
dato1 >= "2017-11-06" & dato1 < "2019-09-06" ~ 8.800, dato1 >= "2019-09-06" ~    8.800))
```

Compute mean CV for each method/instrument

```
df_cv %>% filter(!is.na(CV_kreat)) %>% group_by(method_kreat) %>%
  summarise(mean(CV_kreat)) %>% ungroup()
#Likewise for the other analytes.
```

Define functions

Functions to gather data in the same cell

```
conc <- function(x, y) paste ( x, " (",y, ")", sep = "")              #Combine mean (sd)
concq <- function(x, y, z) paste (x, " (",y, ", ",z, ")", sep = "") #Combine median (.25, .75), beta (95% CI)

backtp <- function(x) (exp(x)-1)*100 #Back-transform log-transformed numbers to percent
```

Table 8, Subjects and samples

Table 8, part 1

```
Table_8_a <- df %>% summarise("Subjects, n"  = n_distinct(id), "Samples, n"   = n_distinct(sample_id),
                  "samples"      = n_distinct(sample_id), across(kreat, ~n())) %>%
  mutate("FA, n (%)" = conc(sum(!is.na(df_FA$FA)),  signif(sum(!is.na(df_FA$FA))/sum(samples)*100, 3)),
"B12, n (%)"   = conc(sum(!is.na(df_B12$B12)), signif(sum(!is.na(df_B12$B12))/sum(samples)*100, 3)),
 "tHcy, n (%)"  = conc(sum(!is.na(df_tHcy$tHcy)), signif(sum(!is.na(df_tHcy$tHcy))/sum(samples)*100, 3)),
"MMA, n (%)"   = conc(sum(!is.na(df_MMA$MMA)),
                      signif(sum(!is.na(df_MMA$MMA))/sum(samples)*100, 3)),
"Kreat, n (%)" = conc(sum(samples), signif(sum(samples)/sum(samples)*100, 3))) %>%
#Use the "samples" variable to count kreat samples because all is.na(kreat) are filtered out and kreat is
registered for each row of blood sample results that are registered in separate rows.
  dplyr::select(1, 2, 5:9) %>% pivot_longer(cols = 1:7, names_to = "variable", values_to = "value",
                                                    values_transform = list(value = as.character)) %>%
  rename("Variable" = variable, "Value" = value)
```

Check for NA to allow calculation of percentage in the next part of the table (below this code chunk)

```
fct_count(df$agegr) #No NA, divide by number of subjects to obtain percentage
fct_count(df$region) #Several NA, divide by total region observations

df %>% group_by(id) %>% filter(row_number() == 1) %>%
           filter(!is.na(region)) %>% ungroup() %>% n_distinct() #626916
```

Table 8, part 2

```
Table_8_b <- df %>%  group_by(id) %>% filter(row_number() == 1) %>%
  summarise(female_1 = length(id[sex == "Kvinne"]),
            male_1   = length(id[sex == "Mann"]),          agegr1_1 = length(id[agegr == "18-29"]),
            agegr2_1 = length(id[agegr == "30-39"]),     agegr3_1 = length(id[agegr == "40-50"]),
            east_1   = length(id[region == "East" & !is.na(region)]),
            south_1  = length(id[region == "South" & !is.na(region)]),
            west_1   = length(id[region == "West" & !is.na(region)]),
            mid_1    = length(id[region == "Mid" & !is.na(region)]),
            north_1  = length(id[region == "North" & !is.na(region)])) %>%
  summarise(female_2 = sum(female_1),    male_2    = sum(male_1),             agegr_1_2 = sum(agegr1_1),
            agegr_2_2 = sum(agegr2_1),         agegr_3_2 = sum(agegr3_1),
            east_2    = sum(east_1),           south_2   = sum(south_1),          west_2    = sum(west_1),
            mid_2     = sum(mid_1),            north_2   = sum(north_1)) %>%
  mutate(#Divide by total nr. of subjects
    "Female, n (%)"= conc((female_2), signif(female_2/675189*100, 3)),
    "Male, n (%)"  = conc((male_2),     signif(male_2/675189*100, 3)),
    "18-29, n (%)" = conc((agegr_1_2), signif(agegr_1_2/675189*100, 3)),
    "30-39, n (%)" = conc((agegr_2_2), signif(agegr_2_2/675189*100, 3)),
    "40-50, n (%)" = conc((agegr_3_2), signif(agegr_3_2/675189*100, 3)),
    #Divide by total region observations
    "East, n (%)"  = conc((east_2), signif(east_2/626916*100, 3)),
    "South, n (%)" = conc((south_2), signif(south_2/626916*100, 3)),
    "West, n (%)"  = conc((west_2), signif(west_2/626916*100, 3)),
    "Mid, n (%)"   = conc((mid_2), signif(mid_2/626916*100, 3)),
    "North, n (%)" = conc((north_2), signif(north_2/626916*100, 3))) %>%
  dplyr::select(11:20) %>% pivot_longer(cols = 1:10, names_to = "variable", values_to = "value",
                                                  values_transform = list(value = as.character)) %>%
  rename("Variable" = variable, "Value" = value)
```

Table 8, merged. Find n subjects by metabolite in "Modified dataframes" section and add manually.

```
Table_8 <- rbind(Table_8_a, Table_8_b)
write_xlsx(Table_8, "Table_8.xlsx")
print(Table_8)
```

Geometric man age (geometric sd)

```
exp(mean(log(df$age))) #34.8
exp(sd(log(df$age)))   #1.32
```

Table S1, Characteristics of the excluded

Preparations

```
#Select only columns that are in the original df to allow the use of "anti_join"
df_short <- df %>% dplyr::select(1:15)
df_ex <- anti_join(df_original, df_short, by = "id")
names(df_ex)[5] = "total_rekkefolge"
names(df_ex)[6] = "rekkefolge_per_aar"
```

Count excluded subjects and samples

```
df_ex %>% distinct(id) %>% count() #394,187
df_ex %>% distinct(sample_id) %>% count() #2,012,668
#Count n_su per analyte
df_ex %>% filter(!is.na(FA)) %>% distinct(id) %>% count() #318,282
#Likewise for the other analytes.
```

Median and mean samples per year. Add region, age group and method variables as in main data frame.

```
df_ex %>% summarise(Median = median(rekkefolge_per_aar)) #1
df_ex %>% summarise(Mean = mean(rekkefolge_per_aar)) #1,44
```

Table S1

Create Table S1 using *df_ex* with the same method as for Table 8.

Table 9, Geometric and adjusted mean (EMM)

Table 9, part 1: Number of measurements per 5-year period

```
#Enter metabolite exclusions manually because the total dataframe must be used.
Table_9 <- df %>% group_by(yeargr) %>%
 summarise("FAn"    = sum(!is.na(FA)),                "B12n"   = sum(!is.na(B12) & B12 < 800),
           "tHcyn"  = sum(!is.na(tHcy) & tHcy < 50),  "MMAn"   = sum(!is.na(MMA) & MMA < 0.50),
           "Kreatn" = sum(!is.na(kreat))) %>%
 mutate("Period, y" = yeargr,      #Divide by total number of samples for each metabolite
        "FA, n " = conc(FAn, signif(FAn/(sum(!is.na(df_FA$FA)))*100, 3)),
        "B12, n " = conc(B12n, signif(B12n/(sum(!is.na(df_B12$B12)))*100, 3)),
        "tHcy, n " = conc(tHcyn, signif(tHcyn/(sum(!is.na(df_tHcy$tHcy)))*100, 3)),
        "MMA, n " = conc(MMAn, signif(MMAn/(sum(!is.na(df_MMA$MMA)))*100, 3)),
        "Kreat, n " = conc(Kreatn, signif(Kreatn/sum(!is.na(df$kreat))*100, 3))) %>%
 dplyr::select(7:12) %>% ungroup()
Table_9 <- transpose(Table_9)
colnames(Table_9) <- c('1','2','3','4')
```

Table 9, part 2: Geometric mean (geometric sd) for each metabolite

```
Table_9_geo <- as.data.frame(bind_rows(list(
  df_FA %>% group_by(yeargr) %>% summarise(
  "FA_1" = signif(exp(mean(log(FA))), 4), "FA_2" = signif(exp(sd(log(FA))), 4)) %>%
  mutate("FA, nmol/L" = conc(FA_1, FA_2)) %>%
  dplyr::select(4) %>% as.tibble() %>% transpose(),

  df_B12 %>% group_by(yeargr) %>% summarise(
  "B12_1" = signif(exp(mean(log(B12))), 4), "B12_2" = signif(exp(sd(log(B12))), 4)) %>%
  mutate("B12, nmol/L" = conc(B12_1, B12_2)) %>%
  dplyr::select(4) %>% as.tibble() %>% transpose(),

  df_tHcy %>% group_by(yeargr) %>% summarise(
  "tHcy_1" = signif(exp(mean(log(tHcy))), 4), "tHcy_2" = signif(exp(sd(log(tHcy))), 4)) %>%
  mutate("tHcy, nmol/L" = conc(tHcy_1, tHcy_2)) %>%
  dplyr::select(4) %>% as.tibble() %>% transpose(),

  df_MMA %>% group_by(yeargr) %>% summarise(
  "MMA_1" = signif(exp(mean(log(MMA))), 4),"MMA_2" = signif(exp(sd(log(MMA))), 4)) %>%
  mutate("MMA, nmol/L" = conc(MMA_1, MMA_2)) %>%
  dplyr::select(4) %>% as.tibble() %>% transpose(),

  df %>% group_by(yeargr) %>% filter(!is.na(kreat), kreat > 0) %>% summarise(
  "kreat_1" = signif(exp(mean(log(kreat))), 4), "kreat_2" = signif(exp(sd(log(kreat))), 4)) %>%
  mutate("kreat, nmol/L" = conc(kreat_1, kreat_2)) %>%
  dplyr::select(4) %>% as.tibble() %>% transpose())))

colnames(Table_9_geo) <- c('1','2','3','4')
```

Table 9, part 3: Estimated marginal means (EMM, adjusted for egfr, sex and age group, with id and method as random terms)

```
FA_emm <- df_FA %>% lmer(log(FA) ~ yeargr + egfr + sex + agegr + (1|id) + (1|method_FA),
      data = ., REML = F)

FA_emm_df <- as.data.frame((FA_emm) %>%
   emmeans("yeargr", cov.reduce=function(x) unique(x), rg.limit = 600000) %>%
   as_tibble() %>% mutate(across(c(emmean, asymp.LCL, asymp.UCL),  ~ exp(.))))

FA_emm_t_ <- FA_emm_df %>% mutate(emmean = round(emmean, 2),
           asymp.LCL = round(asymp.LCL, 2), asymp.UCL = round(asymp.UCL, 2),
           "FA emmean" = (concq(emmean, asymp.LCL, asymp.UCL))) %>%  dplyr::select(7)
FA_emm_t <- transpose(FA_emm_t_)
rownames(FA_emm_t) <- colnames(FA_emm_t_)
colnames(FA_emm_t) <- rownames(FA_emm_t_)

#Likewise for the other analytes.
```

Table 9, merged

```
Table_9 <- rbind(Table_9, Table_8_geo, FA_emm_t, B12_emm_t, tHcy_emm_t, MMA_emm_t, kreat_emm_t)
write_xlsx(Table_9, "Table_9.xlsx")
print(Table_9)
```

Table 9, p-values for the change in estimated marginal means from 2000-2004 to 2015-2019

```
FA_emm %>% summary()

#p-value: <0.001, coefficient: -0.06056
(exp(-0.06056)-1)*100
#Percentage change in FA from 2000-2004 to 2015-2019: -5.68%

#Likewise for the other analytes.
#B12: p-value: <0.001, coefficient: 0.08367. Change in B12 from 2000-2004 to 2015-2019 = 9.32%
#tHcy: p-value: <0.001, coefficient: -0.1179. Change in Hcy from 2000-2004 to 2015-2019 =  -10.98%
#MMA: p-value: <0.001, coefficient: 0.04390. Change in MMA from 2000-2004 to 2015-2019 = 4.53%
#kreat: p-value: <0.001, coefficient: -0.2113. Change in kreat from 2000-2004 to 2015-2019: -18.7%
```

Table S2, Geometric and adjusted mean for each year

Identical to Table 10, but grouped by "year" instead of "yeargr".
Time trends - S2

```
#FA: p-value: <0.001, coefficient: -0.01080. Percentage change in folate: -1.07%
#B12: p-value: <0.001, coefficient: 0.01844. Percentage change in B12 = 1.86%
#tHcy: p-value: <0.001, coefficient: -0.01765. Percentage change in Hcy = -1.75%
#MMA: p-value: <0.001, coefficient: 0.002373. Percentage change in MMA = 0.24%
#kreat: p-value: <0.001, coefficient: -0.01566. Percentage change in kreat: -1.55%
```

Raincloud plots, Distribution of metabolite concentrations in 5-year intervals by sex

Reorder 5-year intervals

```
df_FA <- df_FA %>% mutate(yeargrplot = case_when(
   year <= 2004                    ~ "2000-2004",      year >= 2005 & year <= 2009 ~ "2005-2009",
   year >= 2010 & year <= 2014 ~ "2010-2014",      year >= 2015                    ~ "2015-2019"))
df_FA$yeargrplot <- factor(df_FA$yeargrplot, ordered = T,
                           levels=c('2015-2019', "2010-2014", '2005-2009', '2000-2004'))

#Repeat for the other analyte dataframes.
labels_sex <- c("Kvinne" = "Women", "Mann" = "Men")
```

Raincloud plot folate

```
ggplot(df_FA, aes(yeargrplot, FA, fill = yeargrplot, color = yeargrplot)) +
  ggdist::stat_halfeye(alpha = 0.5, justification = -0.1, scale = 0.3) +
  ggdist::stat_dotsinterval(quantiles = 100, alpha = 0.5, justification = 0.1, side = "bottomright", scale = 0.25)  +
  labs(title = "Distribution of S-Folate levels by sex from 2000 through 2019", y = "S-Folate (nmol/L)", x = "Yea
rs") +
  geom_hline(yintercept = 10, color = "#8c8c8c") +
  annotate("text",x = 5, y = 7.5, label = "10", size = 3) +
  theme_minimal() +
  theme(axis.title.y = element_blank(), legend.position = "none", plot.title.position = "plot") +
  scale_color_viridis(discrete = TRUE) +
  scale_fill_viridis(discrete = TRUE) +
  facet_wrap(~ sex, labeller = as_labeller(labels_sex)) +
  coord_flip()
ggsave("Raincloud S-Folate by sex.png")
```

Repeat for B12, tHcy and MMA with respective cutoff lines, titles and labels.

Raincloud plot of folate status in women by age group

```
df_female <- df_FA %>%
  filter(sex == "Kvinne")

ggplot(df_female, aes(yeargrplot, FA, fill = yeargrplot, color = yeargrplot)) +
  ggdist::stat_halfeye(alpha = 0.5, justification = -0.1, scale = 0.3) +
  ggdist::stat_dotsinterval(quantiles = 100, alpha = 0.5, justification = 0.1, side = "bottomright", scale = 0.25) +
  labs(title = "Distribution of S-Folate levels in women by age group from 2000 through 2019", y = "S-Folate (n
mol/L)", x = "Years") +
  geom_hline(yintercept = c(10, 25.5), color = "#8c8c8c") +
  annotate("text",x = 5, y = 7.5, label = "10", size = 3) +
  annotate("text", x = 5, y = 22, label = "25.5", size = 3) +
  theme_minimal() +
  theme(axis.title.y = element_blank(), legend.position = "none", plot.title.position = "plot") +
  scale_color_viridis(discrete = TRUE) +
  scale_fill_viridis(discrete = TRUE) +
  facet_wrap(~ agegr) +
  coord_flip()

ggsave("Raincloud S-Folate women agegr.png")
```

Table S4, Folate trend in women

Time trend in females. Put together table manually.

```
FA_trend_f <- df_female %>%
  lmer(log(FA) ~ yeargr + agegr + egfr + (1|id) + (1|method_FA), data = ., REML = F)

FA_trend_f_i <- df_female %>%
  lmer(log(FA) ~ yeargr*agegr + egfr + (1|id) + (1|method_FA), data = ., REML = F)

anova(FA_trend_f, FA_trend_f_i) #Interacation gave a little lower AIC and BIC. Use Fa_trend_f_i

FA_trend_f_i %>% summary()
#Change for 18-29 year old women
(exp(-0.09871)-1)*100 #= -9.4%

#Change for 30-39 year old women
(exp(-0.09871+0.04282+0.07067)-1)*100 #= 1.5%

#Change for 40-50 year old women
(exp(-0.09871+0.09296-0.002251)-1)*100 #= -0.8%
```

Table S5, Folate status in women

Prevalence of folate deficiency and suboptimal folate concentrations in women. Put together manually.

```
#All age groups:
df_female %>% nrow() #427969
df_female %>% filter(FA < 25.5) %>% nrow() #361028
df_female %>% filter(FA < 10) %>% nrow() #94644
361028/427969*100 #84.4% below 25.5 nmol/L
94644/427969*100 #22.1% below 10 nmol/L

#18-29 (Likewise for the other two age groups):
df_female %>% filter(agegr == "18-29") %>% nrow() #138621
df_female %>% filter(agegr == "18-29" & FA < 25.5) %>% nrow() #120169
df_female %>% filter(agegr == "18-29" & FA < 10) %>% nrow() #35958
120169/138621*100 #86.7% below 25.5 nmol/L
35958/138621*100 #25.9% below 10 nmol/L

#Two youngest age groups (Most of childbearing age):
df_female %>% filter(agegr != "40-50") %>% nrow() #264458
df_female %>% filter(agegr != "40-50" & FA < 25.5) %>% nrow() #223264
df_female %>% filter(agegr != "40-50" & FA < 10) %>% nrow() #62120
223264/264458*100 #84.4% below 25.5 nmol/L
62120/264458*100 #23.5% below 10 nmol/L
```

Table 10, Time trend analysis

Linear mixed effects regression models (LMER) for folate

```
#Crude
FA_crude <- df_FA %>% lmer(log(FA) ~ yeargr + (1|id), data = ., REML = F)
#Adjusted
FA_adj <- df_FA %>% lmer(log(FA) ~ yeargr+ egfr +sex + agegr +(1|id) + (1|method_FA), data =., REML=F)

#Sex
FA_sex_u <- df_FA %>% lmer(log(FA) ~ yeargr*sex + (1|id), data = ., REML = F)
FA_sex_a <- df_FA %>%
    lmer(log(FA) ~ yeargr*sex + agegr + egfr + (1|id) + (1|method_FA), data = ., REML = F)
summary(FA_sex_u) #Men ***
summary(FA_sex_a) #Men ***

#Age
FA_age_u <- df_FA %>% lmer(log(FA) ~ yeargr*agegr + (1|id), data = ., REML = F)
FA_age_a <- df_FA %>%
    lmer(log(FA) ~ yeargr*agegr + sex + egfr + (1|id) + (1|method_FA), data = ., REML = F)
summary(FA_age_u) #Both***
summary(FA_age_a) #30-39**, 40-50***

#Region
FA_region_u <- df_FA %>% lmer(log(FA) ~ yeargr*region + (1|id), data = ., REML = F)
FA_region_a <- df_FA %>%
    lmer(log(FA) ~ yeargr*region + sex + agegr + egfr + (1|id) + (1|method_FA), data = ., REML = F)
summary(FA_region_u) #West *, South **, Mid ***, North ***
summary(FA_region_a) #West **, South ***, Mid ***, North ***
```

Extract coefficients with confidence intervals and gather in a table.

```
T10_FA <- as.data.frame(bind_rows(list(
  #Crude
  as.tibble(tidy(FA_crude, effects ="fixed", conf.int = T, conf.method = "profile"))[4,] %>%
    mutate("estimate_bt" = (exp(estimate)-1)*100,
           "conf.low_bt" = (exp(conf.low)-1)*100, "conf.high_bt" = (exp(conf.high)-1)*100) %>%
    mutate_at(3:12, round, 2) %>%
    mutate("beta (95% CI)" = concq(estimate_bt, conf.low_bt, conf.high_bt)) %>%
    dplyr::select(13),

  #Adjusted
  as.tibble(tidy(FA_adj, effects ="fixed", conf.int = T, conf.method = "profile"))[4,] %>%
    mutate("estimate_bt" = (exp(estimate)-1)*100,
           "conf.low_bt" = (exp(conf.low)-1)*100, "conf.high_bt" = (exp(conf.high)-1)*100) %>%
    mutate_at(3:12, round, 2) %>%
    mutate("beta (95% CI)" = concq(estimate_bt, conf.low_bt, conf.high_bt)) %>%
    dplyr::select(13),

  #-------------------------- By sex
  #Sex unadjusted female
  as.tibble(tidy(FA_sex_u, effects ="fixed", conf.int = T, conf.method = "profile"))[4,] %>%  #P-verdi: 0
    mutate("estimate_bt" = (exp(estimate)-1)*100,
           "conf.low_bt" = (exp(conf.low)-1)*100, "conf.high_bt" = (exp(conf.high)-1)*100) %>%
    mutate_at(3:12, round, 2) %>%
    mutate("beta (95% CI)" = concq(estimate_bt, conf.low_bt, conf.high_bt)) %>%
    dplyr::select(13),

  #Sex adjusted female
  as.tibble(tidy(FA_sex_a, effects ="fixed", conf.int = T, conf.method = "profile"))[4,] %>%  #P-verdi: 0
    mutate("estimate_bt" = (exp(estimate)-1)*100,
           "conf.low_bt" = (exp(conf.low)-1)*100, "conf.high_bt" = (exp(conf.high)-1)*100) %>%
    mutate_at(3:12, round, 2) %>%
    mutate("beta (95% CI)" = concq(estimate_bt, conf.low_bt, conf.high_bt)) %>%
    dplyr::select(13),

  #Sex unadjusted male
  as.tibble(tidy(FA_sex_u, effects ="fixed", conf.int = T, conf.method = "profile"))[c(4, 5, 8),] %>%
#Sjekker p-verdi, ser at den er *** for alle beta
    dplyr::select(term, estimate, conf.low, conf.high) %>%
    pivot_wider(names_from=c(term), values_from=c(estimate, conf.low, conf.high)) %>%
    summarise("estimate" = sum(across(1:3)),
        "conf.low" = sum(across(4:6)),"conf.high" = sum(across(7:9))) %>%
    mutate_all(~backtp(.)) %>%
    mutate_at(1:3, round, 2) %>%
    mutate("beta (95% CI)" = concq(estimate, conf.low, conf.high)) %>%
    dplyr::select(4),

  #Sex adjusted male
  as.tibble(tidy(FA_sex_a, effects ="fixed", conf.int = T, conf.method = "profile"))[c(4, 5, 9),] %>%
#Sjekker p-verdi, ser at den er *** for alle beta
    dplyr::select(term, estimate, conf.low, conf.high) %>%
    pivot_wider(names_from=c(term), values_from=c(estimate, conf.low, conf.high)) %>%
    summarise("estimate" = sum(across(1:3)),
        "conf.low" = sum(across(4:6)), "conf.high" = sum(across(7:9))) %>%
```

```
  mutate_all(~backtp(.)) %>%
  mutate_at(1:3, round, 2) %>%
  mutate("beta (95% CI)" = concq(estimate, conf.low, conf.high)) %>%
  dplyr::select(4),
#Likewise for remaining subgroups
)))
```

Gather effects with confidence interval in a table.

```
T10_FA <- T10_FA %>%
 mutate("Model" = c("Crude", "Adjusted", "Female, unadjusted", "Female, adjusted",
        "Male, unadjusted", "Male, adjusted", "18-29 unadjusted", "18-29 adjusted",
        "30-39 unadjusted", "30-39 adjusted", "40-50 unadjusted", "40-50 adjusted",
        "East unadjusted", "East adjusted", "South, unadjusted", "South, adjusted",
        "West unadjusted", "West adjusted", "Mid unadjusted", "Mid adjusted",
        "North unadjusted", "North adjusted")) %>% dplyr::select(2, 1)

write_xlsx(T10_FA, "Table_10_FA.xlsx")
```

Repeat regression analysis and combination to table for B12, tHcy and MMA.

Table 10 Compare models

```
anova(FA_crude, FA_adj)             #(crude = Model 1, adj = Model 2)
anova(FA_sex_u, FA_sex_a)           #(sex_a = Model 3)
anova(FA_age_u, FA_age_a)           #(age_a = Model 4)
anova(FA_region_u, FA_region_a) #(region_a = Model 5)
#All models significantly different when adjusted. BIC lower -> better fit.

#Repeat for the models for the other analytes.
#B12: All models significantly different when adjusted. BIC lower -> better fit.
#tHcy: All models significantly different when adjusted. BIC lower -> better fit.
#MMA: All models significantly different when adjusted. BIC lower -> better fit.
```

Table 10 n

```
#Folate
fct_count(df_FA_su$agegr)
#18-29: 172637    30-39: 142733       40-50: 164417
df_FA %>% group_by(agegr) %>% distinct(sample_id) %>% count() %>% ungroup()
#18-29: 228399    30-39: 225021    40-50: 297727

fct_count(df_FA_su$region)
#East: 328889    South: 11711    West: 51200    Mid: 46145   North: 6131
df_FA %>% group_by(region) %>% distinct(sample_id) %>% count() %>% ungroup()
#East: 530194    South: 16094    West: 70854    Mid: 68622   North: 8327

#Likewise for the other metabolites.
```

Gather the individual metabolite tables to Table 4 and fill in n manually.

Table 11, Combined biomarker status

Count and create table manually. Also compute percent manually.

```
#Combined folate and Hcy status
df %>% filter(FA < 10 & tHcy < 50) %>% nrow()              #24769
df %>% filter(FA < 10 & tHcy < 11) %>% nrow()              #8008
df %>% filter(FA < 10 & tHcy >= 11 & tHcy < 50) %>% nrow()  #16761
df %>% filter(FA >= 10 & tHcy < 50) %>% nrow()             #83903
df %>% filter(FA >= 10 & tHcy < 11) %>% nrow()             #49617
df %>% filter(FA >= 10 & tHcy >= 11 & tHcy < 50) %>% nrow()  #34286

#Count subjects and samples folate and tHcy
df %>% filter(!is.na(FA) & !is.na(tHcy) & tHcy < 50) %>% distinct(id) %>% count
df %>% filter(!is.na(FA) & !is.na(tHcy) & tHcy < 50) %>% distinct(sample_id) %>% count
n_su: 87992  n_sa: 108672

#Likewise for Folate and B12, B12 and Hcy, B12 and MMA.
```

Combined folate + B12 + Hcy status

```
df %>% filter(FA < 10 & B12 < 148 & tHcy < 50 & tHcy >= 11) %>% nrow()  #160 (78.8%)
df %>% filter(FA < 10 & B12 < 148 & tHcy < 11) %>% nrow()              #43 (21.2%)


df %>% filter(FA < 10 & B12 < 221 & tHcy < 50 & tHcy >= 11) %>% nrow()  #3064 (76.8%)
df %>% filter(FA < 10 & B12 < 221 & tHcy < 11) %>% nrow()              #927 (23.2%)


df %>% filter(FA < 10 & B12 < 148 & tHcy < 50) %>% distinct(id) %>% count() #201
df %>% filter(FA < 10 & B12 < 148 & tHcy < 50) %>% distinct(sample_id) %>% count() #203


df %>% filter(FA < 10 & B12 < 221 & tHcy < 50) %>% distinct(id) %>% count() #3794
df %>% filter(FA < 10 & B12 < 221 & tHcy < 50) %>% distinct(sample_id) %>% count() #3991
```

Table S3, Deficiency

```
Table_S3_ <- df %>% group_by(yeargr, sample_id) %>%
 summarise(FA_norm_1   = length(sample_id[FA >= 10 & !is.na(FA)]),
           FA_def_1    = length(sample_id[FA < 10 & !is.na(FA)]),
           B12_norm_1  = length(sample_id[B12 >= 221 & !is.na(B12) & B12 < 800]),
           B12_low_1   = length(sample_id[B12 < 221 & B12 >= 148 & !is.na(B12)]),
           B12_def_1   = length(sample_id[B12 < 148 & !is.na(B12)]),
           tHcy_norm_1 = length(sample_id[tHcy < 11 & !is.na(tHcy)]),
           tHcy_high_1 = length(sample_id[tHcy >= 11 & !is.na(tHcy) & tHcy < 50]),
           MMA_norm_1  = length(sample_id[MMA < 0.27 & !is.na(MMA)]),
           MMA_high_1  = length(sample_id[MMA >= 0.27 & !is.na(MMA) & MMA < 0.50])) %>%
 summarise(FA_norm_2   = sum(FA_norm_1),                 FA_def_2    = sum(FA_def_1),
           B12_norm_2  = sum(B12_norm_1),            B12_low_2   = sum(B12_low_1),
           B12_def_2   = sum(B12_def_1),                 tHcy_norm_2 = sum(tHcy_norm_1),
           tHcy_high_2 = sum(tHcy_high_1),           MMA_norm_2  = sum(MMA_norm_1),
           MMA_high_2  = sum(MMA_high_1)) %>%
 mutate("FA >= 10 nmol/L, n (%)"  = conc(FA_norm_2, signif(FA_norm_2/(FA_norm_2+FA_def_2)*100, 3))
,
       "FA < 10 nmol/L, n (%)"  = conc(FA_def_2, signif(FA_def_2/(FA_norm_2+FA_def_2)*100, 3)),
       "B12 >= 221 pmol/L, n (%)"= conc(B12_norm_2, signif(B12_norm_2/(B12_norm_2+B12_low_2+B12_d
ef_2)*100, 3)),
       "B12 148-221 pmol/L, n (%)" = conc(B12_low_2, signif(B12_low_2/(B12_norm_2+B12_low_2+B12_def
_2)*100, 3)),
       "B12 < 148 pmol/L, n (%)" = conc(B12_def_2, signif(B12_def_2/(B12_norm_2+B12_low_2+B12_def_2)
*100, 3)),
       "tHcy < 11, n (%)"   = conc(tHcy_norm_2,
signif(tHcy_norm_2/(tHcy_norm_2+tHcy_high_2)*100, 3)),
       "tHcy >=11, n (%)"   = conc(tHcy_high_2,
signif(tHcy_high_2/(tHcy_norm_2+tHcy_high_2)*100, 3)),
       "MMA < 0.27, n (%)"  = conc(MMA_norm_2, signif(MMA_norm_2/(MMA_norm_2+MMA_high_2)*10
0, 3)),
       "MMA >= 0.27, n (%)" = conc(MMA_high_2, signif(MMA_high_2/(MMA_norm_2+MMA_high_2)*100
, 3))) %>%
 dplyr::select(1, 11:19) %>% ungroup()

Table_S3 <- transpose(Table_S3_)
rownames(Table_S3) <- colnames(Table_S3_)
colnames(Table_S3) <- rownames(Table_S3_)
Table_S3 <- data.frame(rownames(Table_S3), Table_S3)

write_xlsx(Table_S3, "Table_S3.xlsx")
print(Table_S3)
```

Table 12, Correlation analyses

Create dummy variables

```
cor_df <- df %>% mutate("sex_male" = ifelse(sex == "Mann", 1, 0),
       "agegr_30-39" = ifelse(agegr == "30-39", 1, 0), "agegr_40-50" = ifelse(agegr == "40-50", 1, 0),
       "method_FA_XP" = ifelse(method_FA == "Siemens ADVIA Centaur XP", 1, 0),
       "method_FA_XPT" = ifelse(method_FA == "Siemens ADVIA Centaur XPT", 1, 0),
       "method_B12_XP" = ifelse(method_B12 == "Siemens ADVIA Centaur XP", 1, 0),
       "method_B12_XPT" = ifelse(method_B12 == "Siemens ADVIA Centaur XPT", 1, 0),
       "method_tHcy_Centaur" = ifelse(method_tHcy == "Siemens ADVIA Centaur", 1, 0),
       "method_tHcy_XP" = ifelse(method_tHcy == "Siemens ADVIA Centaur XP", 1, 0),
       "method_tHcy_XPT" = ifelse(method_tHcy == "Siemens ADVIA Centaur XPT", 1, 0),
       "method_MMA_LCMSMS" = ifelse(method_MMA == "LC-MSMS", 1, 0),
       "method_MMA_UPLC" = ifelse(method_MMA == "UPLC-triple quadropol MS", 1, 0))

#Create an own cor_df_MMA using df_MMA to exclude observations before 2004.
```

Create data frames for correlation analyses

```
cor_FA <- as.data.frame(cor_df %>% filter(!is.na(FA) & !is.na(tHcy) & tHcy < 50))
cor_FA_B12 <- as.data.frame(cor_df %>% filter(!is.na(FA) & !is.na(B12) & B12 < 800))
cor_B12_tHcy <- as.data.frame(cor_df %>% filter(!is.na(B12) & B12 < 800& !is.na(tHcy)& tHcy < 50))
cor_B12_MMA <- as.data.frame(cor_df_MMA %>% filter(!is.na(B12) & B12 < 800))
cor_B12_tHcyMMA <- as.data.frame(cor_df_MMA %>%
                                   filter(!is.na(B12) & B12 < 800 & !is.na(tHcy) & tHcy < 50))
```

Count number of measurements in each correlation analysis

```
cor_FA %>% distinct(id) %>% count() #su: 87992
cor_FA %>% distinct(sample_id) %>% count() #sa: 108672
#Likewise for the other correlation dataframes.
```

Partial correlation of folate with tHcy, controlling for sex, agegr, egfr and method

```
pcor.test(cor_FA$FA, cor_FA$tHcy, cor_FA[, c(16, 26:30, 33:35)])
```

Likewise for the other correlation analyses. Gather results to a table manually.

Frequency of biomarker analyses ordered together

```
#Folate and tHcy
(cor_FA %>% distinct(sample_id) %>% count())/(df_FA %>% nrow)*100 #14.5%

#Folate and B12 (How many folate samples were ordered when B12 was ordered)
(cor_FA_B12 %>% distinct(sample_id) %>% count())/(df_B12 %>% nrow)*100 #53.7%

#B12 and MMA
(cor_B12_MMA %>% distinct(sample_id) %>% count())/(df_B12 %>% nrow)*100 #8.1%

#B12, tHcy and MMA
(cor_B12_tHcyMMA %>% distinct(sample_id) %>% count())/(df_B12 %>% nrow)*100 #5.4%
```