Graduate thesis

NTNU
Norwegian University of Science and Technology
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Department of Clinical and Molecular Medicine

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Prevalence of folate and vitamin B12 deficiency during pregnancy among healthy, Norwegian women: association to pregnancy outcomes

Graduate thesis in Programme of Professional Study, Medicine
Supervisor: Unni Syversen
Co-supervisor: Miriam K. Gustafson
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Table of content

Abstract

Introduction

Deficiency of folate and vitamin B12 is suggested to affect millions of pregnant women worldwide. The vitamins are essential for embryogenesis, and hypovitaminosis B has been linked to various adverse pregnancy outcomes. In this study we have investigated the serum levels of folate and vitamin B12 in 2. and 3. trimester of pregnancy, and the association with preterm birth and low birth weight. Moreover, the association between these vitamins and BMI and physical activity was studied. Finally, we investigated whether there was a tendency for a general hypovitaminosis among our participants.

Methods

This is a secondary analysis of a two-armed, two-center randomized controlled trial which examined the impact of antenatal exercise on gestational diabetes as the primary outcome. The study included 855 healthy and well-educated women, 19-46 years of age from the two cities Trondheim (n=660) and Stavanger (n=195). The participants were randomized to a 12-week regular exercise program during pregnancy or to standard antenatal care. Clinical data and blood samples were collected in the second and third trimester. The participants answered a questionnaire, addressing sociodemographic and lifestyle factors.

Results

Mean serum folate level was 23.2 ± 7.8 nmol/L and 16.0 ± 6.5 nmol/L and mean vitamin B12 level 265 ± 91 pmol/L and 220 ± 70 pmol/L in the second and third trimester, respectively. Only two women had folate deficiency in the second and four and third trimester, whereas 16 (n=133) and 34% (n=249) displayed vitamin B12 deficiency in the second and third trimester. Women being vitamin B deficient in the second trimester, tended to remain deficient at the end of the pregnancy. Levels of both folate and vitamin B12 were negatively correlated to BMI. Participants in Trondheim had higher folate levels than in Stavanger. Loss to follow-up was 15% (n=131).

Conclusion

We observed a high prevalence of vitamin B12 deficiency in the third trimester, whereas the prevalence of folate deficiency was very low. Deficiency may be associated with adverse maternal and perinatal outcomes and can have a long term health consequences for the offspring. Therefore, our findings are of concern and these observations need to be elucidated.

1.0 Introduction

1.1 Background

Deficiency of folate (vitamin B9) and vitamin B12 in pregnancy remains a global concern (1). Folate deficiency has, however, decreased since the early 2000s, due to folic acid supplements/fortifications during pregnancy(2). Vitamin B12 deficiency is more frequent (3), and has been reported in 20-30% of pregnant women worldwide, compared to 6-12% in the general adult population (4). Both folate and vitamin B12, play important roles in pregnancy, and are crucial for the rapid cell division in embryogenesis (5). Serum levels of the vitamins do normally decrease during pregnancy, attributed to increased fetal demand (1, 6). Folate and vitamin B12 deficiency have been associated with a spectrum of pregnancy adverse outcomes, including low birth weight (LBW <2500g). LBW has been associated with obesity, type 2 diabetes, and metabolic syndrome, as well as osteoporosis in adulthood (7). Hence, optimizing vitamin B-concentrations in the periconceptional period and during pregnancy is of significance (8).

1.2 Function and metabolism of folate and vitamin B12

B vitamins in general are cofactors for the enzymes involved in the energy-producing metabolic pathways of carbohydrates, fats and proteins (9). They also play an important role in maintaining functions of the nervous system (10). Both folate and vitamin B12 are essential for the synthesis of DNA, RNA, lipids, and protein in the cellular cytoplasm. Moreover, the vitamins are necessary cofactors for the conversion of homocysteine to methionine, being an important methyl donor required for the synthesis of neurotransmitters and phospholipids(11, 12).

The major dietary sources of folate are liver, citrus fruits, dark green leafy vegetables and legumes (13). Synthetic folic acid is found in fortified foods and dietary supplements. Folic acid is absorbed in the proximal part of the jejunum and transported across enterocytes and metabolized to 5-methyltetrahydrofolate. It is further exported into the portal vein, and to the liver where folate undergoes first-pass metabolism to a more active form, which improves the vitamins availability (14). After this process, folate is transported to circulating blood and equilibrates in tissues. Folate is reabsorbed through the enterohepatic circulation (13). Body stores are relatively small, and folate deficiency occurs frequently in the absence of supplementation (14).

Vitamin B12, also referred to as cobalamin, is a micronutrient pivotal for cellular growth, differentiation and development (15). Vitamin B12 is synthesized by certain bacteria in the gastrointestinal tract of animals (12). Dietary sources of vitamin B12 include meat (especially beef, pork), liver, poultry, eggs, milk products and seafood (clams, oysters, mackerel, and salmon) (16). There are no naturally occurring bioactive forms of vitamin B12 from plant sources, therefore vegetarians/vegans are at risk for developing deficiency (12). In the ventricle, vitamin B12 is released from the food due to the acid environment and is bound to R protein (haptocorrin). Thereafter, vitamin B12 binds to intrinsic factor, which is secreted by parietal cells in the gastric mucosa. Intrinsic factor is required for absorption of vitamin B12. Like folate, vitamin B12 is stored in the liver, secreted in the bile and reabsorbed via the enterohepatic circulation (11). The stores in the liver can normally sustain physiologic needs for multiple years, if very limited levels of vitamin B12 (12).

1.3 Folate, role in health and disease

Folate deficiency is caused by various factors, such as dietary insufficiency, malabsorption (e.g., due to alcoholism), increased demand (e.g., in pregnancy) or medications (e.g., anti-folate drugs). Poor folate status may result in impairment of DNA and protein synthesis, as well as gene expression. Megaloblastic anemia is a hallmark of folate deficiency. Low folate status has also been associated with an increased risk of cardiovascular disease and cancer (especially colorectal cancer) and cognitive dysfunction (13, 17). Moreover, folate deficiency has been linked to a lower cognitive score among children (1). Low folate levels are related to anemia during childhood and adolescence and might predispose children to infections and diseases (18).

The need for folate increases during pregnancy. Folate deficiency is associated with pregnancy complications (spontaneous abortions, bleeding, preeclampsia, intrauterine growth restriction and abruptio placentae), preterm birth and LBW. Moreover, folate deficiency may contribute to development of neural tube defects (NTD) in embryogenesis (19, 20), and has also been associated with orofacial anomalies (19), and congenital heart defects (1, 21). Folate supplementation has shown a positive effects on NTDs and other congenital anomalies (1, 13, 22), and it has been reported to increase birth weight in studies in Africa and India (1, 23). A folate rich diet alone is not considered to provide the desired risk reduction of NTD, hence supplements of 400 µg/day have been recommended during the periconceptional period and during the first trimester of pregnancy (2). However, the compliance is poor, as demonstrated in a Norwegian study, where 70% of women took supplementation during pregnancy, and only 30% started preconceptionally (23).

Several countries, among others USA, Canada, Brazil and Chile, introduced mandatory enrichment of flour and other grain products in 1998. Thereafter, the incidence of anencephalus has been reduced by 16% and spina bifida by 31% in the US (2). Consequently, NTDs attributed to folate deficiency decreased by about 50% over 10 years. However, the number of NTDs caused by vitamin B12 deficiency tripled during this time period (24).

Many European countries have chosen not to institute a folic acid fortification program because of concerns of unintended consequences, according to the “precautionary principle” (25). Studies from the US. indicate that fortified food has resulted in approximately twice as high intake of folate as anticipated. No adverse effects have been observed in women of childbearing age who have participated in trials (2). A serious concern of high folate intake is the potential of masking vitamin B12 anemia. When the warning symptom remains absent, critical low levels of vitamin B12 may develop and result in severe neurological damage (2).

1.4 Vitamin B12, role in health and diseases

Deficiency is usually caused by malabsorption of vitamin B12, whereas dietary inadequacy is common in the elderly and vegetarians/vegans. Moreover, obesity, bariatric surgery, inflammatory bowel disease, Helicobacter pylori infection, use of metformin or proton pump inhibitors are risk factors of deficiency (4). The lack of intrinsic factor, associated with the autoimmune disease pernicious anemia, is a common cause of hypovitaminosis B12 in the western part of the world (11). Vitamin B12 deficiency is mainly characterized by megaloblastic anemia, but may also manifest as microangiopathic hemolytic anemia and thrombocytopenia (26). Additional symptoms of deficiency include fatigue, weakness, constipation, loss of appetite, weight loss, difficulty maintaining balance, depression, confusion, dementia, poor memory, numbness and tingling in the hands and feet. Early diagnosis of vitamin B12 deficiency is important to avoid irreversible damage (11).

During pregnancy, vitamin B12 absorption increases to cover the requirements of the fetus and mother and is transferred to the fetus when crossing the placenta (11, 27). As mentioned earlier, consuming a long-term, predominantly vegetarian diet, implies an increased risk of deficiency (11, 28). However, a decline in plasma level is also common in pregnant women with an adequate diet, due to alterations in haptocorrin-bound vitamin B12 and hemodilution among others (28).

Vitamin B12 deficiency has been associated with increased risk of pregnancy outcomes, including spontaneous abortion, pregnancy loss, intrauterine growth restriction, preterm birth (PTB, <37 weeks of gestation), LBW and NTDs (27, 29, 30). A moderate association has also been shown between low maternal vitamin B12 levels and other birth defects, such as orofacial clefts and heart defects (1, 27). Exclusively breastfed infants are dependent on the vitamin B12 content in breast milk. Hence, if the mother eats no or little meat, the amount of vitamin will be limited, and the offspring is at risk for deficiency within months after birth. This can lead to long-term deficits in growth development in children (30). Children who suffer delay as a result of severe vitamin B12 deficiency tend not to fully recover (1). Despite evidence of negative effects of vitamin B12 deficiency, the role of supplementation in pregnancy is uncertain (31).

In this student thesis, we aimed to examine the prevalence of vitamin B12 and folate deficiency in 850 healthy, well-educated, pregnant participants. Furthermore, we wanted to explore the association of vitamin levels to different variables, such as BMI, exercising PTB and birth weight.

2.0 Methods

2.1 Study design and participants

This is a secondary analysis of a two armed, two-center randomized controlled trial which examined the impact of antenatal exercise on gestational diabetes as the primary outcome. Pregnant participants attending routine ultrasound examination at 18 weeks of gestation at Trondheim University Hospital and Stavanger University Hospital, were enrolled in this study. The recruiting enrolled from April 2007 to September 2009 (32). Eligible participants were healthy, pregnant, aged 18 years or older with a singleton live fetus. In total, 855 women were included. A total of 724 (85%) completed the study. In accordance with The American College of Obstetrics and Gynecologists, exclusion criteria were pregnancy complications, high risk for PTB or diseases that could hinder participation. Participants living far from the hospital were excluded (33). Health effects of a 12-week regular exercise program during pregnancy were compared with standard antenatal care. Clinical data and blood samples were collected at inclusion before the randomization (18–22 week of gestation), and after the intervention (32–36 week of gestation). Analyses of folate, B12, 25(OH)D, vitamin D-binding protein, calcium, phosphate, magnesium and PTH have been performed.

2.2 Data collection

The participants were recruited consecutively, and the clinical data and blood samples were collected in the second and third trimester (18-22 and 32-36 week of gestation). Body weight and height were measured at inclusion. Questionnaires regarding sociodemographic variables, medical history, diet, childbirths, smoking behaviour and physical activity were completed (34).

2.3 Serum analyses

The blood samples were collected after fasting and the sera were stored at -80 Celsius, at the Department of Laboratory Medicine, Trondheim University Hospital. Folate and vitamin B12 were analyzed in 2009, while 25-hydroxyvitamin D (25(OH)D) and trans retinoic acid (vitamin A) in 2015. All the assays, including vitamin B12 and folate, were delivered by Roche Diagnostics Ltd., Switzerland. 25(OH)D were analyzed by electrochemiluminescence immunoassay (ECLIA), while vitamin A was measured by high performance liquid chromatography (HPLC).

2.4 Definition of vitamin deficiency

There are no universal cut-offs for assessment of folate and vitamin B12. WHO recommends the concentrations for defining deficiencies based on metabolic indicators to be: <10 nmol/L (4 ng/mL) for serum folate and <150 pmol/L (203 pg/mL) for plasma vitamin B12 (1). In our study population we decided to use the reference values from Trondheim University Hospital, as follows: serum folate ≥7.0 nmol/L and serum vitamin B12 186-645 pmol/L. Deficiency is classified <7 nmol/L of folate and <186 pmol/L of vitamin B12 (35). Vitamin A deficiency is defined as <0.70 μmol/L and inadequacy is defined as ≤1.05 μmol/L (36). Vitamin D deficiency is defined as <30 nmol/L and insufficiency is defined as ≤50 nmol/L (35).

2.5 Ethics

This is a secondary analysis of "the training in pregnancy" (TRIP) study. The TRIP study was approved by the Regional Committee for Medical and Health Research Ethics (REK 4.2007.81) and performed in accordance with the Declaration of Helsinki. The trial is registered in the ClinicalTrials.gov (NCT 00476567).

2.6 Statistical analyses

Statistical analyses of data were done by using the SPSS statistics Version 27.0 (Armonk, NY: IBM Corpversion). Independent- and One sample T-test was used for normally distributed variables. Correlations between the vitamin levels, BMI, exercising and birth weight were assessed using Pearson's correlation coefficient. Our data are presented with percentage or as means ± standard deviation (SD). Histograms are used to portray the normal distribution of numerical data, and Box-plot are used for quartiles, means and outliers. Level of statistical significance was set at $p < 0.05$.

3.0 Results

3.1 Maternal characteristics

Table 1 shows the baseline clinical and demographic characteristics of the participants (n=855). Mean inclusion point was at week 20.0 ± 1.7 of pregnancy and mean age at delivery was 30.5 ± 4.3 years. At inclusion, mean BMI was 24.8 ± 3.2 kg/m2. Two of the participants were classified as underweight (BMI ≤18.5), 33% (n=281) as overweight (BMI 25-29.9), and 7% (n=63) were classified as obese (BMI ≥30). LBW was found in 4% (n=30) of the offsprings (37), 94% (n=803) had normal birth weight and 2% (n=20) had fetal macrosomia (birth weight ≥4500g) (38). The participants were well-educated. Nine of the women reported smoking at inclusion. During pregnancy, 26% (n=200) and 60% (n=453) reported their health status to be respectively "very good" and "good", only 3% (n=20) and 0.1% (n=1) reported a "bad" and "very bad" health status. Loss to follow-up was 15% (n=131).

Table 1: *Maternal characteristics at inclusion*

Maternal characteristics	**Total (*n*=855)**	**Trondheim 63°N (*n*=660)**	**Stavanger 58°N (*n*=195)**
Age (years)	30.5 ± 4.3	30.4 ± 4.3	30.6 ± 4.5
Gestational length at inclusion (weeks)*	20.0 ± 1.7	20.0 ± 1.7	20.7 ± 1.5
Marital status *n* (%)**			
Married/cohabitant	834 (98)	645 (98)	189 (97)
Single	20 (2)	14 (2)	6 (3)
Educational level *n* (%)			
Elementary school	5 (0.6)	3 (0.5)	2 (1)
High School	90 (11)	64 (910)	26 (13)
University	760 (89)	593 (90)	167 (86)

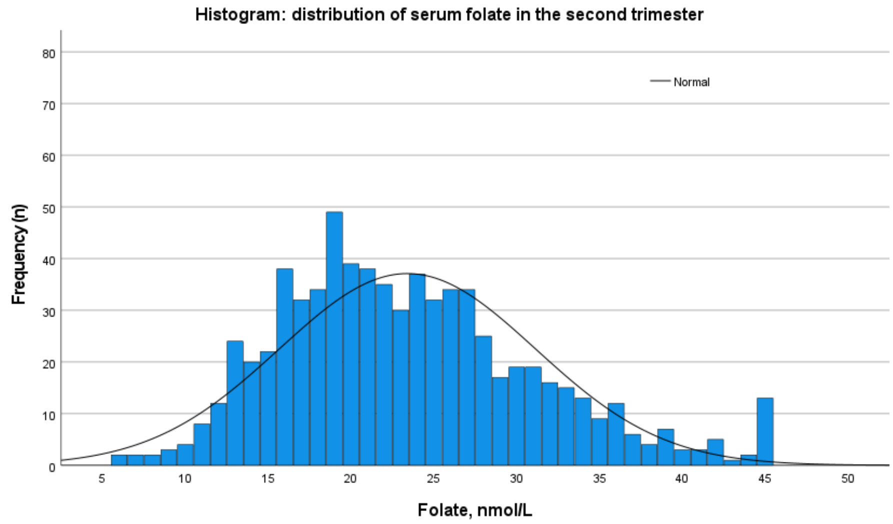
Paid work or self-employed *n* (%)**	793 (93)	614 (93)	184 (94)
Parity *n* (%)			
0	486 (57)	374 (57)	112 (57)
1	254 (30)	199 (30)	55 (28)
2	90 (11)	68 (10)	22 (11)
3+	25 (3)	19 (3)	6 (3)
Smoking *n* (%)**	9 (1)	5 (0.8)	4 (2)
Inclusion body mass index (kg/m^2)**	24.8 ± 3.2	24.9 ± 3.3	24.7 ± 3.0
Blood pressure (mmHg)			
Systolic	109 ± 9	109 ± 9	109 ± 9
Diastolic	69 ± 8	69 ± 8	67 ± 8
Exercised regularly pre-pregnancy *n* (%)	610 (71)	476 (72)	134 (69)
Preterm birth *n* (%)	32 (4)	27 (4)	5 (3)

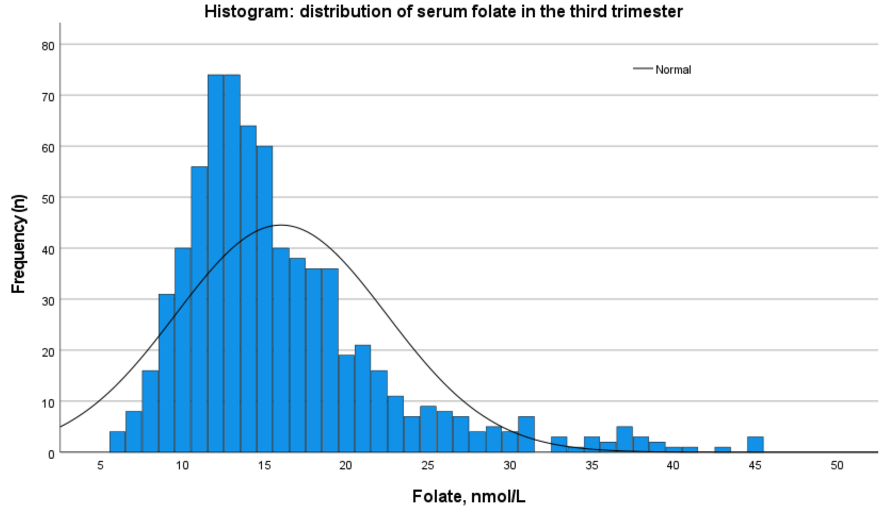
*Ten participants from Trondheim and two from Stavanger are missing.
**One participant from Trondheim is missing.
***Fourteen participants from Trondheim and five participants from Stavanger are missing.

3.2 Folate measurements

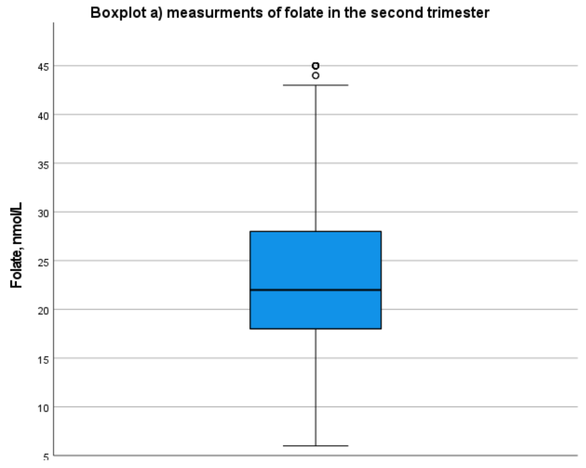
Mean serum level of folate in the second trimester (n=852) was 23.2 ± 7.8 nmol/l (range: 6-45 nmol/L). Only 0.2% (n=2) had deficiency. Mean serum levels of folate in the third trimester (n=722) was 16.0 ± 6.5 nmol/L (range: 6-45 nmol/L), 0.6% (n=4) had deficiency. Average serum folate level dropped by 31% (19.6 nmol/L) from second to third trimester (graph 1 and 2). There was a significant positive correlation between serum levels of folate in the second and third trimester (r=0.620, p<0.001).

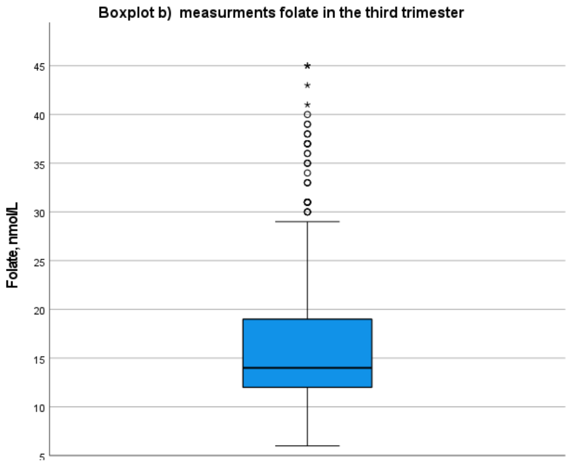
Graph 1a + 1b: *Histogram, distribution of serum folate levels in 2. and 3. trimester, respectively.*





Graph 2a + b: *Boxplot of serum folate levels in 2. and 3. trimester. The median and 25th to 75th percentile are represented with the box and the data range between the whiskers. Outliers (o) and extremes (*) are marked.*

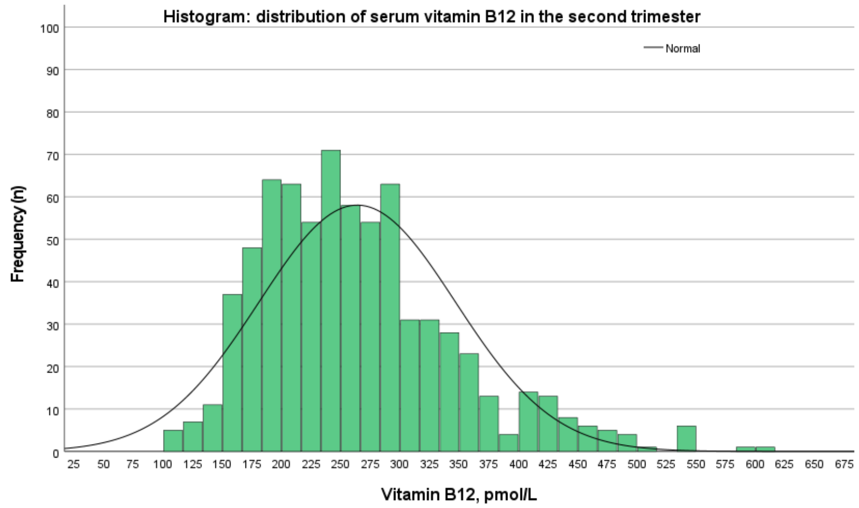


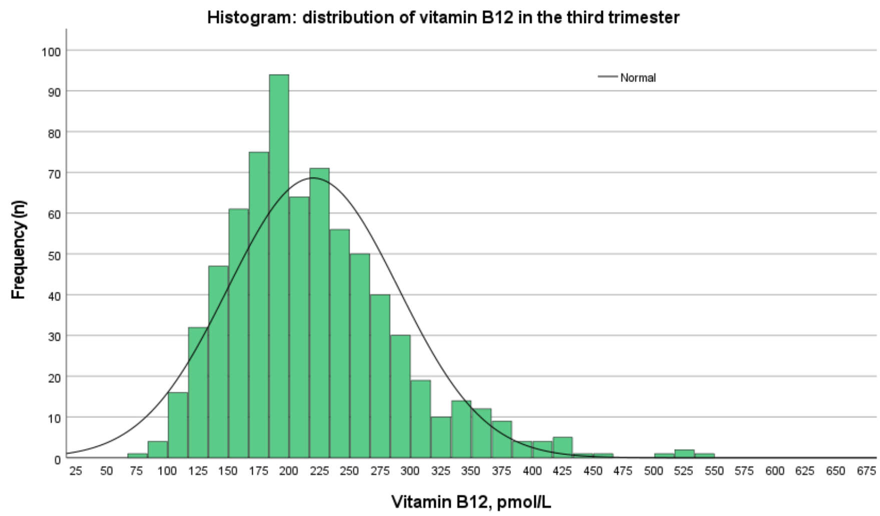


3.3 Vitamin B12 measurements

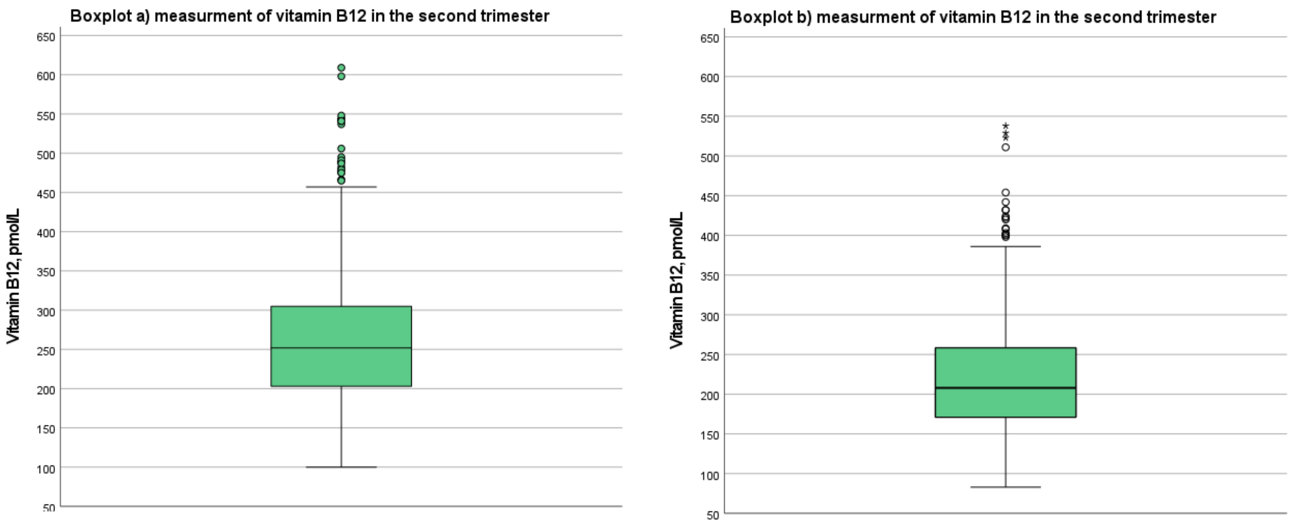
Mean serum level of vitamin B12 (n=854) in the second trimester was 265 ± 91 pmol/L (range: 100-1476 pmol/L), 16% (n=133) had deficiency and one woman had higher serum levels than recommended. Mean serum level of vitamin B12 in the third trimester (n=724) was 220 ± 70 pmol/L (range: 83-538 pmol/L), 34% (n=249) exhibited vitamin B12 deficiency. Average serum vitamin B12 decreased by 17% (46 pmol/L) from the second to third trimester. See graph 3 and 4. A significant correlation between serum levels of vitamin B12 in the second and third trimester ($r=0.849$ $p<0.001$) was observed.

Graph 3a + 3b: *Histogram with distribution of serum B12 levels in 2. and 3. trimester, respectively. In graph 3a, an extreme value of 1476 pmol/L is excluded.*





Graph 4: *Boxplot a and b of serum vitamin B12 in the second (a) and third (b)trimester. The median and 25th to 75th percentile are represented with the box and the entire data range between the whiskers. Outliers (o) and extremes (*) are marked. In boxplot a, an extreme value of 1476 pmol/L was excluded.*



3.4 Geographical differences in folate and vitamin B12 levels

Women in Trondheim had higher levels of folate than women in Stavanger both in the second (mean 23.5 vs. 22.2 nmol/L, p=0.053) and third trimester (mean 16.3 vs. 14.9 nmol/L, p=0.020). See Table 2. No significant difference was found between levels of vitamin B12 in the second and third trimester. See Table 3.

Table 2: *Differences in serum folate levels (nmol/L) between Trondheim and Stavanger*

Trimester	**Trondheim**, mean serum folate (nmol/L) ± SD (n)	**Stavanger**, mean serum folate (nmol/L) ± SD	**All*** mean serum folate (nmol/L) ± SD
Second	23.5 ± 7.8 (657)	22.2 ± 7.8 (195)	23.2 ± 7.8 (n=852)
Third	16.3 ± 6.4 (579)	14.9 ± 6.4 (143)	16.0 ± 6.5 (722)
Both trimesters	19.9	18.6	19.6

*Mean folate levels for all study participants.

Table 3: *Differences in serum vitamin B12 (pmol/L) between Trondheim and Stavanger*

Trimester	**Trondheim**, mean serum vitamin B12 nmol/L ± SD (n)	**Stavanger**, mean serum vitamin B12 nmol/L ± SD (n)	**All*** mean serum vitamin B12 nmol/L ± SD (n)
Second	267 ± 95 (659)	260 ± 77 (195)	265 ± 91 854)
Third	220 ± 71 (580)	219 ± 68 (144)	220 ± 70 (724)
Both trimesters	243	239	242

*Mean vitamin B12 levels for all study participants.

3.5 Correlation between vitamin levels and regular exercise during pregnancy

The majority of the participants, 71% (n=610), practiced regular exercise prior to pregnancy, 52% (n=444) exercised during the second trimester and 64% (n=489) in the third trimester. There was a positive correlation between regular exercise in the second trimester and folate levels (r=0.078, p=0.023), but no correlation to vitamin B12 levels (r=0.037, p=0.283) in the same period. No correlation was found between regular exercise in the third trimester and folate (r=0.044, p=0.233) and vitamin B12 levels (r=0.068, p=0.067). See table 4a and b.

Table 4a and b: *Mean serum folate (nmol/L) and vitamin B12 (pmol/L) values in participants who did or did not exercise regularly during the second and third trimester*

Folate (nmol/L)	**Yes (n)**	**No (n)**
In 2. trimester	22.5 ± 7.6 (411)	23.9 ± 8.0 (444)
In 3. trimester	15.6 ± 6.6 (272)	16.2 ± 6.4 (489)

Vitamin B12 (pmol/L)	**Yes (n)**	**No (n)**
In 2. trimester (855)	263 ± 110 (411)	266 ± 83 (444)
In 3. trimester (761)	220 ± 70 (272)	213 ± 64 (489)

3.6 Correlation between folate, vitamin B12 and BMI

A negative correlation was observed between folate and BMI in the second (r=-0.130, p<0.001) and third trimester (r=-0.119, p=0.001). A negative correlation was also seen between vitamin B12 and BMI, r=-0.110 (p=0.001) and r=-0.156 (p<0.001). See table 5.

Table 5. *Mean serum levels of folate (nmol/L) and vitamin B12 (pmol/L) according to categories of BMI*

BMI - second trimester	**Serum folate (nmol/L)**	**Serum vitamin B12 (pmol/L)**
≤ 18.5 *(n=1)*	19.0	364
18.5-24.9 *(n=509)*	24.1	272
25.0-29.9 *(n=280)*	21.8	260
≥30.0 *(n=62)*	22.6	231
BMI – third trimester		
≤ 18.5 *(n=0)*	-	-
18.5-24.9 *(n=195)*	16.8	234
25.0-29.9 *(n=415)*	16.1	217
≥30.0 *(n=116)*	14.7	207

3.7 Vitamin status in correlation to birth weight

Distribution of offspring's birth weight is presented in table 6. No correlations were seen between levels of folate and birth weight in second (r=-0.020, p=0.558) and third trimester (r=-0.045, p=0.228), and for vitamin B12 during the same period, respectively r=0.013 (p=0.712) and r=-0.059 (p=0.111).

No significant differences were seen between birth weight and serum folate/vitamin B12 levels measured in the third trimester: normal birth weight vs. LBW (p=0.697, p=0.252), normal birth weight vs. macrosomia (p=0.527, p=0.519) and LBW vs. macrosomia (p=0.493, p=0.430), respectively.

Table 6: *Mean serum levels of folate (nmol/L) and vitamin B12 (pmol/L) measured in the third trimester according to weight group of the offspring.*

Weight group (n)	Mean serum folate (nmol/L) ± SD	Mean serum vitamin B12 (pmol/L) ± SD
<2500g (30)	16.7 ± 7.5	250 ± 84
2500-4500g (803)	16.0 ± 6.4	219 ± 70
>4500g (20)	15.0 ± 6.0	230 ± 48

3.8 Vitamin status and preterm birth

Thirty-two (4.1%) of the participants had PTB. These women displayed significantly lower folate levels in the second trimester compared to the total study population (p<0.001) and significantly higher in the third trimester (p=0.001). Vitamin B12 levels were higher in women with PTB compared to the total in the second (p=0.164), and third trimester (p<0.001), although not significant in the second trimester. See table 7.

Table 7: *Mean serum levels of folate (nmol/L) and vitamin B12 (pmol/L) in women with preterm birth compared to total study population*

	PTB group, folate	All participants, folate	PTB group, B12	All participants, B12
Second trimester (n=31)	22.1	23.2	261	265
Third trimester (n=21)	16.8	16.0	229	220

3.9 Relation between folate, vitamin B12 and levels of vitamin A (retinol) and D (25(OH)D)

One of the two who were folate deficient at inclusion, continued being deficient in the third trimester. Of those who were vitamin B12 deficient at inclusion (n=133), 94% (n=133) persisted with low levels in the third trimester. None were simultaneously both folate and vitamin B12 deficient in the second trimester. Two of those being folate deficient in the third trimester (n=4) also had vitamin B12 deficiency.

Table 8 shows the frequency of hypovitaminosis A and D in our population. The two participants being folate deficient in the second trimester, had also low values of vitamin D.

Of the four being folate deficient at the end of pregnancy, one had vitamin A inadequacy and three had vitamin D insufficiency. The relation of hypovitaminosis B12 and low levels of vitamin A and D are presented in table 9.

Table 8*: Frequency of hypovitaminosis A and D during pregnancy. This table shows the percentage and number of cases (n) of those having hypovitaminosis A (inadequacy ≤1.05 µmol/L) and D (insufficiency ≤50 nmol/L) in the second and third trimester.*

	Vitamin A Inadequacy % (n)	**Vitamin D** insufficiency % (n)
2. trimester	9 (79)	27 (232)
3. trimester	45 (325)	34 (246)

Table 9. *Vitamin B12 deficiency in relation to hypovitaminosis A and D. This table shows the percentage and number of cases (n) with vitamin B12 deficiency and simultaneous vitamin A inadequacy (≤1.05 µmol/L) and vitamin D insufficiency (≤50nmol/L).*

	Vitamin B12 deficiency % (n)	**Vitamin B12** deficiency + **vitamin A** inadequacy % (n)	**Vitamin B12** deficiency + **vitamin D** insufficiency % (n)
2. trimester	16 (133)	0 (0), 13 (17)	8 (11), 32 (43)
3. trimester	34 (249)	0.2 (3), 42 (104)	13 (32), 41 (102)

Of the 249 women with vitamin B12 deficiency in third trimester, 104 had also vitamin A inadequacy and 102 had concomitant vitamin D insufficiency. In total, 45 participants had low levels of all three vitamins in the third trimester.

Both folate and vitamin B12 were correlated to each other in the second (r=0.079, p=0.020) and third trimester (r=0.133, p<0.001). A strong correlation was discovered between folate and vitamin D levels in both trimesters, with r=0.153 (p<0.001) and r=0.136 (p<0.001). Vitamin B12 levels were correlated to vitamin D levels in the second (r=0.097, p=0.005) and third trimester (r=0.146, p<0.001). For vitamin A, a correlation to folate levels was also seen at both time points, respectively with r=0.086 (p=0.012), r=0.080 (p=0.031), but no significant correlation was observed between vitamin A and B12 levels.

4.0 Discussion

This study, including 855 participants, is the largest and most comprehensive study conducted in Norway on folate and vitamin B12 status during pregnancy. We observed a low prevalence of folate deficiency in both trimesters, only two women in second and four in third trimester. Vitamin B12 deficiency was seen in 16 and 34% in the second and third trimester, respectively. Participants from Trondheim displayed significantly higher folate levels in the third trimester compared to Stavanger. Women displaying folate and vitamin B12 deficiency in second trimester, tended to remain deficient at the end of the pregnancy. Among those with vitamin B12 deficiency, a substantial number also had hypovitaminosis A or D in the third trimester. A negative correlation was observed between BMI and levels of folate and vitamin B12. Folate and vitamin B12 levels were inconsistently correlated to physical activity. No correlation was found between folate or vitamin B12 levels and birth weight and preterm birth. There were no cases of NTDs.

4.1 Prevalence of folate and vitamin B12 deficiency

The prevalence of folate deficiency was low both in the second and third trimester (0.2 and 0.6%). The low deficiency rate was somewhat unexpected, since a previous study of the same population, showed that they did not adhere to the recommendations of daily folate intake (32). Moreover, another Norwegian study observed that only 30% of the women started taking folic acid preconceptionally (23). Still, the fact that folate deficiency was very rare in our population, implies that they had a sufficient dietary intake of folate at least during second and third trimester. The Norwegian Directorate of Health recommends folic acid supplementation preconceptionally and during pregnancy (39). Folate deficiency was less common in our population than in most other studies. In a study conducted in Switzerland in 2001, folate deficiency occurred in 4% of the pregnant women (40). A similar prevalence (3%) was observed in countries with folic acid fortification, like Mexico, Costa Rica and Argentina (41). In an Ethiopian study, including participants whose diet was based on either maize or enset, only 2% had low plasma folate. (Gibson et al., 2008). In a Turkish study, where the majority received folic acid supplements (85%), deficiency was not observed (42). However, folate deficiency is still prevalent in India, where a deficiency rate of 25% was reported among pregnant women (31).

On the other hand, vitamin B12 deficiency during pregnancy is more common, with an estimated prevalence of 20-30% worldwide (15). We observed a high prevalence in third trimester, by 34%. A Canadian study showed that 17% had vitamin B12 deficiency in the second trimester (43); this is in accordance with our findings. In contrast, a very high prevalence of deficiency, up to 60%, was found in the Indian subcontinent. This may be explained by a predominantly vegetarian diet (4). Another study observed deficiency in 71% of pregnant Turkish women (42), in spite of the rareness of vegetarianism in the population (4). The high rates of low vitamin B12 that were reported in studies from Turkey, and also in Greece, may be linked to high obesity rates in these regions (4). In contrast, in Thailand and Sudan, <8% had low serum vitamin B12 levels, these findings were perceived to be a consequence of high consumption of fish and animal/fermented products (4).

4.2 Alterations in vitamin levels throughout pregnancy

We observed a decline in folate and vitamin B12 levels by 31 and 17% between the second to third trimester. This is in accordance with previous studies on vitamin status during pregnancy (6). The decline may be attributed to alterations during pregnancy such as weight gain, and hemodynamic changes, including alteration in blood composition and blood volume. Furthermore, estrogen exposure causes the concentration of haptocorrin and thus haptocorrin-bound vitamin B12 to decrease (44). These physiological changes will in turn lead to alterations of several serum parameters. A pregnant population can therefore have low total vitamin values without having a functional deficiency (44).

4.3 Geographical differences

The two-center concept gave us the opportunity to examine the geographical differences in vitamin levels between Trondheim and Stavanger. There was no significant difference between maternal levels of vitamin B12 throughout the pregnancy. Interestingly, mean serum folate level was significantly higher among participants from Trondheim in the third trimester. It is reasonable that this may be attributed to nutritional factors. Alcohol consumption is shown to have a negative effect on folate absorption (45). Surprisingly, 35% of the participants reported alcohol consumption during pregnancy (32). Unfortunately, we did not have data on the differences in general nutrition between the two groups, or whether participants in Trondheim consumed less alcohol than the participants from Stavanger.

4.4 Relation between folate/vitamin B12 and vitamin A and D

Vitamin A and D levels have previously been analyzed in the same population during pregnancy (34, 46). In third trimester, as many as 45% had inadequate vitamin A levels and 34% vitamin D insufficiency. We observed that women who displayed folate and vitamin B12 deficiency in the second trimester, tended to remain deficient at the end of the pregnancy. Moreover, those with vitamin B12 deficiency were more prone to exhibit low levels of vitamin D in both trimesters. In third trimester, 34% of the women displayed vitamin B12 deficiency, of these, 42% had vitamin A deficiency or inadequacy and 41% had vitamin D deficiency or insufficiency. This suggests that many of the study participants had an inadequate diet. Our findings are of concern, as a substantial number had low levels of one or more of these vitamins that are of significance for fetal development. This may translate to adverse health outcomes in adulthood. As elaborated on in the introduction, offspring of vitamin B12-deficient women are at increased risk for developmental abnormalities and anemia (1, 27, 30). We have previously shown that low maternal levels of vitamin A are associated with lower peak bone mass in the offspring at the age of 26 years, which implies increased fracture risk in the future (7). Others have shown an association between vitamin D levels and offspring peak bone mass (47).

The same pattern of concomitant hypovitaminosis was seen in women with folate deficiency, however, given the very small proportion of women displaying folate deficiency, it is difficult to draw any firm conclusion. Although, adherence to recommendations regarding folate seemed to be low (23), almost all study subjects had satisfactory serum levels in second and third trimester. However, we do not have data on folate levels the first months of pregnancy which are critical with respect to fetal development.

4.5 Folate and vitamin B12 levels and relation with BMI and exercise

We observed an inverse correlation between folate and vitamin B12 levels and BMI. This is in concordance with previous studies showing that a high maternal BMI is associated with lower levels of folate and vitamin B12 (48, 49). This negative correlation, has been suggested to be linked to intake of processed meat products which have reduced bioavailability of vitamin B12. A high consumption may thus increases the risk of both vitamin B12 deficiency and metabolic diseases. Sukumar et al (49) found that women with B12 insufficiency had higher odds of obesity and gestational diabetes (49). On the other hand, a prospective study in Bangladesh observed no significant difference in folate and B12 levels between participants who were underweight and overweight early in pregnancy (50).

Folate and vitamin B12 are involved in regulation of energy metabolism. Notably, the two vitamins are required as cofactors for energy production and the rebuilding and repair of muscle tissue by physical activity (51). In the present study, 71% of the participants reported to exercise regularly prior to pregnancy, 52% during the second trimester and 64% in the third trimester. The high rate of exercise is attributed to the fact that this was a RCT where the main outcome was to study the effect of an exercise program on the occurrence of gestational diabetes (32). Hence, the percentage of women who exercised was higher than in other studies, for instance the Norwegian Mother and Child cohort study, where the proportion of women who exercised (≥3 times a week) was 46% before pregnancy, and 28 and 20% in weeks 17 and 30, respectively. Moreover, the prevalence of training during pregnancy varies widely from studies reporting <15 % (23) to 66% (52). There is little information on the effects of exercise training on folate and vitamin B12 status, neither in the pregnant or non-pregnant state. We were not able to demonstrate clear tendencies to give a conclusion to whether exercise in pregnancy affects the vitamin B levels or not, and the existing literature and data on this question are sparse and tend to have contrary findings. A Korean study that, concluded that regular exercise decreased folate and increased B12 levels in rats. However, no significant changes in folate and vitamin B12 concentrations were observed by increasing duration of acute aerobic exercise (51). Another study, focusing on the effect of training and vitamin B levels among elderly, did not find any significant differences in folate or vitamin B12 levels in those who exercised regularly and those who did not (53).

4.6 Birth weight and preterm birth and association to folate and vitamin B12 levels

As mentioned, vitamin B is crucial for the rapid cell division in embryogenesis (5). It has been speculated that low levels affect fetal growth and placentation (4, 54), which may be a potential mechanism in development of LBW and PTB. However, we observed no significant correlation between LBW and levels of folate and vitamin B12. On the other hand, a systematic review from 2020, showed an inverse association between maternal folic acid supplementation and incidence of LBW and a positive association with birth weight in low- and middle-income countries (55). Two recent systematic reviews concluded with a possible association between low levels of vitamin B12 and LBW (4, 56). Most of the studies that found an association between LBW and B12 deficiency were conducted in India. As already mentioned, India has one of the highest prevalence in the world of B12 deficiency, due to a predominantly vegetarian diet. They also have among the highest incidences of LBW (4, 56). In other words, vitamin B12 deficiency may be a confounding factor for contribution to LBW in offspring in Indian subcontinent (4, 56).

A large number of studies have examined the association between folate deficiency (1, 57, 58), folate intake and preterm birth, with conflicting results. Maternal folate deficiency plays a contributory role in preterm birth, according to experimental animal studies (20). Several RCTs have attempted folic acid supplementation to prevent PTB, but systematic reviews and meta-analyses concluded that intervention was unsuccessful (59). However, a recent meta-analysis reported that increasing levels of vitamin B12 were associated with a reduced risk of PTB (56). About 4% of the participants (n=32) in our study experienced a PTB. We observed significantly lower levels of both folate and vitamin B12 in the second trimester in women with PTB compared to the whole population, however, not significant for the latter. In third trimester, the opposite pattern was seen, with elevated levels of both vitamins compared to the total population. We speculate that the low concentrations in second trimester are more likely to exert effects that could result in preterm birth. There was a large dropout rate of 35% within the PTB group. With only a few participants left in the PTB group, the results are vulnerable.

4.7 Significance of the study

In this large study of pregnant women, the participants were healthy and well-educated. In spite of that, we observed a high prevalence of vitamin B12 deficiency in third trimester, whereas folate deficiency was very rare. Previously, we have shown high prevalence of vitamin A inadequacy and vitamin D insufficiency in these women. The fact that a substantial number of women had concomitant vitamin B12 deficiency and hypovitaminosis A or D is of concern. Given that maternal hypovitaminosis may affect the fetus negatively, and induce health effects in the future, our findings are of concern. It is likely that hypovitaminosis during pregnancy is even more prevalent among less educated women. Thus, our data highlight the need for increased attention regarding requirement of vitamin B12, as well as vitamin A and D during pregnancy among policy-makers, physicians and the general population. The authorities' recommendations should be revisited, and strategies to ensure adherence should be implemented.

4.8 Strengths and limitations

A strength of this study is the large number of participants included (n=855), and the high follow-up rate (85%). Moreover, blood sampling at two time points during pregnancy was done in both the second and third trimester, the same instruments and procedures were followed. The two-centered concept enabled comparison between different geographical regions of Norway. However, the fact that participants were healthy, well-educated, with low-risk pregnancies may have affected the generalizability (33).

There are no standardized methods for measurements of folate and vitamin B12, which makes it more challenging to interpret and compare results from other regions of the world. There were few cases of LBW and PTB, therefore this study is not powered to detect small, but meaningful differences between these outcomes in associations to vitamins levels. Therefore, these associations need to be investigated in future RCTs to assess the validity of our findings.

5.0 Conclusion

In this comprehensive study of well-educated, healthy women, we observed a very low prevalence of folate deficiency, whereas vitamin B12 deficiency was highly prevalent, reaching 34% in third trimester. Of those with vitamin B12 deficiency, 18% had concomitant hypovitaminosis A or D. BMI was negatively correlated to levels of folate and vitamin B12. Vitamin B deficiency may be associated with adverse maternal and perinatal outcomes and can have a long term impact into adulthood. Therefore, our findings are of concern and these observations need to be elucidated.

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