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Lifestyle and genetic determinants of folate and vitamin B_{12} levels in a general adult population

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Danish legislation regarding food fortification has been very restrictive resulting in few fortified food items on the Danish market. Folate and vitamin B_{12} deficiency is thought to be common due to inadequate intakes but little is known about the actual prevalence of low serum folate and vitamin B_{12} in the general population. The aim of the present study was to evaluate the folate and vitamin B_{12} status of Danish adults and to investigate associations between vitamin status and distinct lifestyle and genetic factors. The study included a random sample of 6784 individuals aged 30–60 years. Information on lifestyle factors was obtained by questionnaires and blood samples were analysed for serum folate and vitamin B_{12} concentrations and several genetic polymorphisms. The overall prevalence of low serum folate ($<6{\cdot}8$ nmol/l) was 31·4 %. Low serum folate was more common among men than women and the prevalence was lower with increasing age. Low serum folate was associated with smoking, low alcohol intake, high coffee intake, unhealthy diet, and the TT genotype of the methylenetetrahydrofolate reductase (MTHFR)-C677T polymorphism. The overall prevalence of low serum vitamin B_{12} (<148 pmol/l) was 4·7 %. Low serum vitamin B_{12} was significantly associated with female sex, high coffee intake, low folate status, and the TT genotype of the MTHFR-C677T polymorphism. In conclusion, low serum folate was present in almost a third of the adult population in the present study and was associated with several lifestyle factors whereas low serum concentrations of vitamin B_{12} were less common and only found to be associated with a few lifestyle factors.

Serum folate: Serum vitamin B_{12}: Lifestyle: Genetics: General adult population

Fortification of cereal products with folic acid – the synthetic analogue to folate used in food fortification and nutritional supplements – was introduced in North America in 1998 to reduce the risk of pregnancies affected by neural tube defects. The mandatory fortification programme has caused a more than 100 % increase in median serum folate levels among the US population[1]. In European countries where no mandatory food fortification with folic acid or other B-vitamins has been introduced, low status of folate is believed to be common[2]. Besides the increased risk of neural tube defects, insufficient status of folate and/or related B-vitamins might contribute to the risk of several other diseases: CVD[3], neurological diseases[4] and certain cancers[5–7].

The B-complex vitamin folate facilitates the transfer of one-carbon units and is essential as a cofactor or coenzyme in a variety of biological processes: for example, synthesis and repair of DNA, regulation of gene expression, amino acid metabolism, neurotransmitter synthesis, and the formation of myelin[8]. Vitamin B_{12} (cobalamin) is required as a cofactor for the two enzymes methionine synthase and methylmalonyl CoA mutase. Methionine synthase catalyses the remethylation of homocysteine to methionine which is also dependent on folate; thus, folate and vitamin B_{12} intersect in this metabolic process[9]. Severe folate deficiency leads to megaloblastic anaemia while vitamin B_{12} deficiency may cause neurological symptoms in the form of myelin degeneration and irreversible cognitive impairment that is seen with or without the coincidence of anaemia.

The Danish legislation regarding food fortification has been very restrictive compared with most other European countries and no fortification with folate or vitamin B_{12} has been allowed until now. However, little is known about the actual prevalence of low folate and vitamin B_{12} status in the general Danish population, and it is not known if subgroups of the population owing to lifestyle factors or genetics are particularly at risk of insufficient folate and/or vitamin B_{12} status. Since there are no food items fortified with folate or vitamin B_{12} on the Danish market, Denmark offers a unique setting for studies of the effect of lifestyle and genetics on folate and vitamin B_{12} status. The aim of the present study was to evaluate the folate status and the vitamin B_{12} status of a general adult population in Denmark. In addition,

Abbreviations: MTHFR, methylenetetrahydrofolate reductase; SNP, single nucleotide polymorphism; TCN, transcobalamin gene.

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we investigated the influence of different lifestyle factors and polymorphisms of genes involved in the metabolism of folate and/or vitamin B_{12} on serum levels of folate and vitamin B_{12}.

Methods

Study population

The individuals included in the present study were participants in the Inter99 study. The study design and characteristics of the participants have been described in detail elsewhere[10]. In brief, an age- and sex-stratified random sample of 13 016 men and women born in 1939–40, 1944–5, 1949–50, 1954–5, 1959–60, 1964–5 and 1969–70 and living in eleven municipalities in the South-Western part of the former Copenhagen County was drawn from the Civil Registration System and invited to a health examination. A total of 12 934 individuals were eligible for invitation of whom 6784 (52·5 %) participated. In general the participation rate was higher in women than in men, and it increased with increasing age. In addition, non-responders had more hospital admissions related to chronic diseases such as diabetes and CVD[10]. The examinations of participants in the present study were completed from March 1999 to January 2001. The health examination included a self-administered questionnaire, a physical examination and various blood tests.

The Inter99 study has been approved by the ethics committee of Copenhagen County (KA 98 155) and the National Board of Health and the study was registered in the ClinicalTrials.gov (NCT00289237). An informed consent has been obtained from all participants.

Measurements of folate and vitamin B_{12}

After an overnight fast blood samples for measurements of serum levels of folate and vitamin B_{12} were collected into tubes and left for clotting before centrifugation. Serum samples were stored at −20°C until the analyses were performed in 2008. Serum folate and vitamin B_{12} concentrations were measured by using a competitive chemiluminescent enzyme immunoassay (Immulite® 2000 System; Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Serum levels of folate and vitamin B_{12} were successfully measured for 6371 (93·9 %) and 6216 (91·6 %) of the participants, respectively.

We used the following standard cut-offs: <6·8 nmol/l for low serum folate and <148 pmol/l for low serum vitamin B_{12}[11]. In addition, we used a lower cut-off value for folate deficiency (<4·0 nmol/l) in some analyses. This cut-off value is used to define folate deficiency in some clinical settings in Denmark.

Genotyping

Participants were genotyped for the following genetic variants with potential relevance to the metabolism of folate and/or vitamin B_{12}: two single nucleotide polymorphisms (SNP) in the methylenetetrahydrofolate reductase gene (MTHFR-C677T (rs1801133) and MTHFR-A1298C (rs1801131)), one in the methionine synthase gene (MTR-A2756G (rs1805087)), one in the methionine synthase reductase gene (MTRR-A66G (rs1801394)), one in the betaine:homocysteine methyltransferase gene (BHMT-G742A (rs3733890)) and two polymorphisms in the transcobalamin gene (TCN2-C6776G (rs1801198) and TCN2b (rs9606756)). The above-mentioned SNP were genotyped by TaqMan allelic discrimination (KBiosciences, Hoddesdon, Herts, UK). All genotyping success rates were above 96·2 % with a mismatch rate below 0·52 % in 384 duplicate samples and none of the observed genotype distributions deviated significantly from Hardy–Weinberg equilibrium ($P>0·2$).

Lifestyle factors

BMI was calculated as weight divided by height squared. Height and weight were measured wearing light clothes and no shoes. Participants were divided into categories based on their BMI according to the following criteria recommended by WHO[12]: underweight (BMI < 18·5 kg/m^2); normal range (BMI ≥ 18·5 to 25 kg/m^2); overweight (BMI ≥ 25 to 30 kg/m^2); obese (BMI ≥ 30 kg/m^2). The self-administered questionnaire provided information on several lifestyle factors. Smoking status was defined in four categories: never smokers; ex-smokers; occasional smokers (<1 g tobacco or cigarettes per d); daily smokers (≥1 g/d). Total alcohol intake was calculated by summation of self-reported weekly intake of beer, strong beer, wine, dessert wine and spirits. Thus, one beer, one glass of wine, or one glass of spirits was approximated to one standard unit (defined as 12 g pure alcohol) and a strong beer was calculated as 1·5 standard units. Five categories were defined from the calculated alcohol intake: 0 units/week; 1–7 units/week; 8–14 units/week; 15–21 units/week; 22 or more units/week. In addition, beer and wine intakes were divided into four categories: 0 units/week; 1–3 units/week; 4–7 units/week; 8 or more units/week. Five categories were defined from self-reported daily intake of coffee: 0 cups/d; 1–3 cups/d; 4–6 cups/d; 7–9 cups/d; 10 or more cups/d. A variable of total physical activity based on information on commuting and leisure time physical activity were used to form four groups of distinct levels of physical activity: 0–2 h/week; 2–4 h/week; 4–7 h/week; 7–12 h/week[13]. Dietary habits were measured using a validated dietary quality score developed from a forty-eight-item FFQ. The dietary score was developed as a crude index of overall quality of the dietary habits and a scoring system was used to divide the participants into three groups: healthy, average, and unhealthy dietary habits[14]. Dietary intake of folate and vitamin B_{12} was estimated by calculations based on a 198-item FFQ[15]. The study participants were divided into quartiles from their estimated intake of folate and vitamin B_{12}.

Statistical analyses

The statistical program SAS (version 9.2; SAS Institute Inc., Cary, NC, USA) was used for all analyses. For categorical outcome variables (low folate status and low vitamin B_{12} status), χ^2 tests were used to assess differences between groups. Multivariable logistic regression models were used to estimate OR for the independent effects of different lifestyle factors on low serum folate and vitamin B_{12} when taking into account possible confounding by other considered

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variables. Since the total alcohol variable includes both beer and wine intake, which may be reverse in their associations with folate and vitamin B_{12} status, beer and wine intake were included in the adjusted models whereas total alcohol intake was omitted except from models estimating the effect of total alcohol intake itself. In these models, none of the other alcohol-related variables was included. Potential effect modifications by genetic variants were evaluated by assessing the *P* values of the respective interaction terms (for example, MTHFR-C677T × lifestyle) in the regression models corrected for multiple testing (Bonferroni). *A priori* we decided only to test first-order interactions between SNP and each of the considered lifestyle factors whereas gene–gene and lifestyle–lifestyle interactions were not evaluated. *P* values of likelihood ratio tests were used to test for statistical significance in all logistic regression analyses.

One-way ANOVA was used to detect differences in continuous outcome variables (serum concentrations of folate and vitamin B_{12}) between study groups. To achieve normal distribution of the continuous outcome variables, measurements of serum folate and vitamin B_{12} were log-transformed. Multiple linear regression models were used for adjustment for potential confounding. Results were computed as percentage differences compared with the reference group corresponding to the back-transformed β-coefficients from the linear regression analyses on log-transformed outcomes multiplied by 100[16]. All *P* values are two-tailed and statistical significance was defined as $P<0{\cdot}05$.

Results

General characteristics of the study population are shown in Table 1. For serum folate, 95 % of the concentrations were in the range 3·4–26·3 (median 8·6) nmol/l, and 95 % of the serum vitamin B_{12} concentrations were between 128 and 702 (median 281) pmol/l. The prevalence of low serum folate and serum vitamin B_{12} concentrations stratified by sex and age group are shown in Table 2. The overall prevalence of low serum folate (< 6·8 nmol/l) was 31·4 % and it was more common among men than women. In addition, low serum folate was inversely associated with age, with a prevalence of 43·4 % among the 30-year-olds and 18·3 % among those aged 60 years. The overall prevalence of folate deficiency (< 4·0 nmol/l) was 5·1 %, and it was also inversely associated with age, while there was no significant difference between men and women. Correspondingly, the median and geometric mean values of the serum folate measurements were highest among those of oldest age and the measurements were significantly higher for women than men (data not shown). The inverse associations between low folate and folate deficiency and age were seen for both men and women when analyses were stratified by sex (data not shown).

The overall prevalence of low serum vitamin B_{12} (< 148 pmol/l) was 4·7 % and, in contrast to low serum folate, low serum vitamin B_{12} was significantly more common among women than men (8·4 *v.* 4·5 %) (Table 2). Low serum vitamin B_{12} was also inversely associated with age. However, this association was only seen for women, and the association disappeared when the statistical models were adjusted for folate status.

The influence of different lifestyle factors on the prevalence of low serum folate and folate deficiency is presented in Table 3. In crude logistic regression models low serum folate as well as folate deficiency were significantly associated with low folate intake, unhealthy diet, BMI (both underweight and obesity), low physical activity, high daily coffee intake, low total weekly alcohol intake, low weekly beer intake, low weekly wine intake, and daily smoking. In addition, the associations with folate intake, diet, physical activity, coffee intake and alcohol intake (total, beer, and wine) demonstrated dose-dependency. Regarding total alcohol intake, beer intake, and wine intake the dose-dependent associations were inverse, showing decreased risk of low serum folate and folate deficiency with increasing intake. When adjusting for confounding by sex, age and lifestyle factors in multiple logistic regression models, the effect of physical activity disappeared. However, the other associations between lifestyle factors and folate status remained significant even though the estimated OR to some degree were attenuated. Results from linear regression models with serum folate measurements as outcome (data not shown) confirmed the results from the logistic regression models in Table 3. In the crude models all the considered lifestyle factors were associated with folate status while the effect of physical activity disappeared when adjusting for confounding.

When looking at the results from unadjusted statistical models, significant associations were found between the prevalence of low serum vitamin B_{12} and high total alcohol intake, high beer intake, a daily coffee intake of 7–9 cups, and low estimated intake of vitamin B_{12} (Table 4). When adjusting for confounding by sex, age, and all considered lifestyle factors, only the effect from coffee intake and estimated intake of vitamin B_{12} remained significant. The effect of sex seen on serum vitamin B_{12} in Table 2 disappeared in the adjusted models. In addition, the highly significant associations with age indicated in Table 2 vanished when further adjusting for serum folate. There were significant associations between low serum folate and low serum vitamin B_{12} in the logistic regression model ($P<0{\cdot}001$) and between serum folate and serum vitamin B_{12} measurements in the linear regression model ($P<0{\cdot}001$).

Table 1. General characteristics of the population (*n* 6784)

	Prevalence (%)	Missing data (*n*)
Male subjects	48·7	0
BMI ≥ 30 kg/m^2	17·6	4
Daily smokers	35·8	58
Total alcohol intake ≥ 22 units/week	12·4	337
Beer intake ≥ 8 units/week	20·5	337
Wine intake ≥ 8 units/week	16·1	337
Coffee intake ≥ 10 cups/d	12·6	214
Physical activity ≤ 2 h/week	12·9	480
Unhealthy dietary habits	15·7	24
Age (years)		
Median	45	0
2·5, 97·5 percentiles	30, 60	
Folate (nmol/l)		
Median	8·6	413
2·5, 97·5 percentiles	3·4, 26·3	
Vitamin B_{12} (pmol/l)		
Median	281	568
2·5, 97·5 percentiles	128, 702	

Table 2. Serum folate and vitamin B_{12} according to age and sex in a general adult Danish population

		Folate (*n* 6371)				Vitamin B_{12} (*n* 6216)	
		Prevalence < 6·8 nmol/l†		Prevalence < 4·0 nmol/l†		Prevalence < 148 pmol/l†	
	Prevalence (%)*	%	n/n_{total}	%	n/n_{total}	%	n/n_{total}
All		31·4	2003/6371	5·1	325/6371	4·7	312/6216
Sex							
Men	49	32·6	1019/3123	5·0	157/3123	4·5	119/3043
Women	51	30·3	984/3248	5·2	168/3248	8·4	193/3173
P		0·045		0·792		< 0·001	
Age (years)							
30	5	43·4	139/320	7·5	24/320	8·8	28/317
35	10	38·5	253/658	7·5	49/658	6·9	44/638
40	20	38·0	474/1246	5·6	70/1246	5·0	61/1223
45	20	32·5	424/1304	5·8	75/1304	4·8	61/1275
50	21	28·4	379/1334	4·6	61/1334	4·8	63/981
55	16	24·0	244/1017	3·0	30/1017	3·8	37/981
60	8	18·3	90/492	3·3	16/492	3·8	18/476
P		< 0·001		< 0·001		0·004	

* Prevalence in total sample of 6784 subjects.
† Differences in prevalence of low serum folate and vitamin B_{12} between groups were tested by χ^2 statistical tests. *P* values relate to tests for differences between groups.

The only SNP showing significant association with serum folate and serum vitamin B_{12} was MTHFR-C677T (Table 5). The TT genotype of this variant was associated with increased prevalence of low serum folate (OR 2·24; 95 % CI 1·85, 2·70; $P<0·001$)) as well as low serum vitamin B_{12} (OR 1·78; 95 % CI 1·25, 2·54; $P=0·003$)) compared with the CC genotype. Correspondingly, it is seen from Table 5 that the geometric mean values of serum folate and serum vitamin B_{12} were significantly lowered by the TT genotype. None of the other tested SNP was consistently associated with serum folate or serum vitamin B_{12} although the GG genotype of the MTR-A2756G seemed to be associated with a decreased prevalence of low serum folate (OR 0·68; 95 % CI 0·50, 0·92; $P=0·017$). However, no significant effect was seen on the geometric mean values. As expected from the principles of Mendelian randomisation[17], there were no associations between genotypes and the considered lifestyle factors and therefore no statistical models including adjustments for confounding by lifestyle factors were applied. In addition, no significant interactions between SNP and lifestyle factors were found after correction for multiple testing.

Discussion

The present study demonstrates that low folate status is common among Danish adults. Almost a third of the participants had serum folate levels < 6·8 nmol/l and levels < 4·0 nmol/l were found in serum from 5·1 % of the population, whereas the overall prevalence of low serum vitamin B_{12} in the present study was 4·7 %. We found that serum folate was associated with nearly all the considered lifestyle factors in addition to the MTHFR-C677T polymorphism whereas serum vitamin B_{12} was only related to diet, BMI and coffee intake besides the MTHFR-C677T polymorphism.

The serum concentrations of folate measured in the present study may have been influenced by decomposition of folate during storage of the serum samples at −20°C. In contrast, vitamin B_{12} is thought to be more stable and resistant to handling and storage[18,19]. From a number of studies, folate in serum samples is known to be unstable at room temperature wherefore folate measurements are vulnerable to sample processing and delayed freezing[18,20]. In addition, a few studies have demonstrated that folate also deteriorates over time at −20°C[19,21] and thus the prevalence of low serum folate may be overestimated in the present study. In addition, serum folate levels show variation due to recent intake of folate, whereas erythrocyte folate is more stable and reflects average body folate status. Therefore erythrocyte folate would probably have been a better indicator of folate status in the present study, but such measurements were not available. In some clinical settings in Denmark, high total homocysteine (> 15 μmol/l) is used to identify patients with likely folate deficiency. We have previously reported data on total homocysteine measurements in a randomly selected subgroup (*n* 2788) of the population included in the present study, and the prevalence of high total homocysteine in that subgroup was 4·6 %, which was very similar to the prevalence of serum folate concentrations < 4·0 nmol/l (4·8 %) (data not shown). In addition, there was a highly significant association between low serum folate and high total homocysteine ($P<0·0001$). By assuming high total homocysteine (> 15 μmol/l) as the 'gold standard' for defining inadequate folate status, the specificity and sensitivity of using low serum folate (< 4·0 nmol/l) as an indicator of inadequate folate status were 97 and 37 %, respectively (OR 18·3; 95 % CI 12·0, 27·8).

As discussed below, the associations found in the present study may be influenced by the lack of information about use of supplements, which is a limitation of the present study. On the other hand, the reported associations between vitamin levels and lifestyle or genetic factors are probably not affected by the potential decomposition of folate during storage since the decline in absolute concentrations is thought to be non-differential and thus independent of genetic and lifestyle factors[19]. In addition, the previously reported data on total homocysteine showed associations with lifestyle factors and the MTHFR-C677T genotype resembling those found for serum folate in the present study[22].

Table 3. Associations between lifestyle factors and serum folate in a general adult Danish population (*n* 6371)*

Folate...	<6·8 nmol/l						<4·0 nmol/l					
	Prevalence (%)	n/n_{total}†	Crude OR	95 % CI	Adjusted OR‡	95 % CI	Prevalence (%)	n/n_{total}†	Crude OR	95 % CI	Adjusted OR§	95 % CI
BMI (kg/m^2)												
Underweight (<18·5)	41·2	28/68	1·63	1·00, 2·66	1·23	0·68, 2·22	16·2	11/68	3·74	1·91, 7·29	2·42	1·00, 5·82
Normal (≥18·5–25)	30·1	808/2688	1·00	Reference	1·00	Reference	4·9	132/2688	1·00	Reference	1·00	Reference
Overweight (≥25–30)	31·5	792/1602	1·07	0·95, 1·21	1·13	0·99, 1·30	4·6	115/1602	0·93	0·72, 1·20	0·97	0·72, 1·31
Obese (≥30)	34·3	374/1099	1·20	1·03, 1·39	1·36	1·14, 1·62	6·0	66/1099	1·24	0·91, 1·68	1·37	0·96, 1·96
P	0·032		0·035		0·009		<0·001		0·002		0·076	
Smoking												
Daily smokers	37·8	852/2255	1·54	1·36, 1·74	1·54	1·32, 1·79	7·8	176/2255	2·28	1·74, 3·00	2·11	1·51, 2·95
Occasional smokers	27·6	62/225	0·96	0·71, 1·31	1·04	0·73, 1·47	4·0	9/225	1·12	0·56, 2·27	1·59	0·77, 3·31
Ex-smokers	27·9	447/1602	0·98	0·85, 1·13	1·11	0·94, 1·30	3·7	59/1602	1·03	0·73, 1·45	1·25	0·84, 1·85
Never smokers	28·3	634/2239	1·00	Reference	1·00	Reference	3·6	80/2239	1·00	Reference	1·00	Reference
P	<0·001		<0·001		<0·001		<0·001		<0·001		<0·001	
Total alcohol (units/week)												
0	42·3	254/601	1·00	Reference	1·00	Reference	11·0	66/601	1·00	Reference	1·00	Reference
1–7	34·8	948/2721	0·73	0·61, 0·87	0·83	0·67, 1·02	5·6	153/2721	0·48	0·36, 0·65	0·66	0·46, 0·93
8–14	28·8	376/1309	0·55	0·45, 0·67	0·61	0·48, 0·77	2·9	38/1309	0·24	0·16, 0·37	0·35	0·22, 0·55
15–21	27·8	189/680	0·53	0·42, 0·66	0·52	0·40, 0·68	3·8	26/680	0·32	0·20, 0·51	0·35	0·20, 0·61
22+	16·4	125/761	0·27	0·21, 0·35	0·26	0·20, 0·35	2·2	17/761	0·19	0·11, 0·32	0·23	0·13, 0·41
P	<0·001		<0·001		<0·001		<0·001		<0·001		<0·001	
Beer (units/week)												
0	34·9	770/2205	1·00	Reference	1·00	Reference	7·0	154/2205	1·00	Reference	1·00	Reference
1–3	33·3	694/2086	0·93	0·82, 1·05	0·83	0·71, 0·96	4·8	100/2086	0·67	0·52, 0·87	0·75	0·55, 1·01
4–7	31·6	250/791	0·86	0·72, 1·02	0·65	0·53, 0·80	3·8	30/791	0·53	0·35, 0·78	0·53	0·34, 0·84
8+	18·0	178/990	0·41	0·34, 0·49	0·29	0·23, 0·37	1·6	16/990	0·22	0·13, 0·37	0·19	0·10, 0·33
P	<0·001		<0·001		<0·001		<0·001		<0·001		<0·001	
Wine (units/week)												
0	35·8	472/1318	1·00	Reference	1·00	Reference	8·5	112/1318	1·00	Reference	1·00	Reference
1–3	34·2	758/2217	0·93	0·81, 1·07	1·07	0·90, 1·26	5·0	110/2217	0·56	0·43, 0·74	0·79	0·57, 1·08
4–7	25·6	329/1287	0·62	0·52, 0·73	0·78	0·64, 0·95	2·9	37/1287	0·32	0·22, 0·47	0·52	0·33, 0·80
8+	26·6	333/1250	0·65	0·55, 0·77	0·87	0·72, 1·07	3·3	41/1250	0·37	0·25, 0·53	0·60	0·39, 0·91
P	<0·001		<0·001		0·002		<0·001		<0·001		0·010	
Coffee (cups/d)												
0	29·0	220/759	1·00	Reference	1·00	Reference	6·5	49/759	1·00	Reference	1·00	Reference
1–3	29·2	506/1734		0·84, 1·22	1·20	0·97, 1·49	3·6	62/1734	0·54	0·37, 0·79	0·62	0·40, 0·96
4–6	29·6	661/2233	1·03	0·86, 1·23	1·22	0·99, 1·50	4·1	91/2233	0·62	0·43, 0·88	0·71	0·47, 1·07
7–9	34·8	232/666	1·31	1·05, 1·64	1·54	1·19, 2·00	5·6	37/666	0·85	0·55, 1·32	0·87	0·52, 1·46
10+	41·5	323/779	1·74	1·40, 2·15	1·69	1·31, 2·17	9·2	72/779	1·48	1·01, 2·15	1·08	0·68, 1·72
P	<0·001		<0·001		<0·001		<0·001		<0·001		0·042	
Physical activity (h/week)												
0–2	36·7	277/754	1·45	1·17, 1·80	1·06	0·83, 1·36	7·0	53/754	2·00	1·25, 3·19	1·13	0·67, 1·91
2–4	34·0	451/1326	1·29	1·06, 1·57	1·10	0·88, 1·36	5·7	76/1326	1·60	1·03, 2·50	1·08	0·67, 1·76
4–7	30·2	930/3084	1·08	0·91, 1·29	0·99	0·81, 1·20	4·6	141/3084	1·26	0·84, 1·91	0·96	0·62, 1·50
7–12	28·6	219/767	1·00	Reference	1·00	Reference	3·7	28/767	1·00	Reference	1·00	Reference
P	<0·001		<0·001		0·567		0·007		0·008		0·795	
Diet												
Unhealthy	43·6	422/967	3·17	2·57, 3·91	2·20	1·72, 2·83	10·3	100/967	6·20	3·63, 10·60	2·94	1·59, 5·44
Average	31·2	1343/4303	1·86	1·55, 2·22	1·60	1·31, 1·95	4·6	197/4303	2·58	1·54, 4·32	1·84	1·04, 3·23
Healthy	19·6	172/876	1·00	Reference	1·00	Reference	1·8	16/876	1·00	Reference	1·00	Reference

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Table 3. *Continued*

Folate…	< 6·8 nmol/l					< 4·0 nmol/l				
	Prevalence (%)	n/n_{total}†	Crude OR	95 % CI	Adjusted OR‡	95 % CI	Prevalence (%)	n/n_{total}†	Crude OR	95 % CI	Adjusted OR§	95 % CI
P	< 0·001		< 0·001		< 0·001		< 0·001		< 0·001		< 0·001	
Estimated folate intake (μg/d)												
Low (< 272)	38·9	606/1559	2·00	1·71, 2·33	1·58	1·32, 1·90	9·0	141/1559	3·80	2·65, 5·43	3·21	2·05, 5·04
Low–middle (≥ 272–350)	33·6	516/1538	1·59	1·36, 1·86	1·30	1·09, 1·56	4·7	72/1538	1·87	1·27, 2·78	1·84	1·15, 2·95
Middle–high (≥ 350–449)	28·8	451/1567	1·27	1·08, 1·49	1·12	0·93, 1·34	4·1	64/1567	1·63	1·09, 2·439	1·88	1·17, 3·01
High (≥ 449)	24·1	378/1567	1·00	Reference	1·00	Reference	2·6	40/1567	1·00	Reference	1·00	Reference
P	< 0·001		< 0·001		< 0·001		< 0·001		< 0·001		< 0·001	

* *P* values relate to tests for differences between groups.
† Differences in prevalence of low serum folate between groups are tested by χ^2 statistical tests.
‡ Adjusted logistic regression model including all considered lifestyle factors (except from alcohol intake) together with sex and age. To estimate OR for different categories of alcohol intake, beer and wine intake were excluded from the adjusted model. The effects of sex and age were also significant ($P < 0·001$) in the adjusted models. The adjusted models included 5368 participants with complete information on all considered variables.
§ Adjusted logistic regression model including all considered lifestyle factors (except from alcohol intake) together with sex and age. To estimate OR for different categories of alcohol intake, beer and wine intake were excluded from the adjusted model. The effects of sex and age were also significant ($P = 0·012$ and $P = 0·019$, respectively) in the adjusted models. The adjusted models included 5368 participants with complete information on all considered variables.

Lifestyle

As expected, serum folate measurements were positively associated with estimated dietary intakes of folate and consumption of a healthy diet[2,23]. In accordance with other studies, we found an association between smoking and low serum folate[24–26]. The exact mechanisms behind the effect of smoking are not identified but smoking may inhibit enzymes such as methionine synthase[27] or may interact with the remethylation of homocysteine to methionine and thereby possibly alter the ability of the cell to store and metabolise folate[25].

The dose-dependent inverse association between serum folate and coffee intake is also consistent with another recent report[28], and in several other studies, high coffee intake has been associated with high total homocysteine indicating low folate status[22,27,29,30]. However, the present results indicate that the effect of coffee does not manifest itself at low-range folate concentrations since the prevalence of serum folate < 4 nmol/l was not affected by coffee consumption to the same degree. In fact, the results suggested a weak beneficial effect of a low to moderate coffee intake compared with no coffee intake. Our findings support a recent report by Ulvik *et al.*[28] who concluded that coffee consumption preferentially affects the upper, but not the lower, part of the B-vitamin concentration distributions[28]. The mechanisms underlying the effect of coffee consumption are largely unknown. Caffeine has been proposed to inhibit the conversion of homocysteine to cysteine by acting as a vitamin B_6 antagonist[31]. Another hypothesis is that coffee consumption simply causes low folate status by increasing the loss of folate by urinary excretion[28].

Studies examining relationships between alcohol consumption and serum folate or total homocysteine have been inconsistent and the overall results indicate that the relationship is complex[27]. In the present study, we found a dose-responsive positive association between serum folate and both total alcohol intake and beer intake. Consumption of wine was less strongly associated with serum folate. The present results are in line with associations between total homocysteine and alcohol intake previously reported in a subgroup of this cohort[22]. Consumption of alcohol has been suggested to be associated with total homocysteine in a J-shaped manner[27], but in a recent randomised intervention study even a moderate alcohol (red wine or vodka) intake was associated with elevated total homocysteine and decreased levels of folate and vitamin B_{12}[32]. In another intervention study, van der Gaag *et al.*[33] reported decreased folate levels after intake of spirits but they found no effect on vitamin B_{12}. After all, the effect of alcohol seems to depend on the type of alcoholic beverage consumed[27,33], and B-vitamins present in beer[33] may to some degree be responsible for the positive effect of beer drinking on serum folate found in the present study. In addition, this may also explain the association with total alcohol since beer constitutes a major part of the total alcohol intake in this population.

Regarding vitamin B_{12}, we found significant positive effects of estimated intake of the vitamin and a healthy diet. A correlation between vitamin B_{12} intake and vitamin B_{12} status has previously been shown even though the results are not consistent[2]. Besides, the only factors affecting serum

Table 4. Associations between lifestyle factors and serum vitamin B_{12} in a general adult Danish population (*n* 6216)*

	Prevalence < 148 pmol/l (%)	n/n_{total}†	Crude OR	95 % CI	Adjusted OR‡	95 % CI
BMI (kg/m^2)						
Underweight	12·5	8/64	2·93	1·37, 6·28	1·23	0·37, 4·14
Normal	4·7	123/2644	1·00	Reference	1·00	Reference
Overweight	5·0	123/2442	1·09	0·84, 1·40	1·41	1·05, 1·90
Obese	5·3	56/1064	1·14	0·82, 1·57	1·26	0·85, 1·87
P	0·038		0·098		0·147	
Smoking						
Daily smokers	5·3	117/2195	1·15	0·87, 1·51	1·37	0·98, 1·92
Occasional smokers	4·0	9/224	0·85	0·43, 1·71	0·99	0·47, 2·09
Ex-smokers	4·9	76/1566	1·04	0·77, 1·41	1·15	0·81, 1·62
Never smokers	4·7	102/2182	1·00	Reference	1·00	Reference
P	0·689		0·686		0·308	
Total alcohol (units/week)						
0	7·2	43/594	1·00	Reference	1·00	Reference
1–7	4·9	129/2661	0·65	0·46, 0·93	0·79	0·53, 1·19
8–14	4·7	60/1288	0·63	0·42, 0·94	0·91	0·58, 1·45
15–21	3·5	23/654	0·47	0·28, 0·78	0·64	0·35, 1·17
22+	4·0	29/731	0·53	0·33, 0·86	0·90	0·51, 1·57
P	0·024		0·032		0·541	
Beer						
0	5·8	125/2153	1·00	Reference	1·00	Reference
1–3	4·6	94/2049	0·78	0·59, 1·03	0·91	0·66, 1·26
4–7	4·2	32/767	0·71	0·47, 1·05	0·88	0·55, 1·41
8+	3·4	33/959	0·58	0·39, 0·86	0·78	0·48, 1·26
P	0·023		0·022		0·773	
Wine (units/week)						
0	6·1	78/1284	1·00	Reference	1·00	Reference
1–3	4·1	89/2175	0·66	0·48, 0·90	0·71	0·49, 1·01
4–7	5·2	65/1261	0·84	0·60, 1·18	0·98	0·66, 1·47
8+	4·3	52/1208	0·70	0·49, 1·00	0·86	0·56, 1·30
P	0·047		0·052		0·175	
Coffee (cups/d)						
0	6·7	49/728	1·00	Reference	1·00	Reference
1–3	6·2	105/1699	0·91	0·64, 1·30	0·86	0·57, 1·28
4–6	4·2	92/2182	0·61	0·43, 0·87	0·69	0·46, 1·03
7–9	2·3	15/654	0·33	0·18, 0·59	0·34	0·17, 0·66
10+	4·3	33/761	0·63	0·40, 0·99	0·60	0·35, 1·02
P	< 0·001		< 0·001		0·008	
Physical activity (h/week)						
0–2	4·3	32/738	0·92	0·56, 1·50	0·65	0·37, 1·12
2–4	5·6	72/1294	1·19	0·79, 1·80	0·83	0·53, 1·30
4–7	4·9	147/3016	1·04	0·71, 1·51	0·87	0·58, 1·30
7–12	4·7	35/743	1·00	Reference	1·00	Reference
P	0·624		0·628		0·443	
Diet						
Unhealthy	6·3	59/943	1·71	1·10, 2·65	1·63	0·98, 2·72
Average	4·9	207/4208	1·32	0·91, 1·94	1·18	0·78, 1·80
Healthy	3·8	32/851	1·00	Reference	1·00	Reference
P	0·051		0·051		0·122	
Estimated intake of vitamin B_{12} (μg/d)						
Low (< 3·58)	8·47	127/1499	2·53	1·83, 3·51	2·01	1·37, 2·95
Low–middle (≥ 3·58–5·22)	4·88	75/1537	1·40	0·98, 2·01	1·28	0·86, 1·91
Middle–high (≥ 5·22–7·60)	2·71	41/1511	0·76	0·51, 1·15	0·64	0·40, 1·02
High (≥ 7·60)	3·52	54/1532	1·00	Reference	1·00	Reference
P	< 0·001		< 0·001		< 0·001	

* *P* values relate to tests for differences between groups.

† Differences in prevalence of low serum vitamin B_{12} between groups were tested by χ^2 statistical tests.

‡ Adjusted logistic regression model including all considered lifestyle factors (except from alcohol intake) together with sex and age. To estimate OR for different categories of alcohol intake, beer and wine intake were excluded from the adjusted model. The effect of age was also significant (*P*=0·007) in the adjusted models while the sex effect was no longer significant (*P*=0·179). When further adjusting for serum folate, the effect of age disappeared too (*P*=0·106) while the other associations were not affected. The adjusted models included 5243 participants with complete information on all considered variables.

vitamin B_{12} in the present study were obesity and coffee intake. In contrast to serum folate, vitamin B_{12} seemed to be positively associated with coffee consumption. These results do not support previous reports from observational[28] and randomised intervention[34,35] studies showing no associations between coffee consumption and vitamin B_{12}. However, an earlier study[36] indicated that coffee may increase the absorption of vitamin B_{12}, which could explain our findings.

A major limitation of the present study is the lack of information on the use of dietary supplements. It has been estimated that about 50 % of the adult Danish population use multivitamin supplements normally containing both

Table 5. Influence of genetic polymorphisms on serum folate and vitamin B_{12} in a general adult Danish population*

		Folate (nmol/l)				Vitamin B_{12} (pmol/l)			
		< 6·8 nmol/l				< 148 pmol/l			
	Prevalence (%)	Prevalence (%)	n/n_{total}†	Geometric mean‡	95 % CI	Prevalence (%)	n/n_{total}†	Geometric mean‡	95 % CI
MTHFR-(C677T) rs1801133 (*n* 5963/5820)									
CC	50·1	27·5	822/2994	9·3	9·2, 9·5	4·9	142/2920	293	288, 297
CT	41·0	33·9	827/2437	8·6	8·4, 8·8	4·5	107/2385	287	282, 292
TT	8·9	45·9	244/532	7·5	7·2, 7·9	8·4	43/515	274	264, 285
P		< 0·001		< 0·001		0·001		0·004	
MTHFR-(A1298C) rs1801131 (*n* 5985/5843)									
AA	44·0	31·8	833/2620	8·8	8·7, 9·0	4·8	122/2561	289	284, 294
CA	45·0	32·4	878/2708	8·8	8·6, 9·0	5·3	140/2634	286	282, 291
CC	11·0	29·5	194/657	9·1	8·8, 9·5	4·3	28/648	295	286, 304
P		0·360		0·205		0·479		0·279	
MTR-(A2756G) rs1805087 (*n* 5966/5826)									
AA	64·6	31·7	1219/3840	8·8	8·7, 9·0	5·0	186/3754	289	285, 293
GA	31·4	32·9	621/1885	8·8	8·6, 9·0	5·2	96/1834	287	281, 293
GG	4·0	24·1	58/241	9·4	8·8, 10·0	3·8	9/238	295	280, 310
P		0·020		0·172		0·615		0·660	
MTRR-(A66G) rs1801394 (*n* 6019/5873)									
AA	19·1	31·9	365/1144	9·0	8·7, 9·3	4·6	51/1115	289	282, 297
GA	48·2	32·0	931/2906	8·7	8·5, 8·9	5·4	154/2840	287	283, 292
GG	32·7	31·5	620/1969	9·0	8·8, 9·2	4·6	89/1918	289	284, 294
P		0·920		0·065		0·365		0·838	
BHMT-(G742A) rs3733890 (*n* 6019/5874)									
GG	51·2	31·5	972/3082	8·9	8·7, 9·0	4·6	139/3007	287	282, 291
GA	40·5	32·2	785/2441	8·8	8·6, 9·0	5·3	127/2380	289	284, 294
AA	8·3	31·9	158/496	8·9	8·5, 9·3	4·7	23/487	294	283, 305
P		0·886		0·962		0·475		0·458	
TCN2-(C6776G) rs1801198 (*n* 5963/5821)									
CC	31·2	31·0	581/1873	8·9	8·7, 9·2	5·3	97/1829	288	282, 294
GC	49·0	32·4	954/2920	8·8	8·7, 9·0	4·8	136/2835	289	285, 294
GG	19·8	31·0	363/1170	8·8	8·5, 9·1	4·8	56/1157	289	282, 296
P		0·539		0·635		0·722		0·952	
TCN2b rs9606756 (*n* 5995/5846)									
AA	78·0	31·9	1490/4677	8·9	8·7, 9·0	4·8	219/4562	289	285, 292
GA	20·5	31·6	389/1232	8·8	8·5, 9·0	5·7	68/1198	287	281, 294
GG	1·5	31·4	27/86	9·1	8·1, 10·2	2·3	2/86	293	269, 319
P		0·979		0·680		0·244		0·891	

MTHFR, methylenetetrahydrofolate reductase; MTR, methionine synthase; MTRR, methionine synthase reductase; BHMT, betaine:homocysteine methyltransferase; TCN, transcobalamin.

* *P* values relate to tests for differences between groups.

† Differences in prevalence of low serum folate and vitamin B_{12} between groups were tested by χ^2 statistical tests.

‡ Differences in geometric means between groups were tested by one-way ANOVA.

folate and vitamin B_{12}, and 6 % use vitamin B products (either single supplements or complexes)[37]. In a Danish study, supplement use was strongly associated with age and sex, being highest among elderly women[37], and, in general, supplement use seems to be associated with a healthier lifestyle profile and an already adequate nutritional intake[38]. Users of dietary supplements have been found to be less likely to smoke, less likely to be obese, to drink less alcohol, and to exercise more than non-users[37,38]. Therefore, unknown use of folate and vitamin B_{12}-containing supplements may to some degree have influenced the associations with obesity (BMI $\geq$ 30 kg/m^2), diet, age, sex and daily smoking found in the present study. However, the present results regarding lifestyle factors are consistent with previous findings in studies where only non-supplement users have been included or where adjustment for supplementary intake was feasible. Lack of information on use of supplements is unlikely to explain the somehow surprising effects of alcohol since individuals with a high alcohol intake are less likely to use dietary supplements than those with a lower intake so that potential confounding in this case rather would under- than overestimate the association.

It should also be acknowledged that the estimated prevalence of low folate and B_{12} status may be influenced by non-participation in the survey. For example, it has been indicated that non-responders were more likely to smoke and less likely to be overweight than those who participated in the examination[10] and this may have influenced the estimates of associations in the present study.

Genetics

The MTHFR-C677T polymorphism has been extensively studied and the association between the TT genotype and low folate status is well documented[39–42]. Individuals with the TT genotype seem to be particularly susceptible to insufficient status of several B vitamins, and they may need to consume more folate to maintain serum folate levels similar to those found in individuals with the CC/CT genotypes[39,40].

Therefore, these individuals might be candidates for personalised nutritional recommendations. In contrast to previous studies[39,42], we also found a significant association between the MTHFR-C677T polymorphism and vitamin B_{12}.

The remaining SNP included in the present study have been considered as potential risk factors for neural tube defects due to their involvement in the metabolism of folate and some have previously been associated with altered folate status[41,43–45]. In addition, mutations in the transcobalmin gene (TCN2) are known to alter the cellular availability of vitamin B_{12}[45]. However, we found no associations between these SNP and serum concentrations of folate and/or vitamin B_{12}.

Conclusions and perspectives

In conclusion, we found that the prevalence of low serum folate was very common and was significantly associated with several common lifestyle and genetic factors in this general adult population where – by regulation – fortification of foods has not been allowed. Low serum vitamin B_{12} was less common and was only associated with a few lifestyle factors. Thus, the present results suggest that in populations without fortification many lifestyle and genetic factors influence folate levels and thereby that the vitamin status of the general population may be improved by introducing lifestyle changes. Thus, the findings may reinforce some current recommendations, for example, on healthy diet and smoking cessation. However, recommendations on supplementation to subgroups such as TT individuals of the MTHFR-C677T polymorphism are controversial and need further investigations in randomised studies.

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B. H. T., L. L. N. H., A. L. and L. O. contributed to the development of the hypothesis and study design. T. J. was the principal investigator of the Inter99 study and responsible for data collection. M. F. performed the micronutrient analyses. B. H. T. performed the statistical analyses, wrote the first draft and coordinated the completion of the paper. All authors contributed to the interpretation of results, the revision of the manuscript, and have approved the final version of the paper.

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