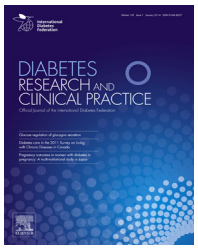
Contents available at ScienceDirect

Diabetes Research and Clinical Practice

journal homepage: www.elsevier.com/locate/diabres

International Diabetes Federation



Vitamin B status in patients with type 2 diabetes mellitus with and without incipient nephropathy

Wilfred A. Nix[a,*], Rudolf Zirwes[b], Volkhard Bangert[b], Raimund Peter Kaiser[c], Matthias Schilling[d], Ulrike Hostalek[e], Rima Obeid[f]

[a] Akademie für Ärztliche Fortbildung in Rheinland-Pfalz, Mainz, Germany
[b] Private Practice, Daun, Germany
[c] Private Practice, Kelberg, Germany
[d] Private Practice, Wittlich, Germany
[e] Merck KGaA, Darmstadt, Germany
[f] Aarhus Institute of Advanced Studies, Aarhus University, D-8000, Aarhus C, Denmark

ARTICLE INFO

Article history:
Received 11 March 2014
Received in revised form
27 May 2014
Accepted 14 September 2014
Available online 8 October 2014

Keywords:
Vitamin B deficiency
Type 2 diabetes
Microalbuminuria
Pyridoxal 5′-phosphate

ABSTRACT

Aim: To investigate the vitamin B status, with particular focus on vitamin B_6, in adults with and without incipient nephropathy secondary to type 2 diabetes mellitus.

Methods: Plasma and/or urine concentrations of vitamins B_6, B_1, B_{12}, related vitamers and biomarkers (including total homocysteine, methylmalonic acid) were measured in 120 adults with type 2 diabetes (including 46 patients with microalbuminuria) and 52 non-diabetic control subjects.

Results: Plasma concentrations of pyridoxal 5′-phosphate (PLP) were significantly lower in patients with type 2 diabetes than in control subjects (median: 22.7 nmol/L, diabetes with microalbuminuria; 26.8 nmol/L, diabetes without microalbuminuria; 39.5 nmol/L, non-diabetic control; $p < 0.0001$). The prevalence of low PLP (<30 nmol/L) was 63%, 58%, and 25% in the diabetes groups with and without microalbuminuria and the control group, respectively. Plasma levels of pyridoxine and pyridoxal were also lower ($p < 0.0001$), but levels of pyridoxamine, pyridoxamine 5′-phosphate, and pyridoxic acid were higher in both groups with diabetes compared to the control group ($p < 0.001$). Thiamine deficiency was highly prevalent in all groups, whereas low vitamin B_{12} and elevated methylmalonic acid were rare. Increased levels of C-reactive protein and soluble vascular cell adhesion molecule-1 were observed in the groups with diabetes ($p < 0.05$, versus healthy control).

Conclusions: Deficiency of vitamin B_6 (PLP, pyridoxine, pyridoxal) and vitamin B_1 (thiamine) was prevalent in type 2 diabetes. Incipient nephropathy was associated with more pronounced alterations in vitamin B_6 metabolism and stronger indications of endothelial dysfunction and inflammation.

* *Corresponding author*. Tel.: +49 6131 2843811; fax: +49 6131 2843810.
E-mail address: nix@arztkolleg.de (W.A. Nix).

http://dx.doi.org/10.1016/j.diabres.2014.09.058

1. Introduction

High blood glucose concentration promotes a variety of reactions, including oxidative stress and formation of advanced glycosylated end products, which have been associated with structural and functional changes in blood vessels that eventually cause dysfunction of several organs, especially the heart, nerves, eyes, and kidneys [1]. Diabetic kidney disease is the most frequent cause of renal failure and end-stage renal disease in developed countries [2,3]. It is characterized by macroalbuminuria of more than 300 mg/day, progressive decline in glomerular filtration rate (GFR), and hypertension. A considerable number of patients newly diagnosed with type 2 diabetes mellitus may already have developed incipient nephropathy (microalbuminuria) due to a preceding period of undiagnosed diabetes and impaired glucose tolerance. Among the participants of the UK Prospective Diabetes Study (UKPDS), the prevalence of microalbuminuria was 6.5% at diagnosis of type 2 diabetes and approximately 25% ten years after the diagnosis [4].

Strict glycemic control does not always inhibit the development of nephropathy and other secondary complications of diabetes, whereas even with suboptimal glycemic control, some patients with long disease duration seem not susceptible to microvascular complications [5]. The aim to prevent or delay the onset of microvascular complications has encouraged the search for other factors (e.g. genetic, metabolic, or nutritional), which may predispose to secondary vascular pathology in diabetes mellitus. The role of vitamin B supplementation in preventing microvascular complications of diabetes has already been a focus of research for some years. Studies on vitamin B_6 vitamers (pyridoxamine, pyridoxal 5′-phosphate) and high-dose vitamin B_1 suggested inhibition of albuminuria in diabetic animal models [6–8]. High-dose thiamine has also proofed successful in decreasing urinary albumin excretion in clinical type 2 diabetes [9]. A combined therapy with vitamin B_1 (thiamine) and vitamin B_6 (pyridoxine) in patients with type 2 diabetes and nephropathy significantly decreased glycation of nuclear DNA in leukocytes [10]. The therapeutic benefit of vitamin B supplementation may be achieved through correcting diabetes-related vitamin B deficiencies, which have been observed in experimental diabetes, especially in tissues where vascular complications develop [11–13].

Evidence for deficiencies of vitamins B_1 and B_{12} in both type 1 and type 2 diabetes mellitus have been described in several recent publications [14–18]. However, data on vitamin B_6 deficiency in patients with type 2 diabetes and on the vitamin B status in diabetic nephropathy are still scarce. The current study was performed to evaluate the vitamin B status in patients with type 2 diabetes mellitus with and without incipient nephropathy. The status of vitamin B_6 was of primary interest.

2. Materials and methods

2.1. Study population

Adults (≥18 years) with type 2 diabetes mellitus with a duration of at least 5 years, glycated hemoglobin (HbA1c) <10% (86 mmol/mol), and a body mass index (BMI) of 19–40 kg/m^2 were recruited through physicians at three general and internal medicine practices in Germany between January and September 2011. At the same time, healthy adults (non-diabetic control subjects) were recruited among office workers, who voluntarily provided information regarding their health situation. For a better representation of the average population, non-diabetic control subjects were also recruited among their relatives and friends. Exclusion criteria were as follows: end stage renal disease with a creatinine clearance of less than 10 mL/min, consumption of more than 50 units of alcohol per week, significant comorbidities, history of renal and/or pancreatic transplant, use of vitamin B supplements (including multivitamin preparations), and participation in an interventional study within 30 days prior to the start of this study. Pregnant or lactating women and women planning to become pregnant were also not recruited. Patients with diabetes were subdivided into two groups based on renal function: patients with microalbuminuria (urinary albumin excretion: 30–300 μg/mg creatinine) and patients without microalbuminuria, i.e. normal urinary albumin excretion (<30 μg/mg creatinine). All patients and control subjects consumed a typical Western European diet, with no special recommendations on food protein content. The study was designed and performed in accordance with the ethical principles stated in the Declaration of Helsinki and was reviewed and approved by the Ethics Committee of the State Chamber of Physicians of Rhineland-Palatinate, Germany. All study participants were informed about the study purposes and plans. Those who agreed to participate gave their written informed consent prior to any investigation.

2.2. Blood and urine sampling

Blood and urine samples were taken in the morning after an overnight fast. Venous blood (5 mL) was drawn from patients with diabetes to determine fasting blood glucose (HemoCue Glucose 201+ analyzer, HemoCue AB), HbA1c (high-performance liquid chromatography, immunturbidimetry), and serum creatinine (Jaffé photometric method). The GFR was estimated according to the Modification of Diet in Renal Disease formula. Micral-Test® (Roche Diagnostics GmbH) strips were used for semiquantitative assessment of urinary albumin concentrations in diabetic patients. Presence of glucose and total protein in spot urine samples of non-diabetic control subjects were assessed by dipstick tests (Siemens Multistix 10 SG, Siemens Healthcare Diagnostics Ltd.).

Blood samples from each patient and control subject were collected in heparinized tubes (5 mL) and in tubes without anticoagulant (10 mL) to determine markers of vitamin B status, lipid metabolism, and hepatic and renal function, C-reactive protein (CRP), and soluble vascular cell adhesion molecule-1 (sVCAM-1). Serum or plasma was separated immediately from blood cells by centrifugation for 10 min at 2000 × g at room temperature; aliquots were snap-frozen and stored at −80 °C until analysis. Spot urine samples (5 mL) were collected from each patient and control subject to determine urinary concentrations of thiamine and vitamin B_6 vitamers. Urine samples were aliquoted, snap-frozen and stored at −80 °C until analysis.

2.3. Analytical methods

Serum total cholesterol, triglyceride, low density lipoprotein (LDL), and high density lipoprotein (HDL) concentrations were determined by using automated enzymatic colorimetric assays; alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a standardized UV kinetic method (cobas® systems, Roche/Hitachi, Roche Diagnostics GmbH, Mannheim, Germany). Cystatin C, sVCAM-1, and CRP were analyzed by quantitative sandwich enzyme immunoassays (Quantikine®, R&D Systems, Abingdon, UK) according to the manufacturer's instructions. Thiamine, thiamine monophosphate (TMP), and thiamine pyrophosphate (TPP) were determined by high-performance liquid chromatography (HPLC, Dionex UltiMate 3000, Thermo Scientific) with fluorimetric detection after pre-column derivatization to the respective thiochromes by using potassium ferricyanide under alkaline conditions [19]. Pyridoxal 5′-phosphate (PLP), pyridoxamine 5′-phosphate (PMP), pyridoxine, pyridoxamine, pyridoxal, and pyridoxic acid were determined by HPLC (Dionex UltiMate 3000, Thermo Scientific) with fluorimetric detection after post-column derivatization using sodium metabisulfite [20]. The stock solutions were calibrated by spectrophotometer using the following molar extinction coefficients ($mM^{-1}\,cm^{-1}$): $\varepsilon_{305} = 1.1$ (PLP), $\varepsilon_{254} = 5.2$ (PMP), $\varepsilon_{308} = 7.0$ (pyridoxine), $\varepsilon_{308} = 7.3$ (pyridoxamine), $\varepsilon_{300} = 5.8$ (pyridoxal hydrochloride), and $\varepsilon_{316} = 5.1$ (pyridoxic acid). Urinary creatinine, plasma methylmalonic acid (MMA), and plasma total homocysteine were determined by using stable isotope dilution analysis liquid chromatography–tandem mass spectrometry as described elsewhere [21]. Vitamin B_{12} (cobalamin) was determined by using a competitive chemiluminiscent immunoassay (ADVIA Centaur®, Siemens Healthcare Diagnostics Inc., Tarrytown, NY, USA) and holotranscobalamin (holoTC) was determined by using a microparticle enzyme immunoassay (AxSYM®, Axis-Shield Diagnostics, Dundee, UK).

2.4. Statistical analysis

The study objectives were analyzed on a per-protocol basis in all participants who had blood and urine samples taken and analyzed. The variable of primary interest was the decrease in plasma PLP levels in patients with type 2 diabetes (with and without microalbuminuria) compared to non-diabetic control subjects. For a statistical power of 0.90, the study group size was determined as $N = 52$ for significance at the 5% level, assuming a mean plasma PLP concentration of 35 nmol/L (SD 20%) in healthy individuals and an estimated decrease in PLP by 20% in patients with diabetes and microalbuminuria. All other variables were considered secondary and were analyzed in a strictly exploratory manner. Non-parametric methods (two-sided Wilcoxon rank sum test or Fisher's exact test) were used to test for differences in demographic characteristics, vital signs at baseline, and analyte concentrations between patients with diabetes and non-diabetic control subjects, for differences in vitamin B_{12} parameters between patients with and without metformin therapy, and for differences in PLP levels between men and women. Pearson's correlation analysis was performed between PLP and age, BMI, and body weight. Statistical analyses were performed using the software SAS® for Windows 9.3.1 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Demographic and medical characteristics of the study population

A total of 174 participants, i.e. patients with type 2 diabetes mellitus with and without microalbuminuria and non-diabetic control subjects, were enrolled in this study. Two patients were excluded from the per-protocol analysis due to violation of inclusion criteria. Relevant characteristics of the three study groups are presented in Table 1. The patients with and without microalbuminuria were similar with regard to age, BMI, smoking history, and alcohol consumption. Control subjects were on average younger than patients with type 2 diabetes. Only 4 patients with diabetes (3.3% of 120 patients) were ≤50 years of age compared to 34 control subjects (65.4% of 52 subjects). In addition, the majority of the patients, i.e. 103 patients with diabetes (85.8%), were overweight or obese ($BMI > 25\ kg/m^2$) compared to 17 control subjects (32.7%). Alcohol consumption was more frequent in the control group than in the groups with type 2 diabetes ($p < 0.05$). Diabetes was well controlled in most patients; the mean HbA1c was 7.3% (corresponding to 65 mmol/mol) in patients with and without microalbuminuria. Mean serum creatinine values and mean GFR did not indicate severe or progressed kidney disease in the patients with type 2 diabetes and microalbuminuria.

The most frequent comorbidities in the patients with diabetes were hypertension and cardiovascular diseases. Medications that may contribute to a vitamin B_{12} deficiency (metformin, proton-pump inhibitors, or ACE inhibitors) were used by 33 patients (72%) with and by 50 patients (68%) without microalbuminuria, and by 2 control subjects (4%).

3.2. Markers of lipid metabolism, liver and kidney function, inflammation, and endothelial dysfunction

The median serum level of total cholesterol was slightly above normal in non-diabetic control subjects but not significantly different compared to the groups with diabetes ($p > 0.05$; Table 2). Other markers of lipid metabolism were within normal reference ranges in all groups; however, triglyceride levels were higher ($p < 0.0001$) and HDL levels were lower ($p < 0.0001$) in the diabetes groups than in the control group. Median ALT and AST concentrations were indicative of normal liver function. Cystatin C, a marker of kidney function, was significantly higher in both diabetes groups compared to the control group ($p < 0.0001$); in patients with type 2 diabetes and microalbuminuria, median cystatin C level was at the upper limit of the normal reference range. Median serum levels of markers indicating inflammation (CRP) or endothelial dysfunction (sVCAM-1) were increased in both diabetes groups compared to the control group ($p < 0.05$).

Table 1 – Characteristics of patients with type 2 diabetes with and without microalbuminuria and non-diabetic control subjects.

Characteristic	Type 2 diabetes with MA (N = 46)	Type 2 diabetes without MA (N = 74)	Healthy control (N = 52)
Sex, male/female (N)	23/23	38/36	18/34
Age (years)	72 (51, 86)***	71 (47, 83)***	43 (19, 73)
BMI (kg/m^2)	30 (21, 39)***	29 (22, 38)***	24 (19, 36)
Smoker, never/former/current (N)	37/7/2*	57/9/8*	42/0/10
Alcohol, none/moderate/excessive[a] (N)	32/14/0*	53/21/0*	22/30/0
Systolic BP (mmHg)	140 (100, 180)***	140 (110, 180)***	121 (100, 150)
Diastolic BP (mmHg)	80 (70, 100)	80 (60, 100)*	80 (55, 93)
Heart rate (bpm)	70 (50, 96)	70 (45, 98)*	68 (42, 92)
Fasting glucose[b] (mmol/L)	8.0 (3.3, 15.0)	7.4 (3.1, 15.2)	All negative
HbA1c (%)	7.3 (5.8, 9.9)	7.1 (5.5, 9.9)	n.d.
Serum creatinine (μmol/L)	84.0 (53.0, 168.0)	79.6 (44.2, 159.1)	n.d.
Urinary albumin/total protein[c] (mg/L)	50 (30, 100)	20 (0, 20)	All negative
GFR (mL/min)	69 (25, 214)	80 (39, 143)	n.d.
Medication, MET/PPI/ACE (N)	17/4/20	37/3/26	0/1/1

Data are presented as median (min, max). ACE = angiotensin-converting-enzyme inhibitor; BP = blood pressure; MA = microalbuminuria; MET = metformin; n.d. = not determined; PPI = proton-pump inhibitor.
[a] Excessive alcohol consumption: >50 unit/week.
[b] Diabetes: plasma glucose; control: glucose in urine, test considered negative below 4–7 mmol/L glucose.
[c] Diabetes: urinary albumin; control: urine total protein, test considered negative below 300 mg/L total protein.
* $p < 0.05$ versus non-diabetic control group.
*** $p < 0.0001$ versus non-diabetic control group.

3.3. *Vitamin B status*

The median plasma or serum concentrations of vitamins B_6, B_1, and B_{12}, their respective vitamers or biomarkers are given in Table 3. The prevalence of low plasma PLP (<30 nmol/L [22]) was 63% (N = 29) in the diabetes group with microalbuminuria, 58% (N = 43) in the diabetes group without microalbuminuria, and 25% (N = 13) in the control group. Median plasma concentrations of the vitamin B_6 vitamers PLP, pyridoxine and pyridoxal were significantly decreased in both diabetes groups compared to the control group ($p < 0.0001$, all parameters); median PLP was decreased by 43% in patients with microalbuminuria and by 32% in patients without microalbuminuria. On the other hand, median plasma levels of pyridoxamine, PMP, and pyridoxic acid were significantly higher in the diabetes groups than in the control group ($p < 0.001$). No significant correlations were found between plasma PLP and age, BMI, or weight in patients with diabetes or in the control group. Significantly higher plasma levels of pyridoxine were found in men compared to women (mean: 42.1 vs. 29.7 nmol/L; $p = 0.0134$) in the group with diabetes and microalbuminura. In the control group, higher urinary excretion of PMP (mean: 30.1 vs. 19.8 μmol/g creatinine; $p = 0.0245$) and pyridoxic acid (mean: 30.1 vs. 19.6 μmol/g creatinine; $p = 0.0218$) was seen in women compared to men. Pyridoxic acid was the major vitamin B_6 metabolite excreted in urine; the excreted amounts of other vitamin B_6 vitamers were small (see Table 4). Median urinary pyridoxic acid as a proportion of total urinary vitamin B_6 was significantly higher in patients with diabetes (with microalbuminuria: 75%, $p < 0.05$; without microalbuminuria: 77%, $p < 0.0001$) than in non-diabetic control subjects (63%). Compared to the control group, median

Table 2 – Serum biochemical parameters of lipid metabolism, hepatic and renal function, inflammation, and endothelial dysfunction.

Parameter	Type 2 diabetes with MA (N = 46)	Type 2 diabetes without MA (N = 74)	Healthy control (N = 52)
Total cholesterol (mg/dL)	193 (124, 262)	201 (111, 321)	206 (136, 297)
Triglyceride (mg/dL)	146 (54, 400)***	131 (42, 399)***	95 (39, 296)
HDL (mg/dL)	43 (12, 90)***	50 (20, 96)***	61 (31, 111)
LDL (mg/dL)[a]	123.0 (63, 220)	126.0 (62, 225)	128.0 (72, 207)
ALT (U/L)	13.0 (3.7, 41.0)***	16.0 (5.8, 42.0)**	20.0 (9.0, 66.0)
AST (U/L)	23.8 (14.5, 46.0)	25.0 (14.1, 90.0)	24.5 (19.0, 57.0)
Cystatin C (μg/mL)	1.09 (0.36, 2.50)***	0.84 (0.47, 2.59)***	0.33 (0.17, 0.82)
CRP (μg/mL)	2.6 (0.1, 73.5)**	1.5 (0.2, 101.5)*	0.8 (0.0, 31.7)
sVCAM-1 (ng/mL)	544 (187, 1546)***	448 (144, 1344)*	356 (169, 729)

Data are presented as median (min, max).
[a] LDL was measured in 73 diabetic patients without microalbuminuria (MA).
* $p < 0.05$ versus non-diabetic control group.
** $p < 0.001$ versus non-diabetic control group.
*** $p < 0.0001$ versus non-diabetic control group.

Table 3 – Plasma concentrations of vitamin B_1 and B_6, serum concentrations of vitamin B_{12}, and plasma concentrations of MMA and total homocysteine.

Parameter	Type 2 diabetes with MA (N = 46)	Type 2 diabetes without MA (N = 74)	Healthy control (N = 52)
Vitamin B_6 (nmol/L)			
PLP	22.7 (4.5, 206.4)***	26.8 (1.3, 166.0)***	39.5 (15.1, 448.5)
Pyridoxine[a]	6.5 (1.1, 372.4)***	7.4 (1.3, 473.6)***	12.4 (6.7, 33.5)
Pyridoxal	11.8 (2.7, 115.1)***	12.4 (3.3, 46.1)***	22.6 (11.2, 119.1)
Pyridoxamine[b]	1.5 (0.1, 21.9)***	1.6 (0.4, 15.9)***	0.8 (0.1, 80.7)
PMP	10.7 (0.9, 56.1)***	9.6 (1.1, 42.2)***	1.4 (0.2, 57.6)
Pyridoxic acid	33.5 (14.1, 486.9)***	30.7 (13.3, 174.7)**	21.1 (10.1, 255.5)
Vitamin B_1 (nmol/L)			
Thiamine	15.4 (6.2, 83.4)***	15.5 (8.3, 55.3)***	10.8 (4.9, 33.9)
TMP	2.3 (0.4, 21.1)***	3.3 (0.4, 11.8)***	5.6 (1.1, 13.2)
TPP	8.9 (5.0, 21.2)*	8.5 (4.1, 16.1)**	6.4 (2.2, 16.8)
Vitamin B_{12}			
Total cobalamin (pmol/L)	223 (101, 553)	218 (102, 1043)	246 (71, 526)
HoloTC (pmol/L)[c]	67 (12, 251)	74 (8, 246)	58 (7, 280)
MMA (μmol/L)	0.25 (0.05, 1.56)	0.22 (0.02, 1.36)	0.21 (0.05, 3.33)
Total homocysteine (μmol/L)	7.1 (3.1, 14.1)**	6.4 (3.4, 13.7)	5.6 (2.4, 19.5)

Data are presented as median (min, max). MA = microalbuminuria; MMA = methylmalonic acid.
[a] Patients with MA: N = 45, patients without MA: N = 71.
[b] Patients with MA: N = 44, patients without MA: N = 66, control subjects: N = 49.
[c] Patients with MA: N = 44, patients without MA: N = 73.
* $p < 0.05$ versus control subjects.
** $p < 0.001$ versus control subjects.
*** $p < 0.0001$ versus non-diabetic control group.

creatinine-corrected urinary pyridoxal levels were decreased in patients with diabetes with or without microalbuminuria by 27% and 17%, respectively ($p < 0.05$); and urinary PMP levels were decreased by 82% and 71%, respectively ($p < 0.0001$).

The prevalence of low plasma thiamine (<70 nmol/L [23]) was 98% in the diabetes group with microalbuminuria and 100% in the diabetes group without microalbuminuria and in the control group. Nevertheless, the median plasma levels of thiamine were increased by 43% in patients with microalbuminuria and by 44% in patients without microalbuminuria compared to the control subjects ($p < 0.0001$). Median TPP levels were also significantly higher ($p < 0.05$) while TMP levels were significantly lower ($p < 0.0001$) in both groups with diabetes compared to the control group. Median urinary thiamine was lowest in patients with microalbuminuria, but there were no significant differences between the diabetes groups and the control group ($p > 0.05$; Table 4).

The prevalence of vitamin B_{12} deficiency (holoTC ≤ 35 pmol/L combined with MMA ≥ 0.30 μmol/L [24]) was 8.7% (N = 4) in the diabetes group with microalbuminuria, 6.8% (N = 5) in the diabetes group without microalbuminuria, and 5.8% (N = 3) in the control group. The median serum concentrations of vitamin B_{12} (cobalamin) and its active form, holoTC, were comparable between the groups with diabetes and the control group ($p > 0.05$). Likewise, median plasma concentrations of MMA, a marker of vitamin B_{12} deficiency, were similar in the

Table 4 – Urinary concentrations of thiamine and B_6 vitamers normalized to urinary creatinine.

Parameter	Type 2 diabetes with MA (N = 46)	Type 2 diabetes without MA (N = 74)	Healthy control (N = 52)
Vitamin B_6 (μmol/g creatinine)			
PLP	0.10 (0.03, 0.38)	0.08 (0.01, 0.86)	0.12 (0.02, 0.66)
Pyridoxine[a]	0.43 (0.01, 4.12)	0.28 (0.01, 7.20)	0.49 (0.02, 5.58)
Pyridoxal[b]	0.38 (0.13, 1.97)*	0.43 (0.16, 2.43)*	0.52 (0.20, 5.18)
Pyridoxamine[c]	0.04 (0.00, 0.75)	0.05 (0.00, 0.25)	0.05 (0.01, 1.89)
PMP[d]	0.16 (0.01, 8.57)***	0.26 (0.02, 8.00)***	0.91 (0.24, 23.17)
Pyridoxic acid	3.74 (2.14, 41.43)	3.99 (2.00, 20.31)	4.02 (2.26, 60.21)
Vitamin B_1 (μmol/g creatinine)			
Thiamine	0.55 (0.21, 11.87)	0.71 (0.18, 1.86)	0.60 (0.27, 4.06)

Data are presented as median (min, max). MA = microalbuminuria.
[a] Patients with MA: N = 45, patients without MA: N = 72, control subjects: N = 51.
[b] Patients with MA: N = 45, patients without MA: N = 72.
[c] Patients with MA: N = 35, patients without MA: N = 61, control subjects: N = 42.
[d] Patients with MA: N = 45, patients without MA: N = 72.
* $p < 0.05$ versus non-diabetic control group.
*** $p < 0.0001$ versus non-diabetic control group.

three groups ($p > 0.05$, comparisons versus control). The median plasma concentration of total homocysteine was significantly higher in diabetes patients with microalbuminuria than in non-diabetic control subjects ($p < 0.001$). Metformin treatment was associated with slightly lower ($p > 0.05$, all comparisons) median serum vitamin B_{12} and holoTC levels in patients with microalbuminuria (metformin vs. no metformin: 188 vs. 227 pmol/L [vitamin B_{12}]; 62 vs. 77 pmol/L [holoTC]) and in those without microalbuminuria (metformin vs. no metformin: 212 vs. 233 pmol/L [vitamin B_{12}]; 63 vs. 79 pmol/L [holoTC]). Plasma concentrations of MMA and total homocysteine were not different ($p > 0.05$) between metformin-treated and non-treated patients.

4. Discussion

The results of our study suggest that type 2 diabetes mellitus is associated with decreased plasma PLP concentrations and alterations in vitamin B_6 metabolism, especially in patients with incipient nephropathy. The measured median plasma PLP levels indicated inadequate vitamin B_6 status in patients with type 2 diabetes with and without microalbuminuria, when applying the proposed cutoff level of 30 nmol/L for normal vitamin B_6 status [22]. Indications for lower blood PLP concentrations in type 2 diabetes compared to non-diabetic controls were also found in a small number of previous studies. In patients with type 2 diabetes with and without retinopathy (mean age: approximately 55 years), mean plasma PLP levels were significantly lower compared to the healthy control group [16]. In a Korean study, mean plasma PLP levels were clinically relevantly (if not significantly) decreased in patients (mean age: 50 years) with newly diagnosed type 2 diabetes [25]. In these studies [16,25], PLP levels were not indicative of vitamin B_6 deficiency—neither in the groups with diabetes nor in the control groups. In our German study population, the prevalence of PLP < 30 nmol/L was more than twice as high among patients with type 2 diabetes than among control subjects (i.e. 63% and 58% in the groups with and without microalbuminuria vs. 25% in the control group). In other studies, the prevalence of plasma PLP concentrations below the cutoff level was lower, i.e. approximately 10% in US patients with type 1 or type 2 diabetes and sensory polyneuropathy (mean age: 64 years) [26], and 10% in Korean patients with newly diagnosed type 2 diabetes [25]. In the Korean study, the prevalence of low plasma PLP was approximately 8% in the non-diabetic control group [25]. The more advanced state of the disease (at least a 5-year diagnosis of type 2 diabetes) of the patients in our study may be a possible explanation for the higher prevalence of PLP deficiency compared to the Korean study. Plasma PLP concentrations may be related to age and sex. A survey of PLP status in the US population found that PLP deficiency was more prevalent in elderly men (≥65 years) than in younger men; whereas in women, PLP deficiency was most prevalent in the 21–44 years age group [27]. Moreover, differences in dietary habits, including protein intake [28], blood albumin levels, smoking, alcohol consumption, use of certain drugs, or presence of autoimmune disorders may have an influence on the vitamin B_6 status. We found no correlation between plasma PLP and either age, BMI, or weight and detected no differences between men and women with regard to plasma PLP in any of the study groups. Age and sex were not matched between the control and the diabetes groups in the current study. The study did not include very young participants. Although few differences between men and women were found in urinary vitamin B_6 metabolites, we cannot rule out the source of these differences. Protein intake may be one factor that affects vitamin B_6 requirements. However, patients were not recommended to follow a protein-deprived diet. Therefore, there is no reason to consider dietary protein as an explanation for differences in vitamin B_6 markers between the study groups.

Plasma PLP is a commonly used marker to evaluate vitamin B_6 status. Previous studies in diabetic patients have rarely investigated other metabolic forms of vitamin B_6. Apart from the significantly lower PLP levels in patients with type 2 diabetes, we also observed significantly lower median levels of pyridoxine and pyridoxal, whereas median levels of pyridoxamine, PMP, and pyridoxic acid were significantly higher in the groups with diabetes than in the control group. These results indicate that there may be alterations in the vitamin B_6 metabolic pathways in type 2 diabetes. The underlying mechanisms require further investigation. One potential reason for the higher plasma PMP level may be an enhanced release of PMP from the liver into the blood. Or, PMP may accumulate as a result of a decreased rate of conversion to PLP, a reaction catalyzed by the vitamin B_2 (flavin-mononucleotide)-dependent pyridoxine 5-phosphate oxidase in the liver. The same enzyme also catalyzes the conversion of pyridoxine 5′-phosphate to PLP. Whether its function is affected in any way in diabetes is speculative; further evidence is missing as we did not determine pyridoxine 5′-phosphate concentrations in this study. Nevertheless, a decreased rate of conversion may contribute to PLP deficiency in type 2 diabetes, independent from dietary intake of vitamin B_6. We found significantly higher plasma levels of pyridoxic acid (the major end product of vitamin B_6 metabolism) in the patients with diabetes, especially in the group with microalbuminuria. Increased plasma concentrations of pyridoxic acid have previously been found in elderly people and in patients with renal insufficiency [29–31]. Accumulation of pyridoxic acid may be the result of impaired renal function. However, urinary concentrations of pyridoxic acid were not significantly different in diabetes patients and control subjects. Increased catabolism of PLP is another possible explanation for the accumulation of plasma pyridoxic acid, especially in combination with a decrease in PLP [21,29].

Possible mechanisms by which vitamin B_6 deficiency may be associated with the development of microalbuminuria and diabetic nephropathy in type 2 diabetes are (1) a potential role of PLP deficiency in inflammatory processes [32,33], or (2) through increased blood homocysteine levels [34]. It has been recognized in recent years that inflammatory processes play an important role in the progression of diabetic nephropathy [35,36]. CRP is a marker of inflammation and a risk predictor for cardiovascular disease [37]. Low plasma PLP was found to be associated with elevated CRP (independent of plasma homocysteine) in an elderly population (Framingham Heart Study cohort) and in nephropathy secondary to type 2 diabetes

[32,38]. In line with this, we found that median CRP was highest in the patients with diabetes and microalbuminuria, which was also the group with the lowest median PLP level. The question whether PLP deficiency directly contributes to the inflammatory process or is a result of it remains unclear. However, current research provides evidence that PLP deficiency is caused by a mobilization of PLP at sites of inflammation and by its increased utilization as a coenzyme in inflammation-related pathways [32,39].

Total homocysteine is a proposed biomarker for cardiovascular disease [40]; elevated plasma levels are associated with inflammation and oxidative stress and thus with endothelial dysfunction [41]. PLP, vitamin B_{12}, and folate (vitamin B_9) are cofactors in pathways of homocysteine metabolism. Deficiency of one or more of these cofactors results in accumulation of homocysteine [42]. Increased plasma total homocysteine levels have been observed in patients with chronic kidney disease and were associated with nephropathy in type 2 diabetes [43–45]. In our study, median total homocysteine was not elevated in any of the groups; in fact, the values were rather low compared to the mean homocysteine levels ranging between 9.0 and 15.1 μmol/L found in predominantly middle-aged European populations [46]. Nevertheless, in our study, median homocysteine levels were significantly higher in the patients with diabetes and microalbuminuria than in the control subjects. Possible explanations for the relative increase in homocysteine in this group may include the higher prevalence of low PLP levels or a decreased elimination of homocysteine from the blood due to incipient renal impairment [47]. The median GFR was slightly lower in the patients with microalbuminuria than in the patients without microalbuminuria. Other factors, such as folate status (not determined in this study), age, and gender, may also have influenced the total homocysteine level.

Elevated blood levels of total homocysteine and MMA are sensitive indicators of vitamin B_{12} deficiency [48]. Median plasma concentrations of these biomarkers did not differ between patients with type 2 diabetes and the control subjects. The prevalence of functional vitamin B_{12} deficiency (e.g. elevated MMA and lowered holoTC) was below 10% in both diabetes groups. In accordance with an earlier report on patients with type 2 diabetes [18], metformin usage was not associated with significantly higher MMA or total homocysteine in the current study. However, in contrast to the study by Obeid et al. [18], metformin treatment was associated with only a slight decrease in plasma vitamin B_{12} and holoTC, which may be explained by the small sample size or renal insufficiency in the current study (higher creatinine and lower GFR compared to the earlier study [18]). In contrast to our findings, retinopathy secondary to type 2 diabetes was associated with vitamin B_{12} deficiency and elevated levels of plasma total homocysteine, but not with PLP deficiency in an Indian study population [16]. Therefore, diabetes-related comorbidities may affect vitamin biomarkers and add challenge to their diagnoses.

Thiamine is another B vitamin for which low plasma concentrations have been reported in patients with diabetes [14,49]. Our study confirmed thiamine deficiency in patients with type 2 diabetes, irrespective of whether incipient nephropathy was present or not. An unexpected finding was the even higher level of thiamine deficiency in the non-diabetic control group, since plasma thiamine concentration had been decreased by 75% in patients with type 2 diabetes compared to healthy volunteers in a previous study [14]. Furthermore, vitamin B_1 deficiency is a rare condition in developed countries. Apart from unbalanced diet or certain diseases, alcohol consumption, which was more prevalent in the control group of this study, is usually associated with thiamine deficiency [50]. Low plasma thiamine levels have been linked to an increase in sVCAM-1 (a marker of endothelial dysfunction) in patients with type 1 and type 2 diabetes [14]. Our data do not seem to indicate a direct association between sVCAM-1 and thiamine deficiency. Compared with the non-diabetic control, sVCAM-1 levels were significantly increased in both diabetes groups. Moreover, the median sVCAM-1 level in patients with microalbuminuria was above the upper limit of the normal range, indicating endothelial damage associated with incipient nephropathy.

In conclusion, our study showed that PLP deficiency was prevalent in patients with type 2 diabetes, especially in patients with incipient nephropathy characterized by microalbuminuria. Type 2 diabetes was also associated with other alterations in vitamin B_6 metabolism, including relative increases in PMP and pyridoxic acid, as well as with vitamin B_1 deficiency. Markers of inflammation and endothelial dysfunction may indicate incipient nephropathy in type 2 diabetes. The exact relationships and interactions between vitamin B and inflammation-associated pathways are yet to be discovered. More studies are also needed to clarify the underlying mechanisms that cause deficiency of B vitamins in type 2 diabetes and to evaluate potential benefits and risks of supplementation therapy for the prevention of microvascular complications secondary to diabetes.

Conflict of interest statement

W. Nix served on the speaker's bureau of Merck and received honoraria, U. Hostalek is an employee of Merck KGaA, and R. Obeid received research grant support by Merck. All other authors have no competing interests to declare. W. Nix is the principle investigator of this study and holds the main responsibility for the scientific content. Merck KGaA had no influence on the design and conception of the study.

Declaration of funding

This work was supported by an unrestricted grant by Merck KGaA.

Acknowledgements

The authors thank Prof. Paul J. Thornalley, Dr. Naila Rabbani, and Dr. Attia Anwar, Warwick Medical School, Clinical Science Research Institute, University of Warwick, Coventry, UK, for measuring plasma and urine concentrations of vitamins B_6 and B_1 and related vitamers, and serum concentrations of CRP, cystatin C, and sVCAM-1. The authors also thank Jana Distler

and Dr. Tanja Henrichs at Syneed Medidata GmbH for statistical and medical writing support, which was funded by Merck KGaA.

REFERENCES

[1] Brownlee M. Biochemistry and molecular cell biology of diabetic complications. Nature 2001;414(6865):813–20.

[2] Gilg J, Castledine C, Fogarty D. Chapter 1 UK RRT incidence in 2010: national and centre-specific analyses. Nephron Clin Pract 2012;120(Suppl 1):c1–27.

[3] United States Renal Data System. 2012 USRDS annual data report: atlas of chronic kidney disease and end-stage renal disease in the United States. Bethesda, MD: National Institutes of Health, National Institute of Diabetes and Digestive and Kidney Diseases; 2012, Available at: ⟨http://www.usrds.org/2012/slides/indiv/v2index.html⟩ [last accessed on 25 Feb 2014].

[4] Adler AI, Stevens RJ, Manley SE, Bilous RW, Cull CA, Holman RR. Development and progression of nephropathy in type 2 diabetes: the United Kingdom Prospective Diabetes Study (UKPDS 64). Kidney Int 2003;63(1):225–32.

[5] Keenan HA, Costacou T, Sun JK, Doria A, Cavellerano J, Coney J, et al. Clinical factors associated with resistance to microvascular complications in diabetic patients of extreme disease duration: the 50-year medalist study. Diabetes Care 2007;30(8):1995–7.

[6] Degenhardt TP, Adlerson NL, Arrington DD, Beattie RJ, Basgen JM, Steffes MW, et al. Pyridoxamine inhibits early renal disease and dyslipidemia in the streptozotocin-diabetic rat. Kidney Int 2002;61(3):939–50.

[7] Nakamura S, Li H, Adijiang A, Pischetsrieder M, Niwa T. Pyridoxal phosphate prevents progression of diabetic nephropathy. Nephrol Dial Transplant 2007;22(8):2165–74.

[8] Babaei-Jadidi R, Karachalias N, Ahmed N, Battah S, Thornalley PJ. Prevention of incipient diabetic nephropathy by high dose thiamine and benfotiamine. Diabetes 2003;52(8):2110–20.

[9] Rabbani N, Alam SS, Riaz S, Larkin JR, Akhtar MW, Shafi T, et al. High-dose thiamine therapy for patients with type 2 diabetes and microalbuminuria: a randomized, double-blind placebo-controlled pilot study. Diabetologia 2009;52(2):208–12.

[10] Polizzi FC, Andican G, Çetin E, Civelek S, Yumuk V, Burçak G. Increased DNA-glycation in type 2 diabetic patients: the effect of thiamine and pyridoxine therapy. Exp Clin Endocrinol Diabetes 2012;120(6):329–34.

[11] Reddi AS, Jyothirmayi GN, DeAngelis B, Frank O, Baker H. Tissue concentrations of water-soluble vitamins in normal and diabetic rats. Int J Vitam Nutr Res 1993;63(2):140–4.

[12] Okada M, Shibuya M, Yamamoto E, Murakami Y. Effect of diabetes on vitamin B_6 requirement in experimental animals. Diabetes Obes Metab 1999;1(4):221–5.

[13] Babaei-Jadidi R, Karachalias N, Kupich C, Ahmed N, Thornalley PJ. High-dose thiamine therapy counters dyslipidaemia in streptozotocin-induced diabetic rats. Diabetologia 2004;47(12):2235–46.

[14] Thornalley PJ, Babaei-Jadidi R, Al Ali H, Rabbani N, Antonysunil A, Larkin J, et al. High prevalence of low plasma thiamine concentration in diabetes linked to marker of vascular disease. Diabetologia 2007;50(10):2164–70.

[15] Pflipsen M, Oh RC, Saguil A, Seehusen DA, Seaquist D, Topolski R. The prevalence of Vitamin B(12) deficiency in patients with type 2 diabetes: a cross-sectional study. J Am Board Fam Med 2009;22(5):528–34.

[16] Satyanarayana A, Balakrishna N, Pitla S, Reddy PY, Mudili S, Lopamudra P, et al. Status of B-vitamins and homocysteine in diabetic retinopathy: association with vitamin-B_{12} deficiency and hyperhomocysteinemia. PLoS One 2011;6(11):e26747.

[17] Al-Atta OS, Al-Daghri NM, Alfadda AA, Abd-Alrahman SH, Sabico S. Blood thiamine and its phosphate esters as measured by high-performance liquid chromatography: levels and associations in diabetes mellitus patients with varying degrees of microalbuminuria. J Endocrinol Invest 2012;35(11):951–6.

[18] Obeid R, Jung J, Falk J, Herrmann W, Geisel J, Friesenhahn-Ochs B, et al. Serum vitamin B_{12} not reflecting vitamin B_{12} status in patients with type 2 diabetes. Biochimie 2013;95(5):1056–61.

[19] Sander S, Hahn A, Stein J, Rehner G. Comparative studies on the high-performance liquid chromatographic determination of thiamine and its phosphate esters with chloroethylthiamine as an internal standard using pre- and post-column derivatization procedures. J Chromatogr 1991;558:115–24.

[20] Bisp MR, Bor MV, Heinsvig EM, Kall MA, Nexø E. Determination of vitamin B_6 vitamers and pyridoxic acid in plasma: development and evaluation of a high-performance liquid chromatographic assay. Anal Biochem 2002;305(1):82–9.

[21] Adaikalakoteswari A, Rabbani N, Waspadji S, Tjokroprawiro A, Kariadi SH, Adam JM, et al. Disturbance of B-vitamin status in people with type 2 diabetes in Indonesia—link to renal status, glycemic control and vascular inflammation. Diabetes Res Clin Pract 2012;95(3):415–24.

[22] Leklem JE. Vitamin B-6: a status report. J Nutr 1990;120:1503–7.

[23] Luong KV, Nguyen LT. The impact of thiamine treatment on generalized anxiety disorder. J Clin Med Res 2012;4(3):153–60.

[24] Obeid R, Herrmann W. Holotranscobalamin in laboratory diagnosis of cobalamin deficiency to total cobalamin and methylmalonic acid. Clin Chem Lab Med 2007;45(12):1746–50.

[25] Ahn HJ, Min KW, Cho YO. Assessment of vitamin B_6 status in Korean patients with newly diagnosed type 2 diabetes. Nutr Res Pract 2001;5(1):34–9.

[26] Gorson KC, Ropper AH. Additional causes for distal sensory polyneuropathy in diabetic patients. J Neurol Neurosurg Psychiatry 2006;77(3):354–8.

[27] Morris MS, Picciano MF, Jacques PF, Selhub J. Plasma pyridoxal 5′-phosphate in the US population: the National Health and Nutrition Examination Survey, 2003–2004. Am J Clin Nutr 2008;87(5):1446–54.

[28] Hansen CM, Leklem JE, Miller LT. Vitamin B-6 status of women with a constant intake of vitamin B-6 changes with three levels of dietary protein. J Nutr 1996;126(7):1891–901.

[29] Bates CJ, Pentieva KD, Prentice A, Mansoor MA, Flinch S. Plasma pyridoxal phosphate and pyridoxic acid and their relationship to plasma homocysteine in a representative sample of British men and women aged 65 years and over. Br J Nutr 1999;81(3):191–201.

[30] Coburn SP, Reynolds RD, Mahuren JD, Schaltenbrand WE, Wang Y, Ericson KL, et al. Elevated plasma 4-pyridoxic acid in renal insufficiency. Am J Clin Nutr 2002;75(1):57–64.

[31] Lindner A, Bankson DD, Stehman-Breen C, Mahuren JD, Coburn SP. Vitamin B_6 metabolism and homocysteine in end-stage renal disease and chronic renal insufficiency. Am J Kidney Dis 2002;39(1):134–45.

[32] Friso S, Jacques PF, Wilson PW, Rosenberg IH, Selhub J. Low circulating vitamin B(6) is associated with elevation of the inflammation marker C-reactive protein

independently of plasma homocysteine levels. Circulation 2001;103(23):2788–91.
[33] Sakakeeney L, Roubenoff R, Obin M, Fontes JD, Benjamin EJ, Bujanover Y, et al. Plasma pyridoxal-5-phosphate is inversely associated with systemic markers of inflammation in a population of U.S. adults. J Nutr 2012;142(7):1280–5.
[34] Cuskelly GJ, Stacpoole PW, Williamson J, Baumgartner TG, Gregory III JF. Deficiencies of folate and vitamin B(6) exert distinct effects on homocysteine, serine, and methionine kinetics. Am J Physiol Endocrinol Metab 2001;281(6):E1182–90.
[35] Lim AK, Tesch GH. Inflammation in diabetic nephropathy. Mediators Inflamm 2012;2012:146154.
[36] Wada J, Makino H. Inflammation and the pathogenesis of diabetic nephropathy. Clin Sci (Lond) 2013;124(3):139–52.
[37] Ridker PM, Hennekens CH, Buring JE, Rifai N. C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. N Engl J Med 2000;342(12):836–43.
[38] Friedman AN, Hunsicker LG, Selhub J, Bostom AG. Clinical and nutritional correlates of C-reactive protein in type 2 diabetic nephropathy. Atherosclerosis 2004;172(1):121–5.
[39] Paul L, Ueland PM, Selhub J. Mechanistic perspective on the relationship between pyridoxal 5′-phosphate and inflammation. Nutr Rev 2013;71(4):239–44.
[40] Humphrey LL, Fu R, Rogers K, Freeman M, Helfand M. Homocysteine level and coronary heart disease incidence: a systematic review and meta-analysis. Mayo Clin Proc 2008;83(11):1203–12.
[41] Papatheodorou L, Weiss N. Vascular oxidant stress and inflammation in hyperhomocysteinemia. Antioxid Redox Signal 2007;9(11):1941–58.
[42] Selhub J, Jacques PF, Wilson PW, Rush D, Rosenberg IH. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA 1993;270(22):2693–8.
[43] Atamer A, Kocyigit Y, Ecder SA, Selek S, Ilhan N, Ecder T, et al. Effects of oxidative stress on antioxidant enzyme activities, homocysteine and lipoproteins in chronic kidney disease. J Nephrol 2008;21(6):924–30.
[44] Herrmann W, Schorr H, Obeid R, Makowski J, Fowler B, Kuhlmann MK. Disturbed homocysteine and methionine cycle intermediates S-adenosylhomocysteine and S-adenosylmethionine are related to degree of renal insufficiency in type 2 diabetes. Clin Chem 2005;51(5):891–7.
[45] Li J, Shi M, Zhang H, Yan L, Xie M, Zhuang L, et al. Relation of homocysteine to early nephropathy in patients with type 2 diabetes. Clin Nephrol 2012;77(4):305–10.
[46] de Bree A, van der Put NM, Mennen LI, Verschuren WM, Blom HJ, Galan P, et al. Prevalence of hyperhomocysteinemia, unfavorable cholesterol profile and hypertension in European populations. Eur J Clin Nutr 2005;59(4):480–8.
[47] van Guldener C. Why is homocysteine elevated in renal failure and what can be expected from homocysteine-lowering? Nephrol Dial Transplant 2006;21(5):1161–6.
[48] Savage DG, Lindenbaum J, Stabler SP, Allen RH. Sensitivity of serum methylmalonic acid and total homocysteine determinations for diagnosing cobalamin and folate deficiencies. Am J Med 1994;96(3):239–46.
[49] Saito N, Kimura M, Kuchiba A, Itokawa Y. Blood thiamine levels in outpatients with diabetes mellitus. J Nutr Sci Vitaminol 1987;33(6):421–31.
[50] Maschke M, Weber J, Bonnet U, Dimitrova A, Bohrenkämper J, Sturm S, et al. Vermal atrophy of alcoholics correlate with serum thiamine levels but not with dentate iron concentrations as estimated by MRI. J Neurol 2005;252(6):704–11.