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Disturbance of B-vitamin status in people with type 2 diabetes in Indonesia—Link to renal status, glycemic control and vascular inflammation

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ABSTRACT

Background: Diabetes is associated with mishandling of thiamine in the kidney and development of diabetic nephropathy. The aim of this study is to assess the disturbance of thiamine and other B-vitamin status of patients with type 2 diabetes in Indonesia.
Methods: One hundred and fifteen patients with type 2 diabetes with and without microalbuminuria or albuminuria and 39 healthy people were recruited. After a 2-month washout period for B-vitamin supplementation, markers of vitamins B_1, B_6, B_9 and B_{12}, were determined.
Results: Fractional excretion of thiamine (22.8 versus 33.5%; $P < 0.05$) and urinary excretion of the vitamin B_6 degradation product 4-pyridoxic acid (0.081 versus 0.133 μmol/g creatinine, $P < 0.001$) was increased in patients with type 2 diabetes with respect to healthy controls. There was also increased total plasma cobalamin (398 versus 547 pmol/l, $P < 0.001$) and holotranscobalamin (74 versus 97 pmol/l, $P < 0.001$) in patients with type 2 diabetes. In multiple regression analysis these were linked to HbA1c, duration of diabetes and systolic blood pressure, and fasting plasma glucose, folate and C-reactive protein, respectively.
Conclusions: There was renal mishandling of thiamine, increased degradation of vitamin B_6 and cytosolic metabolic resistance to vitamin B_{12} in patients with type 2 diabetes in Indonesia.

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Abbreviations: ACR, albumin creatinine ratio; ALT, L-alanine:2-oxoglutarate aminotransferase; ARBs/ACEIs, angiotensin receptor blocker/angiotensin converting enzyme inhibitor; AST, aspartate aminotransferase; BMI, body mass index; CRP, C-reactive protein; CVD, cardiovascular disease; DN, diabetic nephropathy; DTT, dithiothreitol; FPG, fasting plasma glucose; $FE_{Metabolite}$, fractional excretion of designated metabolite; eGFR, estimated glomerular filtration rate; LC–MS/MS, liquid chromatographic with tandem mass spectrometric detection; MMA, methylmalonate; 4-PA, 4-pyridoxic acid; PL, pyridoxal; PLP, pyridoxal-5′-phosphate; PM, pyridoxamine; PMP, pyridoxamine-5′-phosphate; PN, pyridoxine; RBC, red blood cell; TFA, trifluoroacetic acid; tHcy, total plasma homocysteine ([homocysteine] + 2 × [homocystine]); TK, transketolase; TMP, thiamine monophosphate; TPP, thiamine pyrophosphate; sVCAM-1, soluble vascular adhesion molecule-1.

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1. Introduction

The Southeast Asia and the Western Pacific regions are at the forefront of the current epidemic of type 2 diabetes. There is approximately 7 million people in Indonesia with diabetes, mostly type 2 diabetes, and this is predicted to rise to 12 million by 2020 [1]. There will likely be a subsequent increased burden of vascular complications of diabetes, including diabetic nephropathy (DN), in this population [2,3]. It is predicted that 25% of newly diagnosed patients with type 2 diabetes will develop early stage DN within the next 10 years [4]. The development of DN exacerbates the increased risk of cardiovascular disease (CVD) in type 2 diabetes [5]. Improvements in understanding of mechanism of development of DN and related treatment are required to decrease morbidity and premature mortality in this increasing high risk group.

Strategies to prevent DN including nutritional and therapeutic support related to B-vitamin supplements have recently emerged. Two independent intervention studies with therapeutic supplements of thiamine (vitamin B_1) have shown decrease in urinary albumin excretion and reversal of microalbuminuria in patients with type 2 diabetes [6,7]. This treatment may work, at least in part, by countering the effects of increased renal clearance of thiamine found in clinical diabetes [8]. Plasma levels of pyridoxal-5′-phosphate (PLP), a B_6 vitamer, were linked inversely to the vascular inflammatory marker C-reactive protein (CRP) in clinical DN [9]. Supplementation of vitamin B_6 by treatment with pyridoxine improved flow-mediated dilatation in type 1 diabetes [10]. Vitamin B_6, vitamin B_9 (folate) and vitamin B_{12} (cobalamin) have been studied in relation to increased homocysteine and risk of CVD [11–13]. In studies of interactions with drug therapy, short-term and long-term treatment with metformin has been linked to decreases in folate (5–7%) and vitamin B_{12} (14–19%) levels in patients with type 2 diabetes receiving metformin therapy [14,15]. A recent finding suggested co-supplementation with B_6, B_9 and B_{12} in patients with type 2 diabetes and advanced DN exacerbates decline in renal function [13] – an association possibly linked to adverse effects of accumulation of folate and cyanide (from cyanocobalamin treatment) in patients with impaired renal function [16,17].

We hypothesised that there may be increased clearance and washout of thiamine in patients with type 2 diabetes in South East Asia and chose to study patients and people Indonesia as typical of the region. We also investigated disturbances in other B-vitamins. In this report we describe the outcome of a multicenter study of B-vitamin status of patients with type 2 diabetes with and without microalbuminuria and albuminuria and healthy people in Indonesia.

2. Methods

2.1. Patients

Patients with type 2 diabetes were recruited from those attending the diabetes clinics at CiptoMangunkusumo Hospital, Jakarta, Soetomo Hospital, Surabaya, Hasan Sadikin Hospital, Bandung and Dr Wahidin Sudirohusodo Hospital, Makassar, Indonesia. Classification of renal function status implemented in the study is defined by the Asian-Pacific Type 2 Diabetes Policy Group and endorsed by the International Diabetes Federation [18]. Inclusion criteria were: diabetic patients – type 2 diabetes with normoalbuminuria (albumin/creatinine ratio ACR <22 and <31 mg/g for men and women, respectively), microalbuminuria (ACR 22–220 and 31–220 mg/g for men and women, respectively) and albuminuria (ACR >220 mg/g and plasma creatinine <2 mg/dl) matched for age (45–65 years) and gender – including a within group gender balance, diabetes duration ≥ 5 years, $HbA_{1c} < 10\%$ and BMI 19–40 kg/m^2). Urinary albumin excretion criteria were confirmed on at least 2 occasions (pre-screening and study entry). A group of healthy people of the same social background as the patients with type 2 diabetes were also recruited. Exclusion criteria were: allergy or intolerance to thiamine, pyridoxine or cobalamin, participation in another clinical study within the last 30 days, women who were pregnant, breast feeding or of child bearing potential not using adequate contraceptive precautions; for diabetic patients, chronic renal insufficiency (plasma creatinine ≥ 2 mg/dl) [19], liver diseases (abnormal liver function tests – serum albumin, alanine transaminase, aspartate transaminase), anemia (Hb <120 g/l), tuberculosis and severe CVD (angina, myocardial infarction and normal electrocardiogram within 2 months of enrolment). Medication use of the patients with type 2 diabetes was: metformin 60%, α-glycosidase inhibitor 28%, sulfonylurea 54%, insulin 18%, angiotensin receptor blockers/angiotensin converting enzyme inhibitors (ARBs/ACEIs) 15% and statins 15%. Assessments for meeting the inclusion criteria were made at the initial pre-screening visit to the participating clinics.

To eliminate effects of vitamin supplementation, the study involved pre-screening where meeting of inclusion and exclusion criteria was assessed followed by a washout period of 2 months for supplements with thiamine, vitamin B_6, folate and vitamin B_{12}. After the washout period, further peripheral venous blood (with heparin anticoagulant) and urine samples were collected after overnight fasting. Plasma and red blood cell (RBC) fractions were prepared immediately and stored at -80 °C until analysis. Ethical approval for the study was given by the University of Warwick Bioethics Committee, UK, and local ethical approval committees at the recruiting hospitals in Indonesia. Enrolment in the study was with informed consent. The reported investigations have been carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

2.2. Biochemical measurements

Thiamine status was assessed by measurement of thiamine, thiamine monophosphate (TMP) and thiamine pyrophosphate (TPP) in plasma, RBCs and urine, and RBC transketolase (TK) activity – all as previously described [8]. B_6 vitamers (pyridoxine PN, pyridoxal PL, pyridoxal-5′-phosphate PLP, pyridoxamine PM, pyridoxamine-5′-phosphate PMP and 4-pyridoxic acid 4-PA) were determined in plasma, RBCs and urine by HPLC with post-column derivatization [20]. RBC activity of L-alanine:2-oxoglutarate aminotransferase (ALT) and aspartate aminotransferase (AST) were determined spectrophotometrically using commercial kits (Thermo Fisher Scientific, Lough-

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borough, UK). Total plasma cobalamin and folate were determined by electrochemiluminescent immunoassay using a Roche Elecsys E170 analyzer (Roche Diagnostics UK, Burgess Hill, UK). Plasma holotranscobalamin or ''active vitamin B_{12}'' was determined by immunoassay using an Abbott AXSYM analyser (Abbott Diagnostics, Maidenhead, UK).

Total plasma homocysteine (tHcy) and methylmalonic acid (MMA) were determined by stable isotopic dilution analysis liquid chromatography with tandem mass spectrometric detection (LC–MS/MS). Stock solutions of homocystine (1 mg/ml in 100 mmol/l HCl), MMA (1 mg/ml in water), [3,3,3′,3′,4,4,4′,4′-d_8]homocystine (1 mg/ml in 100 mmol/l HCl) and [methyl-d_3]MMA (1 mg/ml in water; Cambridge Isotopes, Andover, MA, USA) and 500 mol/l dithiothreitol (DTT) in water were prepared and stored in −20 °C. The working calibration solution was prepared before analysis and further diluted to obtain calibration curve in the range 0–5 nmol homocystine and 0–40 pmol MMA and 100 pmol homocystine-d_8 and MMA-d_3. Isotopic standard (10 μmol/l d_8-homocystine and 10 μmol/l d_3-MMA; 10 μl) was added to plasma (50 μl) and calibrators (50 μl). DTT (5 μl) was added and samples left at room temperature for 30 min on ice to reduce homocystine to homocysteine. Samples were deproteinized by ultrafiltration over 10 kDa microspin filters (10,000 × *g*, 60 min, 4 °C). The ultrafiltrate (50 μl) was then analysed by LC–MS/MS system. The detection conditions were (molecular ion > fragment ion; *m*/*z*): homocysteine, 136.1 > 90.1 and d_4-Hcy, 140.1 > 94.1 (collision energy 11.0 eV and cone voltage 18.0 V, positive ion mode); MMA, 117.0 > 73.0 and d_3-MMA, 120.0 > 76.0 (collision energy 7 eV and cone voltage 20 V, negative ion mode). LC–MS/MS was performed using a Waters Acquity UPLC system with a Quattro Premier XE tandem mass spectrometric detector. For chromatographic separation, two Hypercarb columns (5 μm particle size, dimensions 50 mm × 2.1 and 250 mm × 2.1 mm) in series were used with a 10 mm × 2.1 mm guard column (Thermo Fisher). The mobile phase components were: (A) 0.1% trifluoroacetic acid (TFA) in water and (B) 0.1% TFA in 50% acetonitrile. The elution gradient programme was: 0–10 min, 0–100% B, 10–15 min, 100% B. The flow rate was 0.2 ml/min. Electrospray ionization was performed with source temperature 120 °C, desolvation gas temperature 350 °C, cone gas and desolvation gas flows 102 and 860 L/h, respectively, capillary voltage 3.0 kV and argon collision gas 5×10^{-3} mBar. Retention times, limits of quantification, inter-batch coefficients of variation and recoveries were: homocysteine – 8.8 min, 5 pmol, 1.6% and 106%; MMA – 11.6 min, 2 pmol, 4.3% and 105%.

Clinical indicators of metabolic control were determined: fasting plasma glucose, HbA_{1c}, plasma total cholesterol and HDL cholesterol and triglycerides. LDL cholesterol (LDL) was deduced by the Friedewald equation [21]. Nephropathy status was assessed by measurement of urinary albumin by gel permeation liquid chromatography [22]. Urinary and plasma creatinine were determined by the creatininase method. Markers of endothelial dysfunction, low grade inflammation and renal function – soluble vascular adhesion molecule-1 (sVCAM-1), CRP and cystatin c were determined by enzyme-linked immunosorbent assay (R and D Systems, Abingdon, UK). eGFR was deduced from plasma cystatin c concentration: eGFR = 86.7/[cystatin c (mg/l)] − 4.2 [23].

2.3. Statistical analysis

Data are mean ± SD for parametric data and median (lower–upper quartile) for non-parametric data. Significance of difference between mean changes was assessed by Student's *t* test and one-way ANOVA. Significance of difference between median changes was assessed by Mann–Whitney-*U* test and Kruskal–Wallis test. Bivariate correlate analysis was performed by the non-parametric Spearman method. Factors linked to markers of B-vitamin dysfunction in patients with type 2 diabetes were explored by multiple linear regression analysis. Bonferroni correction was applied to the 4 vitamin B_6 vitamers (PN, PL, PLP and PMP; significance for $P < 0.0125$) and two vitamin B_{12} analytes (total plasma vitamin B_{12} and holotranscobalamin; significance for $P < 0.025$)

3. Results

3.1. Characteristics of patients with type 2 diabetes and healthy people

The characteristics of patients with type 2 diabetes and healthy people recruited for this study are given in Table 1. Study groups of patients with type 2 diabetes had a slightly higher mean age than the healthy controls. For healthy people included in the study, 2 subjects had HbA_{1c} = 6.5% and were excluded from data analysis as meeting the criterion for diagnosis of diabetes by current international guidelines [24]. For the remaining healthy subjects, 22 of 37 had HbA_{1c} in the range 5.7–6.4%, indicative of pre-diabetes by current international guidelines [24]. For patients with type 2 diabetes, 51% had good glycemic control ($HbA_{1c} \leq 7.0$), 46% had moderate glycemic control ($HbA_{1c} > 7.0$ and ≤ 10.0) and only 3% had poor glycemic control; and 31% had hypertension. They had a slightly higher BMI than the healthy controls that was mostly due to high BMI in the albuminuria group. Overall, obesity (BMI > 30 kg/m^2) was low: one healthy control and 11 (10%) patients with type 2 diabetes were obese. All study groups of patients with type 2 diabetes had a similar duration of diabetes. General health was assessed by blood hemoglobin, plasma albumin and plasma ALT and AST. Blood hemoglobin was ≥120 g/l for all male subjects in the study. For women, 70 of the 84 females included in the study had blood hemoglobin content ≥120 g/l, 13 had blood hemoglobin content ≥110 and <120 g/l, and one had low blood hemoglobin content (70 g/l) at pre-screening. Plasma ALT and AST was within the normal range in 148 and 143 subjects, respectively. The 14 patients with blood hemoglobin <120 g/l and 5 patients with abnormal plasma ALT and AST were retained in the analysis as other biochemical indicators were not dissimilar from patients meeting the inclusion criteria.

Markers of vascular inflammation were surprisingly little-changed in patients with type 2 diabetes. For CRP, 7% of healthy controls and 8% of patients with diabetes had levels higher than the upper limit of the normal range (4.50 mg/l); and for sVCAM-1, 10% of healthy controls and 11% of patients with diabetes had levels higher than the upper limit of the normal range (0.88 mg/l). There were small increases of plasma CRP in patients with diabetes and microalbuminuria

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Table 1 – Characteristics of subjects recruited for this study.

Variable	Healthy subjects	Diabetes		
		Normoalbuminuria	Microalbuminuria	Albuminuria
N	37	53	36	26
Gender (M/F)	17/20	23/30	16/20	13/13
Age (years)	52 ± 6	57 ± 5***	58 ± 4***	57 ± 6**
Duration of diabetes (years)	–	8 (5–14)	7 (5–10)	8 (6–10)
BMI (kg/m^2)	23.6 ± 2.9	24.6 ± 3.6	24.9 ± 3.4	26.2 ± 3.5**
Plasma glucose (mM)	4.94 ± 0.60	6.64 ± 2.02***	8.31 ± 3.06***	8.53 ± 3.37***
HbA_{1c} (%)	5.73 ± 0.42	6.79 ± 1.26***	7.36 ± 1.21***	7.70 ± 1.15***
GFR (ml/min)	102 ± 29	108 ± 33	96 ± 26	83 ± 27*
ACR (mg/g)	4.3 (1.9–9.4)	7.8 (2.53–11.6)	80.9 (45.3–136.6)***,†††	891.4 (486.7–2364.9)***,†††,‡‡‡
Plasma creatinine (μM)	74 ± 16	73 ± 20	80 ± 24	93 ± 31**,†
Systolic BP (mmHg)	115 ± 11	123 ± 14**	131 ± 14***,†	127 ± 16**
Diastolic BP (mmHg)	78 ± 7	79 ± 8	83 ± 9*	80 ± 9*
Total cholesterol (mg/dl)	202 ± 38	197 ± 34	204 ± 40	209 ± 50
LDL (mg/dl)	128 ± 33	119 ± 28	121 ± 31	128 ± 42
HDL (mg/dl)	52 ± 10	52 ± 11	49 ± 12	47 ± 9*,†
TG (mg/dl)	103 (76–138)	119 (82–165)	148 (106–204)**,†	147 (101–175)*
Hb (g/l)	139 ± 12	137 ± 13	135 ± 13	139 ± 19
Albumin (g/l)	45 ± 2	46 ± 2	46 ± 3	43 ± 9
Plasma ALT (U/ml)	24 ± 10	24 ± 15	25 ± 13	24 ± 16
Plasma AST (U/ml)	25 ± 6	25 ± 10	26 ± 9	25 ± 8
CRP (mg/l)	1.01 (0.49–2.20)	1.08 (0.38–1.95)	2.15 (0.64–3.05)*,†	1.46 (0.58–2.12)
sVCAM-1 (mg/l)	0.56 (0.48–0.70)	0.56 (0.47–0.65)	0.60 (0.50–0.73)	0.67 (0.78–0.89)**,†††

* Significance: $P < 0.05$ with respect to healthy subjects.
** Significance: $P < 0.01$ with respect to healthy subjects.
*** Significance: $P < 0.001$ with respect to healthy subjects.
† Significance: $P < 0.05$ with respect to patients with type 2 diabetes and normoalbuminuria.
††† Significance: $P < 0.001$ with respect to patients with type 2 diabetes and normoalbuminuria.
‡‡‡ Significance: $P < 0.001$ with respect to patients with type 2 diabetes and microalbuminuria.

and slightly elevated sVCAM-1 in diabetic patients with albuminuria. Use of ARBs/ACEIs and statin therapy was relatively low – although this is still common in developing countries linked to limited access to medicines [25].

3.2. *Thiamine-related variables*

Median plasma thiamine concentration was 10.7 nM in healthy people and was not changed significantly in patients with diabetes with or without microalbuminuria or albuminuria. Similarly RBC concentration of thiamine and activity of TK were unchanged in patients with diabetes. Urinary excretion of thiamine and $FE_{thiamine}$ were increased 62% and 50%, respectively, in patients with type 2 diabetes and normoalbuminuria (Table 2). Comparison of healthy subjects with all patients with diabetes combined showed that the urinary excretion thiamine was not increased in patients with type 2 diabetes whereas $FE_{thiamine}$ was increased (22.8 versus 33.5%; $P < 0.05$).

3.3. *Vitamin B_6-related variables*

B_6 vitamers, PN, PL, PLP and PMP were detected in plasma. In healthy people, PL was the quantitatively most important B_6 vitamer analyte (78% total plasma B_6 vitamers) and others minor – PLP 16%, PN 5% and PMP 1%. Plasma concentrations of B_6 vitamers in healthy people were (nM): PN 3.50 (2.72–5.45), PL 53.5 (45.1–67.3), PLP 10.7 (9.4–16.6) and PMP 0.89 (0.61–1.22). Plasma concentrations of PL and PLP was increased in patients with diabetes ($P < 0.05$, Kruskal–Wallis); plasma PL was

Table 2 – Thiamine-related variables.

Variable	Healthy subjects	Diabetes		
		Normoalbuminuria	Microalbuminuria	Albuminuria
Plasma thiamine (nM)	10.7 (8.5–12.9)	11.5 (8.4–17.6)	12.3 (6.7–22.3)	10.0 (6.1–12 .3)
RBC thiamine (nmol/g Hb)	0.011 (0.006–0.020)	0.013 (0.007–0.024)	0.014 (0.008–0.024)	0.011 (0.006–0.022)
RBC TK activity (U/g Hb)	0.34 ± 0.11	0.35 ± 0.14	0.35 ± 0.14	0.29 ± 0.10
Urinary thiamine (μmol/g creatinine)	0.29 (0.21–0.49)	0.47 (0.26–1.18)*	0.36 (0.16–0.76)	0.26 (0.15–0.51)
$FE_{thiamine}$ (%)	22.8 (17.0–34.7)	34.2 (19.9–54.7)*	34.0 (21.1–41.9)	27.8 (15.6–58.3)

* Significance: $P < 0.05$ with respect to healthy subjects.

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Table 3 – Vitamin B_6-related variables.

Variable	Healthy subjects	Diabetes		
		Normoalbuminuria	Microalbuminuria	Albuminuria
Plasma PL (nM)	53.5 (45.1–67.3)	60.3 (47.5–75.0)	69.4 (46.4–91.5)	68.4 (52.8–98.6)*
Plasma PLP (nM)	10.7 (9.4–16.6)	18.6 (11.7–34.4)**	15.5 (11.7–36.7)*	13.1 (9.4–19.8)
Plasma 4-PA (nM)	7.4 (5.8–12.0)	13.4 (9.5–24.4)***	12.8 (10.0–23.2)***	15.1 (11.5–25.2)***
Urinary PL (μmol/g creatinine)	1.00 (0.77–1.14)	1.11 (0.92–1.28)	1.08 (0.98–1.41)	1.20 (0.97–1.57)**
Urinary 4-PA (μmol/g creatinine)	0.081 (0.063–0.098)	0.142 (0.097–0.201)***	0.137 (0.098–0.201)***	0.106 (0.084–0.183)**
(% total B_6 excretion)	6.5 (5.4–9.0)	10.4 (6.5–13.6)**	9.2 (6.2–13.1)*	6.9 (4.0–9.9)

* Significance: $P < 0.05$ with respect to healthy subjects.
** Significance: $P < 0.01$ with respect to healthy subjects.
*** Significance: $P < 0.001$ with respect to healthy subjects.

increased in patients with diabetes and albuminuria and plasma PLP was increased in patients with diabetes and normoalbuminuria or microalbuminuria (Table 3).

B_6 vitamers were detected in RBCs. In healthy people, PL and PLP were the quantitatively most important B_6 vitamer analytes (31% and 34%, respectively, of total B_6 vitamers) and other vitamers minor – PN 17% and PMP 18%. B_6 vitamer concentrations in RBCs of healthy people were (nmol/g Hb): PN 0.027 (0.015–0.045), PL 0.049 (0.030–0.078), PLP 0.054 (0.039–0.070) and PMP 0.028 (0.020–0.040). The levels were not changed significantly in patients with diabetes with or without microalbuminuria or macroalbuminuria. Related to this, RBC activities of PLP-dependent enzymes, ALT and AST, were 7.89 ± 3.42 and 27.6 ± 9.3 U/g Hb respectively, in healthy people and were also not changed in patients with diabetes (data not shown).

B_6 vitamers were detected in urine of healthy people. PL was the major B_6 vitamer excreted (95% total B_6 vitamer excretion), with others showing only minor excretion – PN 4%, PLP 1% and PMP <1%. Urinary excretions of B_6 vitamers in healthy people were (μmol/g creatinine): PN 0.040 (0.029–0.056), PL 1.00 (0.77–1.14), PLP 0.006 (0.001–0.053), 0.0032 (0.0020–0.0048). Urinary excretion of PL was increased in patients with diabetes, with respect to healthy controls ($P < 0.05$, Kruskal–Wallis), which was attributed to increased urinary excretion of PL in patients with diabetes and albuminuria (Table 3). Fractional excretion of B_6 vitamers in healthy people was (%): FE_{PN} 9.0 (6.1–17.0), FE_{PL} 15.4 (13.4–16.7), FE_{PLP} 0.34 (0.07–1.85) and FE_{PMP} 2.7 (1.9–5.1). Fractional excretion of B_6 vitamers was not changed significantly in patients with diabetes and with or without microalbuminuria or albuminuria (data not shown).

The major degradation product of vitamin B_6, 4-PA, was detected in plasma, RBCs and urine. Plasma 4-PA was increased in patients with diabetes with or without microalbuminuria or albuminuria. RBC content of 4-PA was not changed in patients with diabetes. Urinary excretion of 4-PA was increased in all diabetic patient groups (Table 3).

3.4. *Folate, total homocysteine and vitamin B_{12}–related variables*

The concentration of folate in plasma was not changed in patients with diabetes with or without microalbuminuria, with respect to healthy people, but was increased 14% in patients with diabetes and albuminuria. Total plasma cobalamin was increased 37% in patients with diabetes and microalbuminuria. Plasma holotranscobalamin was increased 36% and 47% in patients with diabetes and microalbuminuria and albuminuria, respectively. Plasma tHcy was increased in patients with diabetes independent of ACR status whereas MMA was not increased in patients with type 2 diabetes (Table 4). Criteria for diagnosing vitamin B_{12} deficiency are plasma holotranscobalamin <50 pmol/l and plasma MMA >0.27 μmol/l [26]. Applying these criteria to the study groups herein, the proportion of patients with type 2 diabetes and healthy subjects with vitamin B_{12} deficiency was 7% and 14%, respectively.

Bivariate correlation analysis and multiple linear regression analysis for B-vitamin-related variables in patients with

Table 4 – Folate, total homocysteine and vitamin B_{12}-related variables.

Variable	Healthy subjects	Diabetes		
		Normoalbuminuria	Microalbuminuria	Albuminuria
Plasma folate (nmol/l)	30.4 ± 7.5	32.7 ± 8.4	33.8 ± 9.8	34.7 ± 10.2*
Total plasma cobalamin (ng/l)	398 (299–604)	545 (380–748)	545 (430–867)**	568 (397–682)
Plasma holotranscobalamin (pmol/l)	74 (51–92)	86 (65–148)	101 (74–210)**	110 (91–200)***
Plasma tHcy (μmol/l)	10.8 (8.6–14.6)	16.3 (10.4–33.2)**	19.2 (10.0–32.1)***	18.8 (12.0–28.1)***
Plasma MMA (μmol/l)	0.19 (0.13–0.29)	0.19 (0.14–0.29)	0.20 (0.16–0.26)	0.20 (0.15–0.25)

* Significance: $P < 0.05$ with respect to healthy subjects.
** Significance: $P < 0.01$ with respect to healthy subjects.
*** Significance: $P < 0.001$ with respect to healthy subjects.

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Table 5 – Multiple linear regression analysis of markers of B-vitamin disturbance in patients with type 2 diabetes.

Dependent variable	Independent variable	Unstandardized coefficient	Standardized coefficient	P
$FE_{Thiamine}$ (%)	HbA_{1c} (%)	7.12 ± 3.23	0.21	0.032
Urinary 4-PA (μmol/g creatinine)	Duration of diabetes (years)	0.08 ± 0.03	0.23	0.014
	Systolic blood pressure (mmHg)	0.02 ± 0.01	0.19	0.045
	Constant	−2.99 ± 1.42		0.038
Plasma holotranscobalamin (pmol/l)	Fasting plasma glucose (mmol/l)	16.4 ± 3.8	0.38	<0.001
	Folate (ng/l)	−2.55 ± 1.09	−0.20	0.022
	CRP (mg/l)	9.55 ± 4.77	0.17	0.048

Variables included in the models (excluding interrelated dependent variables in different statistical models): age, duration of diabetes, fasting plasma glucose, HbA1c, TC, LDL, HDL, TG, systolic and diastolic blood pressure, ACR, CRP, sVCAM-1, folate, GFR.

type 2 diabetes: In bivariate correlation analysis of thiamine metabolites, plasma thiamine correlated positively with urinary thiamine and RBC TK ($r = 0.74$, $P < 0.001$ and $r = 0.26$, $P < 0.01$, respectively). In multiple regression analysis, increased $FE_{thiamine}$ was selected as the dependent variable since it is a critical marker of renal mishandling of thiamine in type 2 diabetes [8,27]. There was a positive regression of $FE_{thiamine}$ on HbA_{1c} in patients with type 2 diabetes (Table 5).

In bivariate correlation analysis of B_6 vitamers, there were positive correlations of plasma PLP with plasma PL and 4-PA ($r = 0.44$ and 0.70, $P < 0.001$), RBC PLP, PL and 4-PA ($r = 0.70$, 0.53 and 0.37; $P < 0.001$) and with urinary 4-PA ($r = 0.70$, $P < 0.001$). Plasma PL correlated positively with plasma sVCAM-1 ($r = 0.29$, $P < 0.01$) and urinary 4-PA correlated positively with HbA_{1c} (0.26, $P < 0.01$). Increased urinary excretion of 4-PA in patients of type 2 diabetes is a marker of increased degradation of vitamin B_6. In multiple regression analysis, urinary excretion of 4-PA was selected as the dependent variable since it was a marker of increased vitamin B_6 degradation in type 2 diabetes. There were positive regressions of urinary excretion of 4-PA on duration of diabetes and systolic blood pressure in patients with type 2 diabetes (Table 5).

In bivariate correlation analysis of vitamin B_{12}-related variables, plasma total vitamin B_{12} correlated positively with holotranscobalamin ($r = 0.76$, $P < 0.001$), plasma glucose and HbA_{1c} ($r = 0.26$ and 0.25, respectively; $P < 0.01$); and correlated negatively with plasma MMA ($r = -0.38$, $P < 0.001$). Plasma holotranscobalamin had similar correlates. Folate correlated positively with TG ($r = 0.26$, $P < 0.01$). In multiple regression analysis, plasma holotranscobalamin was selected as the dependent variable since it was a marker of vitamin B_{12} disturbance in type 2 diabetes. There were positive regressions of plasma holotranscobalamin on fasting plasma glucose and CRP and a negative regression with plasma folate in patients with type 2 diabetes (Table 5).

Other significant outcomes in bivariate correlation analysis were: plasma sVCAM-1 correlated positively with ACR ($r = 0.40$, $P < 0.001$) and negatively with eGFR ($r = -0.27$, $P < 0.01$); and plasma CRP correlated positively with BMI and TG ($r = 0.29$, $P < 0.01$ and $r = 0.25$, $P < 0.01$, respectively).

3.5. *Effect of drug treatment on B-vitamin status*

Patients with type 2 diabetes receiving treatment with metformin did not have any significant change in B-vitamin-related variables, compared to patients with type 2 diabetes not treated with metformin. Patients with type 2 diabetes receiving treatment with α-glucosidase inhibitors had decreased plasma holotranscobalamin, compared to patients not treated with α-glucosidase inhibitors: 80 (66–109) versus 104 (78–209) pM, $P < 0.05$. They also had decreased RBC PLP concentration: 0.042 (0.031–0.064) versus 0.057 (0.040–0.088) pmol/mg Hb ($P < 0.05$). Patients with type 2 diabetes receiving treatment with sulfonylurea hypoglycaemic agents had decreased plasma holotranscobalamin, compared to patients not treated with sulfonylureas: 90 (68–113) versus 119 (78–213) pM, $P < 0.05$. Patients with 2 diabetes receiving treatment with insulin did not have any significant change in B-vitamin-related variables. Patients with 2 diabetes receiving treatment with ARBs/ACEIs had increased plasma thiamine concentration compared to patients with 2 diabetes not treated with ARBs/ACEIs: plasma thiamine concentration – 15.8 (10.8–31.6) versus 10.6 (6.6–16.7 nM, $P < 0.01$. Patients receiving treatment with statins had expected decreased plasma total cholesterol (170 ± 28 versus 205 ± 38 mg/dl, $P < 0.01$) and LDL cholesterol (93 ± 26 versus 124 ± 29 mg/dl, $P < 0.001$) but no significant difference of B-vitamin-related variables.

4. Discussion

Countering the effects of increased renal clearance of thiamine in clinical diabetes by thiamine supplementation is an emerging strategy to improve the treatment of early-stage DN [28]. Studies of thiamine status in healthy people have shown that urinary thiamine normalised to urinary creatinine correlates positively with 24 h urinary excretion of thiamine [29]. Twenty-four hour urinary excretion of thiamine also correlates with dietary intake of thiamine [30]. Urinary thiamine–creatinine ratio measured herein therefore relates to dietary intake of thiamine and was increased approximately 2-fold in patients with type 2 diabetes and normoalbuminuria only. This was also found in previous study of patients with type 2 diabetes in the United Kingdom [8]. This may reflect improved dietary advice and compliance with it of some but not all patients with type 2 diabetes. Herein, however, patients with type 2 diabetes stratified by ACR – normoalbuminuria, microalbuminuria and albuminuria – had similar duration of diabetes, HbA_{1c}, blood pressure and lipids (except triglycerides), suggesting that patients with microalbuminuria and albuminuria had progressed to incipient and

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overt DN more rapidly than those with normoalbuminuria. Patients with microalbuminuria and albuminuria had lower urinary excretion of thiamine than those with normoalbuminuria (0.30 versus 0.46 μmol/g creatinine, $P < 0.025$). This may reflect influence of dietary thiamine on rate of development of DN with higher dietary thiamine intake linked to slower progression of DN. This was found in the prospective Joslin Kidney Study of patients with type 1 diabetes [31].

Thiamine status showed limited disturbance in patients with type 2 diabetes, excepting 62% increase in urinary excretion and 50% increase in $FE_{thiamine}$ in patients with normoalbuminuria. The latter was also typical of all patients with diabetes combined. In healthy people the plasma concentration of thiamine was lower and $FE_{thiamine}$ higher than found previously in a UK study although the urinary excretion of thiamine was similar in the two populations [8]. Plasma thiamine concentration in patients with type 2 diabetes were similar to those found in patients with type 2 diabetes in Pakistan [6]. There was also a marked lower increase in $FE_{thiamine}$ in patients with type 2 diabetes than found in the UK study. The reasons for these disparities are not clear but renal handling of thiamine was nevertheless disturbed with increased washout of thiamine in patients with type 2 diabetes in Indonesia. $FE_{Thiamine}$ was not correlated with urinary excretion of thiamine, suggesting that increase in $FE_{Thiamine}$ was not due to increased dietary thiamine intake. Increased $FE_{Thiamine}$ occurs in both experimental and clinical diabetes and is linked to decreased re-uptake of thiamine from the glomerular filtrate in the renal tubules in the diabetic state [8,27], likely linked to hyperglycemia-induced down regulation of thiamine transporter proteins in renal proximal tubular epithelial cells [32]. The positive regression of $FE_{Thiamine}$ with HbA_{1c} is consistent with this mechanistic interpretation. Thiamine supplements correct associated tissue specific thiamine deficiency in diabetes (reviewed in [28]).

For vitamin B_6, there was no evidence of increased fractional excretion of B_6 vitamers in patients with type 2 diabetes, suggesting renal mishandling similar to thiamine is not present. The finding of increased plasma PLP patients with type 2 diabetes may relate to changes in tissue storage of PLP. The major pools of vitamin B_6 storage are in the skeletal muscle and liver [33]. A minor release of PLP from tissues in the diabetic state may account for this increase. The finding of increased plasma and urinary levels of 4-PA suggests there is increased degradation of B_6 vitamers in patients with type 2 diabetes. Urinary 4-PA normalised to urinary creatinine correlates positively with 24 h urinary excretion of 4-PA [34]. Increased urinary excretion of 4-PA in diabetes, expressed as a percentage of total vitamin B_6 excretion, was not correlated with urinary excretion of pyridoxal, suggesting that increased excretion of 4-PA is not linked to dietary intake of vitamin B_6. 4-PA is formed by oxidation of PL catalysed by tissue oxidases [35]. The correlation of increased urinary excretion of 4-PA with HbA_{1c} suggests that increased B_6 vitamer degradation is linked to metabolic dysfunction in hyperglycemia. Multiple regression analysis revealed associations of urinary excretion of 4-PA with duration of diabetes, suggesting that degradation of vitamin B_6 may increase in severity as diabetes progresses. Increased degradation of vitamin B_6 is a novel aspect of B-vitamin disturbance in diabetes that may be considered in assessing requirement for vitamin supplementation.

Levels of vitamin B_{12} in patients with type 2 diabetes have been studied in relation to mechanistic links to increased plasma tHcy [12,13] and effects of metformin therapy [14,15]. Low plasma holotranscobalamin and high plasma MMA is an indicator of vitamin B_{12} deficiency [26]; plasma holotranscobalamin is considered now to be a better marker of vitamin B_{12} status than total plasma B_{12} concentration [36]. More recently, the concept of metabolic resistance to vitamin B_{12} in diabetes has been proposed to explain increased plasma levels of vitamin B_{12} in the presence of markers of decreased functional activity of the vitamin – increased tHcy, for example [37]. In this study, patients with type 2 diabetes had increased total cobalamin and holotranscobalamin in plasma. There was a concomitant increase in tHcy but not of MMA. Plasma holotranscobalamin in healthy subjects and patients with type 2 diabetes and plasma MMA in all subjects groups were similar to those of a large healthy human population [38], suggesting there was likely limited voluntary supplementation of vitamin B_{12} in the study groups.

Increased plasma holotranscobalamin without decrease or negative correlation with plasma tHcy but with negative correlation with MMA in patients with type 2 diabetes is a marker of vitamin B_{12} disturbance. Plasma holotranscobalamin in healthy subjects correlates negatively with both tHcy and MMA [38,39]. Increased plasma tHcy is thought to reflect decreased methyl transfer to homocysteine mediated by methylcobalamin and catalysed by methionine synthase and decreased transsulfuration to cystathionine catalysed by cystathionine-β-synthase in the cytosol of cells. Decreased methionine synthase activity and transsulfuration was found in patients with type 2 diabetes and microalbuminuria and albuminuria [40]. Formation of MMA is decreased by increased methyl transfer from methylmalonyl-CoA to form succinyl-CoA in mitochondria mediated by the mitochondrial vitamin B_{12} metabolite 5′-deoxyadenosyl-cobalamin. Hence, increased plasma holotranscobalamin and tHcy without increased MMA suggests there may be decreased functional activity of cobalamin, or vitamin B_{12} resistance, in the cytosolic but not in mitochondrial compartments of tissues in patients with type 2 diabetes – ''cytosolic metabolic resistance'' to vitamin B_{12}.

Positive correlations of plasma total cobalamin and holotranscobalamin with plasma glucose and HbA_{1c} suggests hyperglycemia may be linked to impairment of cobalamin metabolism. Multiple regression analysis revealed a positive link of increased plasma holotranscobalamin with fasting plasma glucose and CRP, suggesting both hyperglycemia and vascular inflammation may be linked to metabolic resistance to vitamin B_{12} in type 2 diabetes. Increased plasma total cobalamin and holotranscobalamin in patients with type 2 diabetes may suggest hyperglycemia impairs tissue uptake of vitamin B_{12}. If this is indeed so, our findings are consistent with mitochondria effectively scavenging residual vitamin B_{12} taken up from the cytoplasm to maintain metabolism of MMA. These effects support and advance the concept of metabolic resistance to vitamin B_{12} in diabetes [37].

Vascular inflammation is thought to be a characteristic and mechanistic mediator of vascular disease in patients with type

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2 diabetes [41]. Important markers of vascular inflammation are plasma CRP and sVCAM-1. Plasma CRP has previously been linked to BMI, glucose intolerance and microalbuminuria in Western populations. In this study, plasma CRP was only increased in patients with type 2 diabetes and microalbuminuria. Nevertheless, plasma CRP correlated positively with BMI and TG. The positive link of sVCAM-1 to ACR and negative link to GFR revealed the expected links of the marker of endothelial dysfunction with decline in renal function. Plasma sVCAM-1 was increased in patients with type 2 diabetes and albuminuria compared to healthy people – as found previously [42]. In a similar study of 74 patients with diabetes in the United Kingdom, however, we found 51% of patients had sVCAM-1 levels higher than the normal range maximum whereas only 11% of patients with type 2 diabetes had sVCAM-1 levels above this level herein, which was similar percentage as in healthy controls. Good glycemic control and also genetic and dietary factors in the Indonesian study population may be linked to low vascular inflammation [41]. This study suggests that low grade inflammation is not a marked characteristic of all populations of patients with type 2 diabetes.

A 2-month washout period was included in this study between pre-screening and study entry to minimise the effects of voluntary vitamin supplementation prior to entry into the study. The half-life of thiamine in plasma is relatively short (2 days) [43] and in tissues 9–18 days [44]. For vitamin B_6 it was reasoned that concentrations of B_6 vitamers in human subjects to adjust to new amounts of dietary intake within 10–20 days [33] – which has also been evidenced experimentally for plasma and RBCs pools of PLP [45,46]. The half-life of vitamin B_{12} is *ca*. 400 days [47] and hence the 2-month washout period does not provide correction for tissue loading of vitamin B_{12} by voluntary supplementation. The half-life of holotranscobalamin and total plasma cobalamin, however, is markedly less – *ca*. 3 days [48]. The washout period of 2 months provided for correction of B-vitamin levels after supplementation for thiamine, B_6 vitamers and vitamin B_{12} (plasma holotranscobalamin and total cobalamin only).

A high proportion of healthy people recruited for the study, *ca*. 60%, had HbA_{1c} indicative of pre-diabetes state of impaired glucose tolerance (IGT). The age range was 47–65 years. The prevalence of IGT in Indonesia assessed by oral glucose tolerance test in 2009 for this age group was 13–18% [49]. Analysis of B-vitamin status in healthy people with pre-diabetes IGT showed no significant disturbance of B-vitamin status.

There is a possibility that patients with type 2 diabetes in this study with microalbuminuria and albuminuria developed nephropathy independent of the presence of diabetes. The criteria for patients with type 2 diabetes and microalbuminuria or albuminuria were a documented diagnosis of type 2 diabetes mellitus of $\geq$5 years and presence of microalbuminuria or albuminuria on 2 or more occasions – including at pre-screening and study entry. The Collaborative Study Group, using entry criteria similar to these found that 94% of patients with type 2 diabetes and nephropathy had diabetic glomerulosclerosis on biopsy [50]. The presence of non-diabetic nephropathy was, therefore, likely limited and the outcomes of study groups type 2 diabetes with and microalbuminuria or albuminuria are indeed attributable to diabetic incipient and overt nephropathy, respectively.

Assessing the effects of medication of patients with type 2 diabetes on B-vitamin status, there was no disturbance of B-vitamin status with treatment by metformin, insulin nor statins. Treatment with α-glucosidase inhibitors and sulfonylureas was, however, linked to decreased plasma holotranscobalamin, and treatment with α-glucosidase inhibitors also linked to decreased RBC concentration of PLP. Treatment with ARBs/ACEIs was associated with increased plasma thiamine concentration, suggesting that benefits of these agents may also be associated with decreasing renal clearance of thiamine. The number of patients in this study receiving treatment with ARBs/ACEIs was small, however, and this effect requires confirmation in a larger patient group.

The clinical significance of B-vitamin disturbance in patients with type 2 diabetes found herein may be judged from vitamin supplementation studies. Supplementation with thiamine of patients with type 2 diabetes reversed early-stage kidney disease – a response that may be linked to correcting renal deficiency of thiamine linked to this increased thiamine washout [28]. Previous studies of supplementation with vitamins B_6, B_9 and B_{12} in patients with vascular disease or diabetes found no effect of co-supplementation on death from CVD, decreased risk of stroke and increased risk of unstable angina (HOPE-2) study [12] and patients with type 1 or type 2 diabetes mellitus and advanced DN increased the rate of decline in renal function (DIVINe study) [13]. Folate and cyanide from cyanocobalamin treatment are possible factors linked to adverse effects in the DIVINe study [16,17], which may negate otherwise beneficial effects of vitamin B_6 and B_{12} supplementation, but this requires further investigation. Currently countering the effects of diabetes-induced renal washout of thiamine by thiamine supplementation appears to be the most important disturbance of B-vitamin metabolism requiring correction for improved clinical outcome.

5. Conclusions

This study shows that there was increased $FE_{thiamine}$ in patients with type 2 diabetes – suggesting renal mishandling of thiamine, increased urinary excretion of 4-PA – indicative of increased vitamin B_6 degradation, and increased tHcy without increased MMA in the presence of increased plasma total and active vitamin B_{12}, consistent with cytosolic metabolic resistance to vitamin B_{12}. There were also modest increases of plasma CRP and sVCAM-1 in patients with type 2 diabetes with microalbuminuria and albuminuria, respectively, suggesting limited vascular inflammation. Therefore, in Indonesia patients with type 2 diabetes – typical of the South East Asian region, there is increased washout of thiamine, degradation of vitamin B_6 and cytosolic metabolic resistance to vitamin B_{12} without marked vascular inflammation.

Conflict of interest

The authors have a competing interest to declare. P.J.T. received honoraria from MercK KGaA (Germany) for speaking

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at expert meetings on ''The Role of B-vitamins in Preventing Diabetic Complications'' in 2010 and 2011.

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