Estimated Creatinine Clearance, Homocysteine and High Sensitivity-C-Reactive Protein Levels Determination for Early Prediction of Nephropathy and Atherosclerosis Risk In Type 2 Diabetic Patients

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Abstract Type 2 diabetes mellitus (T2D) patients are increased risk for cardiovascular disease (CVD) and chronic kidney disease (CKD). Many studies had demonstrated that CKD is significantly associated with CVD. We aim to indicate the using estimated creatinine clearance (eCrCl), homocysteine (tHcy), and high sensitivity-C-reactive protein (hs-CRP) levels, may have an impact on the interpretation risk for nephropathy and CVD. eCrCl was using the Cockcroft-Gault formula, eCrCl levels were stratified according to the Kidney Disease Outcome Quality Initiative definition. We measured serum tHcy, hs-CRP, and the other biochemical variables in 54 T2D patients compared with 40 age matched healthy controls (NDM). T2D patients were significantly lower eCrCl than NDM ($P < 0.05$). T2D patients also showed significantly higher in tHcy, hs-CRP, and MDA levels than NDM subjects ($P < 0.05$). The eCrCl was significantly correlated with tHcy and hs-CRP levels in T2D patients ($r = -0.504$, $P < 0.001$; $r = -0.282$, $P = 0.047$). eCrCl had an impact on interpretation for CKD, especially in T2D patients. Decrease eCrCl concomitant with increased in tHcy, hs-CRP, and MDA levels may present a higher risk for future diabetic nephropathy and CVD.

Keywords Estimated creatinine clearance · Homocysteine · Hs-CRP · Chronic kidney disease · Cardiovascular disease · Type 2 diabetes mellitus

List of Abbreviation

T2D	Type 2 diabetes mellitus
CVD	Cardiovascular disease
CKD	Chronic kidney disease
eCrCl	Estimated creatinine clearance
tHcy	Homocysteine
hs-CRP	High sensitivity-C-reactive protein
RI	Renal insufficiency
RF	Renal failure
Glu	Glucose
TC	Total cholesterol
TG	Triglycerides
HDL-C	High density lipoprotein-cholesterol
LDL-C	Low density lipoprotein-cholesterol
KDOQI	Kidney disease outcome quality initiative

Introduction

The number of patients with type 2 diabetes mellitus (T2D) is increasing worldwide. T2D patients are increased risk for cardiovascular disease (CVD) that of the association with several risk factors included hypertension, hyperlipidemia, and obesity. Data from the Framingham study has been shown diabetes to be associated with a 2–4 fold increase in risk CVD [1]. Diabetic nephropathy develops in 25–35% of patients with T2D. Hypertension and proteinuria, which together characterize diabetic nephropathy, are associated with an increased risk of cardiovascular disease [2]. Development of albumin is used as a sensitive clinical risk marker and predictor to identify

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those at risk of future development of renal dysfunction [3, 4]. We reported that renal outcomes assessed at follow-up were development of renal impairment or chronic kidney disease (CKD) assessed as estimated creatinine clearance (eCrCl) < 60 ml/min/1.73 m². Many studies have demonstrated that total homocysteine (tHcy) is a risk factor for CVD in T2D patients [5, 6]. tHcy, a nutritional marker associated with premature atherosclerosis, predicts future risk of CVD in observational studies [7]. Atherosclerosis is considered to be an inflammatory process triggered by response to injury and oxidative stress. Increasing in the plasma inflammation markers were shown to be related with the risk of vascular disease in T2D patients [8, 9]. C-reactive protein (CRP), an inflammatory marker, is well known to be increased in inflammatory conditions [10, 11]. CRP was recommended to measure as the risk for atherosclerosis [12, 13].

Patients renal insufficiency (RI), renal failure (RF) associated with higher levels of tHcy and CRP and have a high prevalence of cardiovascular disease, and it has been proposed that atherosclerosis may promote the progression of renal disease in T2D patients [14–16]. Aim of the present study to indicate the using eCrCl, tHcy, and hs-CRP levels, may have an impact on the interpretation risk for nephropathy and CVD in T2D patients.

Materials and Methods

Subjects

In this cross sectional study, participants consisted with 54 T2D patients (38 female, 12 male) with a median age of 68.5 years (interquartile, 64.5–76.0 years) and 40 age matched healthy volunteers (as NDM) (28 female, 12 male) with a median age of 67 years (interquartile, 63.5–70.8 years). All T2D patients (overt diabetes more than 5 years) were randomized during September 2009 to October 2010 and regularly treat with glycemic lowering, lipid lowering, and anti-hypertensive drug. These participants had no sign of cardiovascular disease, renal damage, and infectious diseases as based on their medical history and a physical examination. The exclusion criteria for entry into the study were sustained heart failure, peripheral vascular disease, acute or chronic infection, cancer, hepatic disease, acute illness and recent myocardial infarction, unstable angina, or stroke. All participants gave written informed consent and the Ethics Committee of the Naresuan University approved the study protocol.

Anthropometric Measurement and Biochemical Measurement

Waist circumference (WC) was measured at the midpoint between the rib cage and the top of the lateral border of the

iliac crest during minimal respiration. Blood pressure (BP) was measured as the mean value of at least two measurements of all participants on the same day with a Terumo digital blood pressure monitor, ES-P110 (Terumo Corporation, Japan). All participants attended for venous blood collection in the morning after 8–12 h overnight fast.

Fasting plasma glucose (Glu), serum BUN, creatinine, total cholesterol (TC), triglycerides (TG), were measured immediately after collection by using enzymatic procedures with a Hitachi 912 auto-analyzer (Roche Diagnostic, Switzerland). High density lipoprotein-cholesterol (HDL-C) was measured by a direct method using polyethylene-glycol pretreated enzymes (Roche Diagnostic, Switzerland). We calculated low density lipoprotein-cholesterol (LDL-C) with Friedewald's formula [17].

Renal Function

All participants had clinically normal renal function, defined as a serum creatinine concentration below 159.12 μ mol/l (1.8 mg/dl) and serum BUN concentration below 7.14 mmol/l (20 mg/dl). We used the Cockcroft-Gault formula to calculate the eCrCl which incorporates age, body weight and sex [18]. The formula is as follows:

$$eCrCl = [(140 - age) * Weight (kg) * constant] / \text{serum creatinine } (\mu\text{mol/L})$$

Where constant is 1.23 for men and 1.04 for women; serum creatinine in μ mol/l.

The classification of eCrCl by stages was done according to the Kidney Disease Outcome Quality Initiative (KDOQI) [19] criteria as follow: Stage I: normal eCrCl (≥ 90 ml/min/1.73 m²); Stage II: mildly eCrCl (60–89 ml/min/1.73 m²); Stage III: moderately eCrCl (30–59 ml/min/1.73 m²); Stage IV: severely eCrCl (< 30 ml/min/1.73 m²), and Stage V: end-stage renal disease: eCrCl (< 15 ml/min/1.73 m²).

Total Homocysteine (tHcy) Assay

tHcy concentrations in the serum was measured by a fluorescence polarization immunoassay (FPIA) on an automated analyzer (AxSYM, Abbotte Diagnostics, USA). The normal range of tHcy was 5–15 μ mol/l. The elevated eHcy level is defined as tHcy level above the upper limit.

High Sensitive CRP Assay

The CRP concentrations were measured by highly sensitive CRP (hs-CRP) with the use of latex-enhanced immunonephelometric assay on the Hitachi 912 auto-analyzer (Roche Diagnostic, Switzerland) that has been standardized

against the World Health Organization reference. The normal range of hs-CRP was <3.0 mg/l.

MDA (As Serum Lipid Peroxidation) Assay

The MDA was determined by the method of Tangvarasitichai et al. [20]. The method is based on the formation of red (pink) chromophore following the reaction of thiobarbituric acid (TBA) with MDA and the other breakdown products of peroxidized lipids called thiobarbituric acid reactive substances (TBARS). One molecule of MDA reacts with 2 molecules of TBA to yield a pink pigment with absorption maximum at 532 nm. The normal range of MDA (TBARS) was <3.5 $\mu\text{mol/l}$.

Statistical Analysis

All variables are expressed as median and interquartile (Q1–Q3). We compared all clinical characteristics of T2D patients with NDM control subjects by using Mann–Whitney test. These T2D patients were stratified to 4 groups by using eCrCl levels according to the KDOQI criteria. We also compared all clinical characteristics of T2D patients in each group by using Kruskal–Wallis test. Bivariate correlation between eCrCl with tHcy, hs-CRP, and the other variables were assessed by using Spearman rank correlation test. All tests were two tailed, and a P value < 0.05 was considered significant. All analysis

was performed using the SPSS computer program version 13.0 (SPSS, Chicago, IL).

Results

All clinical characteristics of the 54 T2D patients and 40 NDM control subjects in the present study are listed and compared as shown in Table 1. Patients with T2D were significantly lower eCrCl ($P < 0.001$) and HDL-C ($P < 0.001$), and higher in tHcy ($P = 0.02$), hs-CRP ($P = 0.004$), MDA ($P < 0.001$), BP, WC, TC, TG, LDL-C, than their control subjects. We stratified the 54 T2D patients into four groups by using eCrCl levels according to the KDOQI definition. Seven (12.96%) T2D patients had stage I: normal eCrCl ≥ 90 ml/min/1.73 m² (G1), 15 (27.78%) T2D patients had stage II: mildly eCrCl 60–89 ml/min/1.73 m² (G2), 28 (51.85%) T2D patients had stage III: moderately eCrCl 30–59 ml/min/1.73 m² (G3), 4 (7.41%) T2D patients had stage IV: severely GFR < 30 ml/min/1.73 m² (G4). We also compared the clinical characteristics of those 4 groups by using the Kruskal–Wallis test. Those were significantly differed in age ($P < 0.001$), CT ($P < 0.001$), eCrCl ($P = 0.01$), tHcy ($P = 0.09$), and hs-CRP ($P = 0.04$). G1 was the youngest group. tHcy and hs-CRP levels were subsequent increased from G2 to G4, and eCrCl level was subsequent decreased from G2 to G4, respectively. As in Table 2, there

Table 1 The baseline characteristics of T2D patients and their NDM control subjects

Parameter	T2D patients ($n = 54$)	NDM patients ($n = 40$)	P value
Age (year)	68.5* (64.5–76.0)**	67.0* (63.5–70.8) **	0.331
Systolic BP (mmHg)	135.0 (121.0–154.3)	126.0 (114.0–135.3)	0.009
Diastolic BP (mmHg)	77.0 (70.8–93.3)	74.0 (70.0–80.0)	0.015
WC (cm)	90.0 (85.0–94.1)	83.0 (77.0–88.0)	<0.001
Fasting Glu (mmol/l)	6.52 (5.27–8.11)	4.40 (4.18–4.95)	<0.001
BUN (mmol/l)	4.64 (3.93–5.82)	3.93 (3.32–4.64)	0.002
CT ($\mu\text{mol/l}$)	79.56 (61.88–97.24)	72.72 (61.88–88.40)	0.190
TC (mmol/l)	5.22 (4.79–5.99)	4.59 (4.26–4.99)	<0.001
TG (mmol/l)	1.93 (1.39–3.90)	1.42 (1.14–1.93)	<0.001
HDL-C (mmol/l)	1.18 (0.98–1.35)	1.55 (1.36–1.76)	<0.001
LDL-C (mmol/l)	2.95 (2.33–3.46)	2.38 (2.03–2.62)	<0.001
eCrCl (ml/min/1.73 m ²)	57.9 (42.3–73.0)	72.8 (62.6–88.0)	0.001
tHcy ($\mu\text{mol/l}$)	16.2 (12.3–19.8)	13.7 (11.3–17.0)	0.020
hs-CRP (mg/l)	3.26 (2.19–5.60)	1.27 (0.61–2.76)	0.004
MDA ($\mu\text{mol/l}$)	3.25 (2.33–3.60)	1.69 (1.27–2.12)	<0.001

* Median

** Interquartile

BP blood pressure, WC waist circumference, Glu glucose, BUN blood urea nitrogen, CT creatinine, TC total cholesterol, TG triglycerides, HDL-C high density lipoprotein cholesterol, LDL-C low density lipoprotein cholesterol, eCrCl estimated creatinine clearance, tHcy total homocysteine, hs-CRP high sensitive C-reactive protein, MDA malondialdehyde

was a trend towards increasing serum tHcy and hs-CRP with decreasing eCrCl in each group. Almost of T2D patients showed mildly and moderately renal insufficiency while BUN and creatinine levels were expressed as normal levels.

The bivariate correlation between eCrCl with tHcy, hs-CRP, and other variables in T2D patients showed that eCrCl was significantly correlated with age ($r = -0.613$, $P < 0.001$), WC ($r = 0.330$, $P = 0.019$), diastolic BP ($r = 0.374$, $P = 0.007$), tHcy ($r = -0.504$, $P < 0.001$)

and hs-CRP ($r = -0.282$, $P = 0.047$) in T2D patients as shown in Table 3 and Fig. 1a, b.

Discussion

T2D patients in the present study were significantly lower in eCrCl levels than NDM controls the same as we reported previously [4]. T2D patients with mild and moderate renal insufficiency have significantly higher in tHcy and hs-CRP

Table 2 All clinical characteristics of the type 2 diabetes patients stratified according to eCrCl levels as KDOQI definition

Parameter	G1 ($n = 7$)	G2 ($n = 15$)	G3 ($n = 28$)	G4 ($n = 4$)	<i>P</i> value
Age (year)	55.0* (50.0–66.0)**	65.0* (58.5–68.2)**	74.0* (69.0–80.0)**	72.5* (67.5–76.8)**	<0.001
Systolic BP (mmHg)	127.0 (111.0–148.0)	141.0 (121.75–162.5)	135.0 (122.5–153.5)	125.0 (90.25–165.8)	0.582
Diastolic BP (mmHg)	85.0 (76.0–104.0)	91.0 (75.3–104.0)	75.0 (69.0–87.5)	70.0 (65.0–87.8)	0.097
WC (cm)	94.5 (90.0–108.0)	89.5 (85.8–97.0)	87.0 (85.0–2.5)	88.5 (66.75–93.8)	0.209
Fasting Glu (mmol/l)	6.71 (5.23–7.37)	6.41 (5.27–7.65)	6.33 (5.25–8.33)	9.16 (5.06–12.91)	0.791
BUN (mmol/l)	5.0 (4.28–6.78)	5.18 (3.93–6.18)	4.64 (3.93–5.89)	2.86 (2.50–4.82)	0.280
CT ($\mu\text{mol/l}$)	88.4 (79.56–106.08)	75.14 (61.88–101.66)	79.56 (61.88–92.82)	79.56 (57.46–95.47)	<0.001
TC (mmol/l)	5.70 (4.90–6.37)	5.41 (4.08–6.77)	4.95 (4.79–5.73)	5.84 (5.43–6.42)	0.286
TG (mmol/l)	2.43 (1.41–3.99)	3.00 (1.39–4.65)	1.81 (1.29–2.74)	3.52 (1.42–5.15)	0.472
HDL-C (mmol/l)	1.33 (1.10–1.50)	1.20 (0.93–1.52)	1.14 (0.98–1.33)	1.10 (0.85–1.21)	0.477
LDL-C (mmol/l)	3.45 (2.85–3.85)	2.65 (1.93–3.43)	2.97 (2.34–3.30)	3.36 (2.84–3.73)	0.211
eCrCl (ml/min/1.73 m^2)	97.71 (91.48–120.93)	70.41 (67.38–79.30)	47.96 (40.17–53.96)	24.88 (22.58–27.05)	<0.001
tHcy ($\mu\text{mol/l}$)	14.41 (10.45–15.39)	14.45 (11.73–17.15)	17.01 (12.39–20.02)	28.45 (20.82–33.68)	0.009
hs-CRP (mg/l)	1.07 (0.71–2.30)	3.56 (1.64–6.56)	3.76 (2.47–5.80)	4.80 (2.39–17.15)	0.04
MDA ($\mu\text{mol/l}$)	2.96 (2.33–5.50)	3.17 (2.33–3.65)	3.55 (2.23–3.39)	3.75 (3.10–5.83)	0.572

* Median

** Interquartile

Comparison between the four groups was compared by Kruskal-Wallis test

G1 = eCrCl ≥ 90 mL/min/1.73 m^2 , G2 = eCrCl 60–89 mL/min/1.73 m^2 , G3 = eCrCl 30–59 mL/min/1.73 m^2 , G4 = eCrCl < 30 mL/min/1.73 m^2

BP blood pressure, WC waist circumference, Glu glucose, BUN blood urea nitrogen, CT creatinine, TC total cholesterol, TG triglycerides, HDL-C high density lipoprotein cholesterol, LDL-C low density lipoprotein cholesterol, eCrCl estimated creatinine clearance, tHcy total homocysteine, hs-CRP high sensitive C-reactive protein, MDA malondialdehyde

Table 3 Bivariate correlation of eCrCl with tHcy, hs-CRP, and others variables in the T2D patients

eCrCl (mL/min/1.73 m ²) with	<i>r</i>	<i>P</i> value
Age (year)	−0.613	<0.001
WC (cm)	0.330	0.019
Diastolic BP (mmHg)	0.374	0.007
tHcy (μmol/l)	−0.504	<0.001
hs-CRP (mg/l)	−0.282	0.047

BP blood pressure, WC waist circumference, tHcy total homocysteine, hs-CRP high sensitive C-reactive protein

levels than NDM in the present study. And age-related increase in tHcy is in agreement with previous reports [21, 22]. The MDA levels, a by-product of lipid peroxidation, as an oxidative stress marker was also significantly elevated in T2D patients. Oxidative stress is thought to be a key component in the pathophysiology of T2D patients. Hyperglycemia was a source of an increase in the production of oxidative free radicals, because oxidative stress is involved in inflammation. Generalized vascular inflammation and active lipid peroxidation (oxidative stress) generate pro-inflammatory and pro-atherogenic [23–25] in early events leading to atherosclerosis. In the present study, increased concentrations of MDA in T2D patients were indicating increased lipid peroxidation. There is increasing evidence that elevations of oxidative stress and associated oxidative damages are mediators of vascular injury in various cardiovascular pathologies including atherosclerosis, and ischemia–reperfusion [26] hs-CRP, the inflammatory marker is also significantly elevated in T2D patients in the present study. It may be from the pathogenesis of atherosclerosis. One possible cause may be from the glomerulosclerosis that results from the influx and accumulation of inflammatory cells (monocytes and macrophages), with mesangial cells responding in the similar manner to vascular smooth muscle cells. Then, renal disease and cardiovascular disease should share similar risk factors. tHcy and hs-CRP are the risk factors for atherosclerosis [27, 28], may be important factors in the progression of renal disease. Our data demonstrated that tHcy and hs-CRP levels were significantly associated with the decline in eCrCl. Then, plasma concentration of tHcy in diabetic patients might also be an effect of abnormal renal clearance of tHcy [29]. Patients with renal failure have a high prevalence of cardiovascular disease, and it has been proposed that atherosclerosis may promote the progression of renal disease [15, 30]. Since tHcy level is markedly elevated in patients with chronic renal failure, it has been presented as a potential factor contributing to the high risk of CVD in these patients [31].

Both tHcy and hs-CRP markers were investigated in diabetic patients as the risk factors of CVD. In our study, no significant relationship could be found between tHcy

Fig. 1 The reverse correlation of tHcy (a) and hs-CRP (b) levels with decreased eCrCl in type 2 diabetes patients ($r = -0.504$, $P < 0.001$, $r = -0.282$, $P = 0.047$), respectively

and hs-CRP. It was obvious that both tHcy and hs-CRP were significantly higher and reverse associated with eCrCl. These may hypothesize in which renal insufficiency leads to elevate of tHcy and hs-CRP, which then leads to progression of renal disease and further increase risk in CVD. On going follow up studies of these patients with renal insufficiency will provide further information on the association between tHcy, hs-CRP, MDA levels and the development of cardiovascular disease.

However, there are several limitations in the present study. The important limitation is that glomerular filtration rate is estimated and not measured by mean of inulin clearance or a radionuclide method. Second, our study is a small sample size and cross-sectional study. Third, vitamin B6, B12 and folic acid status are not assessing. However, all patients had dietary advice in their primary care clinics and none had clinical or hematological evidence of deficiency of these vitamins.

Conclusion

eCrCl had an impact on interpretation risk for CKD or renal insufficiency, especially in T2D patients. Decrease eCrCl concomitant with increased in tHcy, hs-CRP, and MDA levels may present a higher risk for future diabetic nephropathy and CVD.

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Conflict of Interest None.

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