Research Article

Urinary Leukotriene E_4 Is Associated with Renal Function but Not with Endothelial Function in Type 2 Diabetes

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Leukotrienes are inflammatory and vasoactive mediators implicated in endothelium-dependent relaxations and atherosclerosis. Urinary leukotriene E_4 (U-LTE$_4$) is a validated disease marker of asthma and increases also in diabetes and acute coronary syndromes. The aim of the present study was to evaluate the association of U-LTE$_4$ and CRP with endothelial function. Urine samples were obtained from 30 subjects (80% males; median age 65) with type 2 diabetes of at least two years duration and a median glomerular filtration rate (eGFR) of 71 (14–129) mL/min. Reactive hyperemia index (RHI) was used as a measure of microvascular endothelial function, whereas macrovascular endothelial function was determined be means of flow-mediated dilatation of the brachial artery (FMD). Decreased renal function was associated with lower concentrations of U-LTE$_4$. In addition, U-LTE$_4$ was correlated with serum creatinine ($R = -0.572$; $P = 0.001$) and eGFR ($R = 0.517$; $P = 0.0036$). A stepwise multiple linear regression analysis identified eGFR as an independent predictor of U-LTE$_4$ concentrations. In conclusion, the present results did not establish an association of U-LTE$_4$ with endothelial dysfunction. However, eGFR was an independent predictor of U-LTE$_4$, but not CRP, in this cohort, suggesting that GFR should be considered in biomarker studies of U-LTE$_4$.

1. Introduction

Urinary concentrations of the lipid-derived mediator leukotriene E_4 (U-LTE$_4$) may serve as a disease marker in several pathological conditions, such as asthma [1], obstructive sleep apnea [2], and acute coronary syndromes [3]. In addition, U-LTE$_4$ has been associated with diabetes [4], and U-LTE$_4$ also increases gradually with body mass index (BMI) [2]. In the latter contexts, the leukotriene pathway may not only serve as diagnostic markers but could potentially also offer mechanistic insights into the role of inflammation in metabolic disease. For example, targeting either leukotriene receptor signaling or leukotriene synthesis decreases proinflammatory cytokine secretion from visceral fat [5] and protects against insulin resistance in diet-induced obesity [6, 7].

Importantly, type 2 diabetes is associated with endothelial dysfunction, which plays an important role in the development of vascular complications in these patients [8]. The abnormalities in vascular reactivity associated with type 2 diabetes concern both the micro- and macrocirculation [9]. In the microcirculation, endothelial dysfunction is reflected by reduced extremity skin hyperemia, whereas macrovascular endothelial function can be assessed through brachial artery flow-mediated vasodilatation (FMD).

The leukotriene pathway is activated during the atherosclerosis process [10, 11] and affects vascular function both *in vitro* and *in vivo* [12]. Although no previous study has specifically assessed U-LTE$_4$ as a marker of endothelial function, there are several indications for a role of the leukotriene pathway in endothelium-dependent vascular reactivity. For example, leukotrienes induce endothelium-dependent responses in isolated human vessels [12], through the release of nitric oxide [13] and prostacyclin [14]. In addition, salivary leukotriene concentrations were recently associated with an increased pulse wave velocity [15], which may serve as an indirect measure of global endothelial function [16].

Based on the above observations, the aim of the present study was to assess U-LTE$_4$ in relation to micro- and macrovascular endothelial function in subjects with type 2 diabetes and to compare the results with the established inflammatory marker CRP. The results indicate that in this patient group, renal function is an important confounder for the association of U-LTE$_4$, which should be taken into account when using this biomarker.

2. Materials and Methods

2.1. Patients. The present cohort represents the baseline examination of a randomised, double-blind, and placebo-controlled study assessing the effect of the endothelin receptor antagonist bosentan treatment on endothelial function. Fourty-six patients with type 2 diabetes of at least two years duration and microalbuminuria were enrolled into the study, and analyzable urine samples were obtained from 30 subjects for measures of U-LTE$_4$. The main study has been described elsewhere [17]. In brief, patients with type 2 diabetes of at least two years duration and microalbuminuria were recruited from the department of Endocrinology, Metabolism and Diabetes at the Karolinska University Hospital (Stockholm, Sweden) from autumn 2007 to spring 2010. Patients were classified as having diabetes mellitus if fasting blood glucose exceeded 7.0 mmol/L (at least on two occasions) or blood glucose concentration was >11.0 mmol/L two hours after an oral glucose loading (75 g). Albuminuria was defined as urine albumin concentration >20 μg/L or >30 μg/12 h and a ratio of albumin/creatinine >3.0 mg/mmol. Patients were excluded if they had a myocardial infarction or unstable angina within the last three months, decompensated heart failure, changed dose of any vasodilator drug during the preceding six weeks, childbearing potential, impaired hepatic function (defined as twice the normal limit of serum aminotransferases), ongoing treatment with glibenclamide, cyclosporine, or warfarin, or concomitant disease that may have interfered with the possibility for the patients to comply with or complete the study protocol. The study protocol was conducted according to the Declaration of Helsinki and was approved by the local ethics review board. Informed consent was obtained from all patients. The baseline investigations before randomization consisted of assessment of microvascular and macrovascular endothelial function and blood and urine sampling.

2.2. Peripheral Endothelial Function Testing. The investigations were performed following a light breakfast in the morning after 20 min rest with the patient in the supine position. The patients were asked to refrain from caffeine-containing drinks or tobacco consumption and intake of drugs was withheld for the 12 preceeding hours and until after the examinations. Noninvasive determination of digital endothelial function was measured with a pulse amplitude tonometry (PAT) device placed on the tip of each index finger (Endo-PAT2000, Itamar Medical, Caesarea, Israel). The PAT device comprises a pneumatic plethysmograph that applies a uniform pressure to the surface of the distal finger, allowing measurement of pulse volume changes. The inflation pressure of the digital device was electronically set to 10 mm Hg below diastolic blood pressure or 70 mm Hg (whichever was lower). The PAT signal was recorded at baseline and following 5 min arterial occlusion using an inflatable cuff while the contralateral arm served as a control. The blood pressure cuff was inflated 60 mm Hg higher than the systolic pressure or at least 200 mm Hg for 5 minutes. Lack of residual pulsatility was monitored throughout the occlusion period. The postocclusive hyperemia stimulates endothelium-dependent vasodilatation causing an increase in digital pulse amplitude [18, 19]. Pulse amplitude was recorded electronically in both fingers and analyzed by a computerized, automated algorithm (Itamar Medical). The change from the baseline measurement is expressed as the reactive hyperemia index (RHI) which in part reflects vasodilator function of the digital microcirculation [20]. Previous evaluation of this method has demonstrated that RHI is to a large part dependent on NO bioavailability [18]. Endothelium-independent vasodilatation (EIDV) was determined following sublingual administration of nitroglycerine (0.4 mg). The ratio between the maximal pulse amplitude following nitroglycerine administration and baseline pulse amplitude was used for calculating EIDV. The coefficient of variation for Endo-PAT is 12% in our laboratory.

2.3. Flow Mediated Vasodilatation of the Brachial Artery. Macrovascular endothelial function was determined by analysis of flow-mediated dilatation (FMD) of the brachial artery during reactive hyperemia following forearm ischemia described in detail earlier [21] by means of an 8 MHz linear-array transducer connected to an Acuson Sequoia (Acuson Corporation, Mountain View, CA, USA) in parallel with the determination of digital PAT. Baseline images were saved every third second during one minute and a mean value was calculated from these values. The artery was continuously imaged for three minute during the hyperemia following release of the pressure cuff to determine endothelium-dependent vasodilatation. A mean value was calculated from three recordings at maximum dilatation. EIDV was determined following sublingual administration of nitroglycerine. All images were analyzed using proprietary software (Brachial analyzer, Medical Imaging Applications, Iowa City, IA, USA) by a an experienced technician. The coefficient of variation for FMD is 19% in our laboratory.

2.4. Laboratory Investigations. Fasting plasma glucose, Hb1Ac (Mono S), and blood lipids were assessed with standard methods according to local laboratory routines. High-sensitive C-reactive protein was analyzed using turbidimetry (Beckman Coulter, Fullerton CA, USA). Urine was collected in the morning and frozen in aliquots at −80°C until analysis. Urinary creatinine was determined using a standard colorimetric assay. U-LTE$_4$ was measured using enzyme immune assay kits from Cayman Chemicals (Ann Arbor), and results were expressed as pg U-LTE$_4$ per mg U-creatinine as previously described [2]. Glomerular filtration rate (eGFR) was estimated using the Cockroft-Gault formula.

2.5. Statistical Analysis. Data are expressed as median (range) and frequencies are expressed as percentages. Univariate

correlations between either $U\text{-}LTE_4$ or hsCRP and clinic parameters were established by Spearman correlation. A multiple stepwise linear regression was performed to evaluate independent predictors of $U\text{-}LTE_4$ and hsCRP, respectively. $P < 0.05$ was considered significant. Analyses were performed using SigmaPlot version 12 (Systat Software Inc.).

3. Results and Discussion

The clinical and biochemical characteristics of the 30 study subjects (80% males) of the study cohort are shown in Table 1. Stratifying the subjects according to renal function, measured as eGFR, revealed significant differences for flow mediated vasodilatation, consistent with a decreased macrovascular endothelial function in the group exhibiting severe renal function with eGFR <60 mL/min [22]. Of the inflammatory markers, hsCRP remained unchanged between the groups, whereas $U\text{-}LTE_4$ exhibited significantly decreasing concentrations with decreased renal function (Table 1).

The two inflammatory markers hsCRP and $U\text{-}LTE_4$ exhibited a positive correlation ($R = 0.326$), which however did not reach statistical significance ($P = 0.0778$). The univariate analyses for the respective disease markers with the clinical and biochemical characteristics of the study cohort are shown in Table 2. Lipid measurements did not correlate with either hsCRP or $U\text{-}LTE_4$ (Table 2). There were no significant correlations for hsCRP with any of the tested parameters in either the univariate (Table 2) or multivariate (data not shown) analysis. CRP is a well-established biomarker for systemic low grade inflammation and has been associated with arterial stiffness [23], intima media thickness [24], subclinical carotid atherosclerosis [25], and metabolic syndrome [26]. Patients with type 2 diabetes have increased hsCRP which decreases with treatment intensity and correlate positively with a drop in HbA1c [27].

Inflammation may be a key feature of endothelial dysfunction. Previous studies have reported a significant inverse association between microvascular endothelial function during reactive hyperemia and hsCRP in men with stable coronary disease and macrovascular endothelial function measured by FMD in another study [28, 29]. Nevertheless, the lack of correlations with macrovascular endothelial function in the present study is in line with a previous larger cohort of healthy individuals in which brachial artery FMD had no relationship with hsCRP [30]. Furthermore, the present study did not reveal any associations of $U\text{-}LTE_4$ with either macro- or microvascular endothelial function (Table 2).

Several potential confounders should be taken into account when assessing the constituents of the leukotriene pathway as disease markers. For example, an association between $U\text{-}LTE_4$ and obesity was initially demonstrated in subjects with obstructive sleep apnea [2] and was subsequently reported in asthmatics [31]. Likewise, salivary concentrations of leukotriene B_4 ($S\text{-}LTB_4$) associated with BMI and waist to hip ratio in a case control study of hypertension [15]. In contrast to those studies, the present study did not reveal any significant associations between $U\text{-}LTE_4$ and either BMI or waist circumference (Table 2). Likewise, the present cohort lacked an association between $U\text{-}LTE_4$ and

HbA1c (Table 2), which support the findings of a previous study in which $U\text{-}LTE_4$ remained unchanged before and after intense glycemic control in type 2 diabetes [32]. It should be noted that the $U\text{-}LTE_4$ concentrations in the present study were higher compared with what was previously measured in healthy controls and in younger diabetics [4, 33], but similar to those reported patient cohorts of asthma [33], renal failure [34], or acute coronary syndrome [3]. In addition to dissimilar age and pathologies, also alterations of creatinine clearance and the fact that $U\text{-}LTE_4$ was measured using different techniques (ELISA versus liquid chromatographic tandem mass spectrometry) could contribute to higher creatinine-normalized $U\text{-}LTE_4$ concentrations in the present study.

Concentrations of $U\text{-}LTE_4$ were significantly correlated with measures of renal function in terms of plasma creatinine and eGFR (Table 2). The multivariate analysis including all parameters listed in Table 2 confirmed these findings, showing that eGFR was an independent predictor of $U\text{-}LTE_4$ concentrations in these patients (β-coefficient 0.325; $P = 0.011$).

In a rodent model of glomerulonephritis, urinary leukotriene concentrations were elevated at initial stages of disease, followed by a decline at more advanced phases [35]. In addition, renal function is ameliorated by anti-leukotriene treatment in several animal models of renal failure, including proteinuria in diabetic rats [36], renal ischemia reperfusion [37], and drug-induced nephrotoxicity [38]. Previous studies of $U\text{-}LTE_4$ in relation to renal function in humans have, however, generated conflicting results. In a study of patients with hepatorenal syndrome, $U\text{-}LTE_4$ excretion rate fell in parallel with creatinine clearance, indicative of a GFR-dependent renal excretion of this inflammatory marker [39]. In contrast, in another study of kidney transplant recipients, $U\text{-}LTE_4$ was not associated with either serum creatinine or transplant rejection [34]. In the present study of patients with type 2 diabetes of at least two years duration and microalbuminuria, renal function was an independent predictor of $U\text{-}LTE_4$ even when taking into account anthropometric and biochemical variables as well as measures of micro- and macrovascular endothelial function.

In summary, the present study did not reveal any significant associations of $U\text{-}LTE_4$ with measures of endothelial function and identified renal function as an independent predictor of $U\text{-}LTE_4$. Several limitations must, however, be acknowledged. This study represents a limited sample size. In addition, diabetes patients were selected based on existing microalbuminuria. The generalizability of the present findings to larger populations with or without diabetes is hence unknown. Furthermore, the inflammatory parameters measured were limited to hsCRP and $U\text{-}LTE_4$. Notably, no salivary samples were available in this cohort for measures of $S\text{-}LTB_4$, which could provide further information for a potential association of the leukotriene pathway with endothelial dysfunction [15]. Finally, the lack of association of $U\text{-}LTE_4$ with either endothelial function or anthropometric should be interpreted with care given the major confounder of renal function in the present cohort.

TABLE 1: Baseline characteristics of total study group and tertiles based on glomerular filtration rate.

	Total study group $n = 30$	eGFR >90 $n = 8$	eGFR 90–60 $n = 10$	eGFR <60 $n = 12$	P value
Male, %	80	75	80	83	
Age, years	65 (48, 78)	60 (50, 78)	60 (48, 67)	66 (54, 75)	0.29
BMI, kg/m^2	30.7 (25, 40.9)	29.7 (25.0, 36.3)	32.1 (28.1, 35.3)	31.0 (26.2, 40.9)	0.67
Waist circumference, cm	113 (95, 133)	116 (101, 122)	115 (100, 121)	111 (95, 133)	0.55
Systolic blood pressure, mmHg	140 (80, 185)	140 (130, 180)	138 (80, 185)	148 (115, 180)	0.71
Diastolic blood pressure, mmHg	80 (50, 90)	80 (70, 90)	80 (50, 90)	75 (60, 90)	0.46
Creatinine, μmol/L	87.5 (49, 483)	75 (49, 87)	85 (78, 104)	169 (81, 483)	**<0.0001**
eGFR, mL/min	71 (14, 129)	110 (91, 129)	75 (61, 88)	41 (14, 55)	**<0.0001**
Cholesterol, mmol/L	3.6 (2.2, 6.9)	3.7 (3.1, 6.9)	3.4 (2.2, 4.4)	3.7 (2.6, 5.0)	0.31
LDL cholesterol, mmol/L	2.1 (1.0, 5.0)	2.3 (1.8, 5.0)	2.0 (1.1, 3.1)	2.4 (1.0, 3.2)	0.34
HDL cholesterol, mmol/L	0.8 (0.6, 1.4)	0.9 (0.7, 1.4)	0.8 (0.6, 1.4)	0.8 (0.6, 1.2)	0.39
Triglycerides, mmol/L	1.3 (0.8, 2.4)	1.3 (0.8, 2.3)	1.4 (0.8, 2.2)	1.4 (2.4, 0.8)	0.87
HbA1c, mmol/mol	45.4 (27.9, 83.6)	53.6 (28.9, 62.8)	44.3 (27.9, 68.3)	46.5 (29.0, 83.6)	0.56
Peripheral arterial tonometry, RHI	1.60 (1.28, 3.19)	1.50 (1.39, 3.19)	1.58 (1.28, 2.14)	1.66 (1.35, 2.38)	0.84
Flow mediated vasodilatation, %	2.0 (0, 10.9)	1.9 (0.7, 10.9)	4.1 (1.2, 8.7)	1.5 (0, 4.8)	**0.036**
hsCRP, mg/L	1.9 (0.3, 15)	2.3 (0.4, 14)	2.0 (0.3, 6.6)	1.4 (0.3, 15.0)	0.88
U-LTE4 (pg/mg creatinine)	197 (13, 483)	332 (156, 483)	184 (29, 470)	177 (13, 390)	**0.049**

Data shown as median (min, max).

TABLE 2: Univariate spearman correlations between U-LTE$_4$ and CRP with clinical/biological parameters ($n = 30$).

	U-LTE$_4$		CRP	
	R	P	R	P
Sex	−0.347	0.0605	−0.197	0.293
Age	−0.140	0.456	−0.0944	0.616
BMI	−0.187	0.375	0.136	0.520
Waist circumference	0.0518	0.788	−0.170	0.375
Systolic blood pressure	0.0362	0.847	−0.0825	0.662
Diastolic blood pressure	−0.0026	0.989	−0.0087	0.963
Creatinine	−0.572	**0.00103**	−0.0076	0.966
eGFR	0.517	**0.00359**	0.00334	0.985
Cholesterol	0.0301	0.873	−0.169	0.371
LDL	−0.0203	0.914	−0.229	0.220
HDL	0.221	0.238	0.122	0.517
Triglycerides	−0.141	0.455	−0.253	0.175
HBA1c	−0.0167	0.930	−0.310	0.107
Peripheral arterial tonometry	0.0937	0.620	−0.170	0.367
Flow mediated vasodilatation	0.0679	0.729	−0.0199	0.919

Since leukotrienes are undetectable in plasma [40, 41], these findings suggest that other means of leukotriene measures should be considered in such patients groups, such as salivary LTB [15] or assessment of leukotriene synthesis from *ex vivo* stimulated leukocytes [42].

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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4. Conclusion

The present results did not establish an association of either of the two studied inflammatory disease markers, CRP and U-LTE$_4$, with endothelial dysfunction in patients with type 2 diabetes. However, eGFR was an independent predictor of U-LTE$_4$, but not CRP, in this patient cohort. In conclusion, these results indicate that renal function should be taken into consideration when studying U-LTE$_4$ as biomarker.

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