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Association of chronic inflammation and accelerated atherosclerosis among an indigenous black population with chronic kidney disease

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Abstract

Introduction

Inflammation plays a major role in the development of atherosclerosis and cardiovascular morbidity and mortality in chronic kidney disease (CKD) patients. Toll-like receptor-4 (TLR4) is a major receptor for lipopolysaccharides (endotoxin) and other ligands involved in the pathogenesis of inflammation. We determined whether endotoxin levels and the presence of TLR4 polymorphisms are associated with markers of inflammation and atherosclerosis among South African CKD patients.

Materials and methods

Endotoxin, lipopolysaccharide binding protein (LBP), serum CD14 (sCD14), interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1) and carotid intima media thickness (CIMT) were measured in 160 participants (120 CKD patients and 40 controls). Associations between endotoxins and CIMT in the presence of sCD14, IL-8 and MCP-1, were assessed using odds ratios. Participants were screened for the presence of Asp299Gly and Thr399Ile TLR4 polymorphisms, and CIMT and inflammatory markers were compared between subjects with and without TLR4 polymorphisms.

Results

Endotoxin levels correlated with sCD14 ($r = 0.441$, $p<0.001$) and MCP-1 ($r = 0.388$, $p<0.001$) levels while increased CIMT was associated with MCP-1 ($r = 0.448$, $p<0.001$), sCD14 levels ($r = 0.476$, $p<0.001$), LBP ($r = 0.340$, $p<0.001$), and IL-8 ($r = 0.395$, $p<0.001$). Atherosclerosis was associated with endotoxin levels (odds ratio: 4.95; 95% confidence interval: 2.52–9.73; $p<0.001$), and was predicted by higher serum levels of inflammatory

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markers. Analysis of patients with TLR4 polymorphisms showed reduced serum levels of inflammatory markers and CIMT values compared with the patients carrying the wild type TLR4 alleles.

Conclusion

The study demonstrated associations between circulating endotoxaemia, systemic inflammation and accelerated atherosclerosis among South African CKD patients, and showed that the atherogenic predictive power of endotoxaemia was significantly increased by the presence of elevated levels of inflammatory markers. Additional findings, which must be confirmed, suggest that TLR4 polymorphisms are associated with low levels of inflammatory markers and CIMT values.

Introduction

Atherosclerosis is increasingly being recognized as a chronic inflammatory condition and inflammatory mediators are thought to play a role in the pathogenesis of atherosclerosis [1–6]. Previous studies have shown that elevated endotoxin levels are associated with a risk of atherosclerosis [1, 7, 8]. Available evidence suggests that endotoxin may promote accelerated atherosclerosis through its ability to induce factors that play an important role in the endotoxin signalling pathway leading to a persistent chronic inflammatory state [9–11]. Circulating endotoxin binds to lipopolysaccharide binding protein (LBP), which facilitates binding of lipopolysaccharide (LPS) to soluble CD14 (sCD14) via toll-like receptor adaptor molecules, resulting in the downstream activation of nuclear factor- κB (NF-κB) and production of pro-inflammatory mediators [12–14].

Circulating endotoxaemia portends harmful outcomes both on cardiovascular function and structure, thus driving systemic inflammation, oxidative stress and atherogenesis [15]. Subclinical endotoxaemia significantly contributes to systemic inflammation, and thus, is a strong risk factor for atherosclerosis and cardiovascular disease [1]. Although, previous studies have evaluated the association of circulating endotoxaemia with accelerated atherosclerosis, none of these studies were carried out among indigenous black CKD patients with attendant high cardiovascular risk [2, 7].

Toll-like receptor-4 (TLR4) is a major receptor for LPS (endotoxin) and other ligands potentially involved in the pathogenesis of inflammation and vascular remodeling [16–20]. TLR4 is expressed by endothelial cells and monocytes, and its levels are markedly increased in atherosclerotic lesions, particularly in macrophages and endothelial cells [21, 22]. The responsiveness of an individual to LPS via the TLR4 signaling cascades provides an efficient innate immunity, which offers initial benefit but portends chronic vascular damage and increased risk of future atherosclerosis [16, 18].

Two common co-segregating missense mutations, A896G and C1196T resulting in the replacement of aspartic acid by glycine at amino acid position 299 (Asp299Gly) and the substitution of threonine by isoleucine at amino acid position 399 (Thr399Ile), respectively, have been identified in the human TLR4 gene [23]. These polymorphisms have been linked with endotoxin hypo-responsiveness and reduced risk of atherogenesis [13].

Although the TLR4 Asp299Gly polymorphism has been associated with reduced CIMT and lower serum levels of inflammatory cytokines in healthy populations, and reduced risk of

myocardial infarction in Caucasians [16, 24, 25], to date, these studies have provided diverse results on the role of the TLR4 polymorphisms in the development of atherosclerosis [16, 24–30]. In addition, associations between TLR4 polymorphisms and susceptibility for atherosclerosis have not been previously investigated in CKD patients of African ancestry. In this study we determined the relationship between endotoxin-related inflammation and severity of atherosclerosis in South African CKD patients. Additionally, genotyping of TLR4 variants (Asp299Gly and Thr399Ile) was employed to investigate whether genetic variants of TLR4 were associated with reduced inflammatory response and susceptibility for atherosclerosis among indigenous black CKD cohorts.

Materials and methods

The study included 120 CKD patients managed at the Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). Forty age- and gender-matched black Africans who were staff and students at CMJAH were recruited as controls. Exclusion criteria included clinical signs of active or chronic infection, diabetes mellitus, seropositive status for hepatitis B, C and human immunodeficiency virus (HIV), autoimmune disease, liver dysfunction, malignancy, heart failure (New York Heart Association; NYHA III-IV) and use of anti-inflammatory or immunosuppressive therapy at least three months prior to enrolment. Information regarding age, race and tobacco smoking were documented. Blood pressure was recorded at the time of clinic visits in the arm with an Accusson mercury sphygmomanometer whilst the patient was in the sitting position. The average of three readings, 5 minutes apart, was taken as the blood pressure measurement. Mean arterial blood pressure (MABP) was calculated as diastolic blood pressure plus one third pulse pressure. Waist and hip circumferences were assessed using a tape measure. Waist-hip ratio (WHR) was calculated as waist measurement divided by hip measurement. This study was approved by the University of the Witwatersrand Human Research Ethics Committee (Protocol M130127).

Blood sample collection and preparation

Blood for measurement of endotoxin and other inflammatory markers was collected into anticoagulant-free tubes after an overnight fast. Blood samples were allowed to clot at room temperature. Serum samples were separated within 30 minutes by centrifugation at 3000 rpm for 10 minutes, transferred into fresh polypropylene tubes and immediately frozen at −80°C until ready for assay. Serum calcium, phosphate, creatinine and lipid profile were assayed using AdviaR 1800 auto-analyzers (Siemens Healthcare Diagnostics Inc, USA).

Endotoxin levels

Using previously described methods [31], serum endotoxin was quantified using the Limulus amebocyte lysate QCL-1000^{TM} assay (Lonza Walkersville, USA) according to manufacturer's instructions.

Lipopolysaccharide binding protein concentrations

Serum levels of LBP were measured using a commercial human LBP ELISA kit, Hycult HK315 (Hycult biotechnology, Uden, the Nertherlands) according to manufacturer's protocol. Absorbance was measured using an ELx800 Universal plate reader (BioTek Instruments, Inc, VT, USA).

Serum sCD14, IL-8 and MCP-1 concentrations

Serum sCD14, IL-8 and MCP-1 assays were analyzed using Luminex® Performance Assay multiplex kits (R&D Systems, Inc. Minneapolis, USA). Assays were in accordance with the manufacturer's instructions. Data were acquired on the Bio-Plex™ 200 system (Bio-Rad, Texas, USA). Fluorescence intensity for MCP-1, IL-8 and sCD14 were read in the bead region 53, 54 and 59 respectively, and concentrations generated automatically using Bio-Plex manager software, version 5.0 (Bio-Rad Laboratories Inc, Hercules, USA).

DNA extraction and TLR4 genotyping

Genomic DNA was extracted from whole blood using a modified salting out method [32] and the concentrations determined using a NanoDrop™ spectrophotometer (Thermo Scientific, Massachusetts, USA). The TLR4 Asp299Gly and Thr399Ile polymorphisms were determined by utilizing polymerase chain reaction (PCR) which was performed for amplification using optimised primers sets (TLR4-299; Forward: `5′-GAT TAG CAT ACT TAG ACT ACT ACC TCC ATG-3′`, Reverse: `5′-GAT CAA CTT CTG AAA AAG CAT TCC CAC-3′` and TLR4-399; Forward: `5′-GGT TGC TGT TCT CAA AGT GAT TTT GGG AGA A-3′`, Reverse: `5′-ACC TGA AGA CTG GAG AGT GAG TTA AAT GCT-3′`). The PCR was performed using KAPA2G Robust HotStart Ready Mix PCR Kit (Kapa Biosystems, Massachusetts, USA) as per the manufacturer's protocol. The PCR was run on a thermocycler (MJ Mini Thermal cycler, Bio-Rad, USA) according to manufacturer's instructions. The PCR conditions were 3 mins of denaturation at 94˚C, followed by 40 cycles (95˚C for 15 secs, 60˚C for 15 secs, and 72˚C for 30 secs), and finally 72˚C for 60 secs. The PCR products for Asp299Gly and Thr399Ile alleles were digested with restriction enzymes *NcoI* (`5′-C/CATGG-3′`) and *HinfI* (`5′-G/ANTC-3′`) respectively [33]. All the PCR products were visualized on a 2% agarose gel stained with ethidium bromide, with the aid of an image analyser (Gel Doc™ EZ Imager, Bio-Rad, USA). All genotypes were assigned by independent investigators who were blinded to the results of CIMT measurements and markers of immune activation of the participants.

Carotid intima media thickness

Carotid intima media thickness was assessed using high resolution B-mode ultrasonography with the aid of a L3-11 MHz linear array transducer (Philips Corporation USA) as previously described [34]. The CIMT was measured in plaque-free areas and all measurements were performed by the same sonographer who was blinded to the clinical details, laboratory data and results from the genetic analyses of the participants.

Data analysis

Data analyses were performed using the statistical package for social sciences (SPSS) 16 (SPSS, Inc., Chicago IL). Data were presented as means ± SD or medians and interquartile ranges (IQR), where appropriate. Categorical data were compared using chi-square test and continuous data using student *t*-test or Mann-Whitney test. Correlation between continuous variables was examined by the Spearman's rank correlation coefficient. Further analysis was performed after categorising endotoxin levels into two groups (cut-off value of 0.5 EU/ml). The categorisation of endotoxin levels ($\leq$ 0.5 EU/ml and $>$ 0.5 EU/ml) was adopted from a previous study [2]. In addition, CIMT measurements, sCD14, IL-8 and MCP-1 concentrations were subdivided into two groups according to the median values. Subclinical atherosclerosis was defined as CIMT $>$ 0.55 mm. Association between endotoxin and atherosclerosis was assessed using logistic regression analysis.

To determine whether the baseline IL-8 or MCP-1 or sCD14 levels were an effect modifier of the relationship of circulating endotoxin to subclinical atherosclerosis, we performed a stratified multivariable logistic regression analysis, aimed at estimating the atherogenic predictive power of endotoxin in the presence of immune mediators, including IL-8, MCP-1 and sCD14. The models were adjusted for age, blood pressure, smoking, eGFR and cholesterol. Genotype frequencies were compared using Fisher's exact test. A Mann-Whitney test was used to compare levels of markers of inflammation between patients with wild-type TLR4 and those with Asp299Gly allele alone, as well as those with combined TLR4 mutant alleles. A p-value <0.05 was regarded as statistically significant.

Results

The patients' characteristics and laboratory data are summarized in Table 1. At baseline, 22.5% and 23.3% of the CKD patients received angiotensin-II receptor blockers/angiotensin-converting enzyme inhibitors and statins, respectively. Endotoxin concentrations in CKD patients were significantly higher than that of the controls ($p<0.001$). Carotid intima media thickness was significantly higher among CKD patients compared to the controls ($p<0.001$). There were no significant differences in the CIMT values between patients who were treated with ACEI/ARB/statins and those who did not received the treatment [ACEI/ARB: 0.56 (0.49–0.63) vs 0.56 (0.50–0.65), p = 0.566; statins: 0.58 (0.52–0.68) vs 0.55 (0.49–0.63), p = 0.154]. According to the American Society of Echocardiography, 25% of CKD patients had high CVD risk (CIMT values $\geq 75^{th}$ percentile), 46% of patients had average risk for CVD (CIMT values in the 25th to 75th percentile), while 29% of patients presented with CIMT values lower than 25^{th} percentile, considered lower CVD risk.

Table 1. Demographic, clinical and laboratory data of the study population.

Parameter	Patients (N = 120)	Control (N = 40)	P value
Age (years; mean ± SD)	41.1 ± 10.2	42.2 ± 10.1	0.573
Sex (Male/Female)	64/56	22/18	0.709
Smoking (Yes/No)	17/103	2/38	0.121
MABP (mmHg)	134.8 (118.8–150.0)	117.0 (107.8–130.6)	<0.001
Waist-Hip ratio	0.91 (0.87–0.96)	0.88 (0.83–0.93)	0.122
eGFR (ml/min/1.73m^2)	9.0 (4.0–44.0)	96.5 (83.0–119.0)	<0.001
Total cholesterol (mmol/L)	4.20 (3.43–5.18)	4.00 (3.33–4.88)	0.519
HDL (mmol/L)	1.20 (0.90–1.40)	1.25 (1.03–1.40)	0.421
LDL (mmol/L)	2.30 (1.80–3.00)	2.20 (1.60–2.98)	0.456
TG (mmol/L)	1.20 (0.80–1.70)	0.95 (0.63–1.60)	0.172
Endotoxin (EU/ml)	0.54 (0.37–0.73)	0.33 (0.26–0.41)	<0.001
LBP (μg/ml)	131 (104–165)	91 (76–110)	<0.001
sCD14 (μg/ml)	1.74 (1.35–2.25)	1.15 (1.04–1.41)	<0.001
LBP/sCD14 ratio	78.9 (65.4–91.6)	70.9 (60.0–95.4)	0.987
IL-8 (pg/ml)	8.92 (5.06–21.12)	4.69 (3.14–7.21)	<0.001
MCP-1 (pg/ml)	9.00 (4.88–16.75)	3.63 (2.22–7.09)	<0.001
CIMT (mm)	0.56 (0.50–0.64)	0.46 (0.42–0.51)	<0.001

MABP, mean arterial blood pressure; eGFR, estimated glomerular filtration rate (CKD-EPI); HDL, high density lipoprotein; LDL, low density lipoprotein; TG, triglyceride; LBP, lipopolysaccharide binding protein; sCD14, serum CD14I; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1. Result analyzed using Mann-Whitney test, with Chi-Square test for nonparametric data. Continuous data were expressed as mean ± SD or median (IQR) and categorical data as percentages.

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Endotoxin levels, inflammatory markers and CIMT

Endotoxin levels showed a positive correlation with sCD14 ($r = 0.441$, $p<0.001$) and MCP-1 ($r = 0.388$, $p<0.001$). Serum LBP showed a positive correlation with sCD14 ($r = 0.605$, $p<0.001$) and MCP-1 ($r = 0.429$, $p<0.001$). Carotid intima media thickness was also associated with MCP-1 ($r = 0.448$, $p<0.001$), sCD14 levels ($r = 0.476$, $p<0.001$), LBP ($r = 0.340$, $p = 0.001$), and IL-8 ($r = 0.395$, $p<0.001$). Eight patients had carotid plaques; and endotoxin levels were significantly higher among patients with carotid plaques compared to those without plaques (median 0.75 EU/ml; IQR 0.49–1.06 EU/ml versus median 0.53 EU/ml; IQR 0.33–0.71 EU/ml, $p = 0.002$).

Atherosclerotic risk and inflammatory markers

Overall, circulating endotoxin levels were associated with increased risk of atherosclerosis [odds ratio: 4.95; 95% confidence interval: 2.52–9.73; $p<0.001$], with a more than four-fold increase in the risk of subclinical atherosclerosis among those with high endotoxin levels (> 0.5 EU/ml) compared with the reference group of patients with low endotoxin levels ($\leq$ 0.5 EU/ml). Table 2 displays the results of the logistic regression analysis examining the association of subclinical atherosclerosis with combinations of endotoxin and IL-8 or MCP-1 or sCD14 stratum. In the fully adjusted model, patients with coexisting high endotoxin levels and high IL-8 levels or MCP-1 or sCD14 presented with significantly elevated risks of atherosclerosis. However, the risk of atherosclerosis in patients with high endotoxin levels was not influenced by the presence of high IL-8 or MCP-1 or sCD14 levels. Likewise, the risk of atherosclerosis in patients with low endotoxin levels was not influenced by exposure to high levels of IL-8 or MCP-1 or sCD14.

TLR4 genotype distribution

The TLR4 Asp299Gly and Thr399Ile alleles were successfully genotyped in all, except for 4 of the samples in CKD patient group. Co-segregation of the Asp299Gly and Thr399Ile alleles was observed in 5 patients and 1 control, while 4 subjects (2 patients and 2 controls) had an isolated Asp299Gly polymorphism. The genotype distribution of wild-type TLR4 (AA genotype) did not show a significant difference among the patients and control subjects (93.9% vs 92.5%; $P = 0.497$). The occurrence of the G alleles was low (Table 3).

The heterozygous Asp299Gly (AG) allele was present in 7 patients and 3 controls, with minor allele frequency (MAF) of 3% and 3.8% respectively. The frequency of the variant genotype (CT) for TLR4 Thr399Ile allele was 4.3% in the patients and 2.5% in controls (MAF 2.2% and 1.3% respectively), whereas the CC genotype was prevalent in both the patient (96%) and control (98%) groups. The TLR4 Thr399Ile allele was present in only 6 of the genotyped subjects. The genotype of TLR4 polymorphisms in the studied population was in Hardy-Weinberg equilibrium (Asp299Gly: $\chi2 = 0.113$; $P = 0.736$ for patients and $\chi2 = 0.061$; $p = 0.805$ for controls; Thr399Ile: $\chi2 = 0.056$; $P = 0.812$ for patients and $\chi2 = 0.006$; $P = 0.936$ for controls). The GG and TT genotypes for Asp299Gly and Thr399Ile alleles, respectively, were not detected in the studied population.

TLR4 polymorphisms, inflammatory markers and CIMT

Compared with the carriers of the wild-type TLR4, CKD patients with only the Asp299Gly allele and those with the combined Asp299Gly/Thr399Ile alleles had significantly lower serum levels of inflammatory markers and reduced CIMT values (Table 4). However, no association was observed between TLR4 polymorphisms and traditional risk factors for atherosclerosis.

Table 2. Association of interleukin-8, monocyte chemoattractant protein-1 and serum CD14 levels with risk of early atherogenesis.

Categories	Serum concentration of immune mediators (Median; IQR)	Patients with subclinical atherosclerosis	Odds ratio (95% CI)	P value	Adjusted Odds ratio* (95% CI)	P value
Interleukin-8						
Endotoxin ≤ 0.5 EU/ml						
Interleukin-8 ≤ 7.99 pg/ml	4.8 (3.8–6.5)	14/36	1.0 (Reference group)		1.0 (Reference group)	
Interleukin-8 > 7.99 pg/ml	17.5 (10.5–38.8)	11/19	3.7 (1.0–13.7)	0.047	2.3 (0.5–11.1)	0.307
Endotoxin > 0.5 EU/ml						
Interleukin-8 ≤ 7.99 pg/ml	4.6 (3.6–6.5)	9/20	2.3 (0.7–7.4)	0.152	1.9 (0.5–7.4)	0.341
Interleukin-8 > 7.99 pg/ml	20.9 (13.4–122.8)	36/45	11.5 (4.3–31.2)	<0.001	8.5 (2.7–26.3)	<0.001
MCP-1						
Endotoxin ≤ 0.5 EU/ml						
MCP-1 ≤ 8.46 pg/ml	4.4 (2.1–6.6)	13/36	1.0 (Reference group)		1.0 (Reference group)	
MCP-1 > 8.46 pg/ml	15.1 (11.4–18.9)	12/19	6.0 (1.7–21.2)	0.007	3.4 (0.8–15.6)	0.113
Endotoxin > 0.5 EU/ml						
MCP-1 ≤ 8.46 pg/ml	5.8 (4.1–6.6)	9/21	3.0 (0.9–9.6)	0.064	2.3 (0.6–8.5)	0.213
MCP-1 > 8.46 pg/ml	15.6 (10.8–20.1)	36/44	15.6 (5.4–45.2)	<0.001	11.8 (3.5–40.5)	<0.001
Serum CD14						
Endotoxin ≤ 0.5 EU/ml						
Serum CD14 ≤ 1.7 μg/ml	1.2 (1.0–1.4)	16/39	1.0 (Reference group)		1.0 (Reference group)	
Serum CD14 > 1.7 μg/ml	2.1 (1.9–2.4)	9/16	2.2 (0.6–8.7)	0.250	1.3 (0.3–6.1)	0.730
Endotoxin > 0.5 EU/ml						
Serum CD14 ≤ 1.7 μg/ml	1.5 (1.1–1.6)	7/18	1.6 (0.5–4.9)	0.449	1.4 (0.4–4.9)	0.644
Serum CD14 > 1.7 μg/ml	2.2 (2.0–2.6)	38/47	10.9 (4.1–29.4)	<0.001	7.7 (2.6–23.4)	<0.001

Odds ratios, 95% confidence interval and p-values were derived from logistic regression analyses of subclinical atherosclerosis on endotoxin as well as interleukin-8, monocyte chemoattractant protein-1 and sCD14.

*Adjusted for Age, blood pressure, smoking, eGFR and cholesterol.

CIMT = carotid intima media thickness; CI = confidence interval, MCP-1 = monocyte chemoattractant protein-1

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Discussion

We demonstrated that serum levels of inflammatory markers were markedly elevated in CKD patients compared with the controls, and positively correlated with CIMT, a surrogate marker of atherosclerosis. Furthermore, in support of previous studies that showed that LBP (marker of circulating endotoxaemia) was an independent predictor of atherosclerosis, this present study also established an association between elevated LBP levels and CIMT [35, 36]. Taken together, these findings indicate that low-grade inflammation is related to atherogenesis, and as previously reported may trigger a cascade of accelerated atherosclerosis in CKD patients [2].

In addition, we demonstrated that risk of atherosclerosis was associated with circulating endotoxaemia, and further analysis revealed that the risks were predicted by elevated levels of inflammatory markers suggesting that the severity of systemic inflammation plays a critical role in predicting the atherogenic potential of circulating endotoxaemia in CKD patients. This

Table 3. Genotype and allele frequencies of TLR4 polymorphisms in patients and controls.

Genotype	Patients, N = 116[a]	Controls, N = 40	OR (95% CI)
Asp299Gly			
AA (wild-type)	109 (94%)	37 (92.5%)	
AG	7 (6.0%)	3 (7.5%)	
GG	0 (0%)	0 (0%)	
Frequency of the Gly allele, (%)	3.0	3.8	1.06 (0.70–1.62) p = 0.497
Thr399Ile			
CC (wild-type)	111 (95.7%)	39 (97.5%)	
CT	5 (4.3%)	1 (2.5%)	
TT	0 (0%)	0 (0%)	
Frequency of the Ile allele, (%)	2.2	1.3	0.89 (0.61–1.28) p = 0.513

OR = odds ratio; CI = confidence interval.

[a] Four specimens were not genotyped in patient group

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observation corroborated previous reports that identified increased levels of neopterin and soluble interleukin-2 receptors as independent predictors of vascular risk in a prospective population based study [37].

Even though our study is underpowered, we observed that the heterozygous Asp299Gly allele was associated with lower levels of inflammatory markers and reduced CIMT values, thus corroborating the findings of previous studies by Kiechl et al [16] and Ameziane et al [25]. On further analysis, our results suggest that the TLR4 polymorphisms seem to exact a major effect among patients with combined TLR4 Asp299Gly and Thr399Ile polymorphisms. Based on this observation, we hypothesize that TLR4 Asp299Gly and Thr399Ile polymorphisms may have a synergistic effect in our CKD patients.

Table 4. Relationship between TLR4 polymorphisms, traditional risk factors, CIMT and inflammatory markers in CKD patients.

Variables	Wild-Type TLR4 (N = 109)	TLR4 Asp299Gly + Variant (N = 7)	TLR4 Asp299Gly+, Thr399Ile + Variants (N = 5)	P-value	
				Wild-Type vs. Asp299Gly variant	Wild-Type vs. Combined TLR4 Variants Group
Age (Years); Mean ± SD	41.6 ± 10.0	40.1 ± 15.6	35.6 ± 16.4	0.894	0.253
Smoking (Yes/No)	15/94	2/5	2/3	0.285	0.109
Cholesterol (mmol/L)	4.20 (3.40–5.20)	4.40 (3.35–4.80)	4.40 (3.20–4.50)	0.958	0.972
HDL (mmol/L)	1.20 (0.90–1.40)	1.10 (0.95–1.30)	1.10 (1.00–1.30)	0.641	0.829
LDL (mmol/L)	2.30 (1.80–3.00)	2.70 (1.80–3.15)	2.70 (1.80–3.00)	0.655	0.668
TG (mmol/L)	1.20 (0.80–1.70)	1.10 (0.85–1.30)	1.00 (0.70–1.20)	0.475	0.225
MABP (mmHg)	143.0 (130.3–157.3)	137.0 (110.7–143.7)	137.0 (111.0–143.3)	0.181	0.350
Waist-Hip ratio	0.92 (0.87–0.96)	0.89 (0.84–0.92)	0.86 (0.81–0.89)	0.286	0.072
CIMT (mm)	0.57 (0.50–0.65)	0.49 (0.45–0.53)	0.49 (0.47–0.53)	0.009	0.028
IL-8 (pg/ml)	9.36 (5.58–22.33)	4.83 (2.90–6.26)	3.09 (2.70–4.83)	0.007	0.004
MCP-1 (pg/ml)	9.19 (5.12–17.03)	6.69 (2.87–7.45)	3.61 (2.12–7.67)	0.046	0.039
sCD14 (μg/ml)	1.76 (1.39–2.27)	1.30 (1.07–1.65)	1.30 (0.96–1.45)	0.047	0.054
LBP (μg/ml)	131.3 (108.4–165.9)	112.6 (76.1–118.7)	81.7 (70.5–113.6)	0.061	0.087

TLR4 = Toll-like receptor 4; HDL = High density lipoprotein; LDL = Low density lipoprotein; IL-8 = Interleukin-8; TG = Triglycerides; MABP = Mean arterial blood pressure; CIMT = Carotid intima media thickness; MCP-1 = Monocyte chemoattractant protein-1; sCD14 = Serum CD14; LBP = Lipopolysaccharide binding protein

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Although the mechanisms by which TLR4 Asp299Gly and Thr399Ile polymorphisms mediate their synergistic effects are yet to be fully elucidated, newer evidence suggests that both the Asp299Gly and Thr399Ile mutations are within the fourth exon of the TLR4 gene, regulating a ligand-binding region and a coreceptor-binding region, respectively [38]. These two amino acid residues have been proposed to lie on the same side of the TLR4 molecule [39]. It has also been suggested that the Asp299Gly/Thr399Ile double mutant modifies an immunodominant epitope that, in turn, results in reduced expression of mutant TLR4 molecules [40]. Thus, it is possible that reduced expression of mutant TLR4 molecules, which might lead to attenuated TLR4 receptor signaling, could bring about the combined effect of TLR4 Asp299Gly and Thr399Ile polymorphisms observed in this study. Moreover, in TLR4 models, it has also been demonstrated that double mutant transfected cells showed a consistently greater hypo-responsiveness to low dose endotoxin than cells with either of the single polymorphic TLR4 variants [40]. It is also possible that individuals carrying both mutated genotypes of TLR4 alleles, which might be in linkage disequilibrium with mutations in the regulatory region of the TLR4 gene, could develop subnormal TLR4-mediated responses. Nevertheless, to elucidate the precise underlying mechanism would require further study on the stoichiometry, structure, and signaling of the TLR4/MD-2/CD14 complexes, which is beyond the scope of the present study.

The genotype frequencies of the Asp299Gly and Thr399Ile alleles observed in our study were similar to those reported by previous studies carried out in South African populations [41–43]. This study confirms and extends the findings of these previous studies, indicating that homozygote Asp299Gly and Thr399Ile polymorphisms are not common in the South African population.

Remarkably, we observed that only 6 of our cohorts had the heterozygous Thr399Ile polymorphism. This result further confirms the findings of Ferwerda et al. [44] who reported that the TLR4 Thr399Ile polymorphism is prevalent in the European population, but is almost in non-existent in the African populations, and this finding may help to explain our inability to demonstrate any association between the isolated Thr399Ile allele and a decreased risk of atherosclerosis in this study.

This study has its limitations: firstly, the descriptive and cross-sectional nature of this study does not allow for evaluation of cause-and-effect relationship between endotoxaemia-related inflammation and atherosclerosis. A prospective epidemiological study is needed to determine the contributory role of systemic inflammation on the risk of incident atherosclerosis in the South African CKD population. Secondly, our sample size was relatively small; a larger sample size comprising CKD populations from different ethnic groups would provide a more accurate reference database, given the spectrum of genetic diversity across the Southern African subregion. The results of the TLR4 genotyping should therefore be confirmed in a larger CKD population from different ethnic groups to determine if the findings of this study are generalizable. Finally, our genotype analysis was restricted to the 2 common TLR4 SNPs. It is possible that we may have missed some novel polymorphisms that are unique to the South African population and would only be detected by whole genome sequencing analysis. Future studies aiming to sequence the whole genome using methods designed to assess variants that are specific for African populations would be a suitable alternative method for addressing this problem.

In conclusion, we demonstrated associations between circulating endotoxaemia, systemic inflammation and accelerated atherosclerosis among South African CKD patients. Our findings showed that the atherogenic predictive power of endotoxin was significantly increased by the presence of elevated serum levels of inflammatory mediators in South African CKD patients and that TLR4 polymorphisms are associated with low levels of inflammatory markers and CIMT values. These findings have two implications. First, endotoxin may function as a pro-inflammatory mediator of accelerated atherosclerosis in black South African CKD

patients. Second, our findings provide support for a paradigm shift in the search for possible therapeutic targets to reduce atherosclerotic CVD in CKD patients. These might include prevention of endotoxaemia either through treating foci of endotoxin in CKD patients including periodontal disease, catheters and vascular access or by reducing translocation of endotoxin from the gut through reduction of gut venous congestion and/or oedema.

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References

1. Wiedermann CJ, Kiechl S, Dunzendorfer S, Schratzberger P, Egger G, Oberhollenzer F, et al. Association of endotoxemia with carotid atherosclerosis and cardiovascular disease. Prospective results from the Bruneck study. J Am Coll Cardiol. 1999; 34: 1975–81. https://doi.org/10.1016/s0735-1097(99)00448-9 PMID: 10588212
2. Wiedermann CJ, Kiechl S, Schratzberger P, Dunzendorfer S, et al. The role of immune activation in endotoxin-induced atherogenesis. Journal of Endotoxin Research. 2001; 7:322–6. PMID: 11717590
3. Li L, Messas E, Batista EL Jr., Levine RA, Amar S. Porphyromonas gingivalis infection accelerates the progression of atherosclerosis in a heterozygous apolipoprotein E deficient murine model Circulation 2002; 105:861–7.
4. Libby P, Ridker PM, Hansson GK. Inflammation in atherosclerosis: From pathophysiology to practice. J Am Coll Cardiol. 2009; 54(23):2129–38. https://doi.org/10.1016/j.jacc.2009.09.009 PMID: 19942084
5. Kiechl S, Egger G, Mayr M, Wiedermann CJ, Bonora E, Oberhollenzer F, et al. Chronic infections and the risk of carotid atherosclerosis: Prospective results from a large population study. Circulation. 2001; 103:1064–70. https://doi.org/10.1161/01.cir.103.8.1064 PMID: 11222467
6. Geng S, Chen K, Yuan R, Peng L, Maitra U, Diao N, et al. The persistence of low-grade inflammatory monocytes contributes to aggravated atherosclerosis. Nat Commun 2016; 7:13436. https://doi.org/10.1038/ncomms13436 PMID: 27824038
7. Szeto C, Kwan BC, Chow K, Lai K, Chung K, et al. Endotoxaemia is related to systemic inflammation and atherosclerosis in peritoneal dialysis patients. Clin J Am Soc Nephrol. 2008; 3: 431–6. https://doi.org/10.2215/CJN.03600807 PMID: 18256376
8. Gualtero DF, Lafaurie GI, Fontanilla MR. Two-dimensional and three-dimensional models for studying atherosclerosis pathogenesis induced by periodontopathogenic microorganisms. Mol Oral Microbiol 2018; 33(1):29–37. https://doi.org/10.1111/omi.12201 Epub 2017 Nov 6. PMID: 28984079

9. Hauser AB, Stinghen AEM, Goncalves SM, Bucharles S, Pecoits-Filho R. A gut feeling on endotoxaemia:causes and consequences in chronic kidney disease. Nephron Clin Pract. 2011; 118:c165–c72.

10. Stoll LL, Denning GM, Weintraub NL. Potential Role of Endotoxin as a Proinflammatory Mediator of Atherosclerosis. Arterioscler Thromb Vasc Biol 2004 24(12):2227–36. https://doi.org/10.1161/01.ATV.0000147534.69062.dc PMID: 15472123

11. Jiang D, Yang Y, Li D. Lipopolysaccharide induced vascular smooth muscle cells proliferation: A new potential therapeutic target for proliferative vascular diseases. Cell Prolif 2017; 50(2). https://doi.org/10.1111/cpr.12332 Epub 2017 Feb 2. PMID: 28150467

12. Roshan MH, Tambo A, Pace NP. The Role of TLR2, TLR4, and TLR9 in the Pathogenesis of Atherosclerosis. Int J Inflam. 2016; 2016:1532832. Epub 2016 Oct 4. https://doi.org/10.1155/2016/1532832 PMID: 27795867

13. An D, Hao F, Zhang F, Kong W, Chun J, Xu X, et al. CD14 is a key mediator of both lysophosphatidic acid and lipopolysaccharide induction of foam cell formation. J Biol Chem. 2017 292(35):14391–400. https://doi.org/10.1074/jbc.M117.781807 Epub 2017 Jul 13. PMID: 28705936

14. Patel PN, Shah RY, Ferguson JF, Reilly MP. Human experimental endotoxemia in modeling the pathophysiology, genomics, and therapeutics of innate immunity in complex cardiometabolic diseases. Arterioscler Thromb Vasc Biol 2015; 35(3):525–34. https://doi.org/10.1161/ATVBAHA.114.304455 PMID: 25550206

15. Stoll LL, Denning GM, Weintraub NL. Potential Role of Endotoxin as a Proinflammatory Mediator of Atherosclerosis. Arterioscler Thromb Vasc Biol 2004; 24:2227–36. https://doi.org/10.1161/01.ATV.0000147534.69062.dc PMID: 15472123

16. Kiechl S, Lorenz E, Reindl M, Wiedermann C, Oberhollenzer F, Bonora E, et al. Toll-like receptor 4 polymorphisms and atherogenesis. N Engl J Med. 2002; 347:185–92. https://doi.org/10.1056/NEJMoa012673 PMID: 12124407

17. Chow JC, Young DW, Golenbock DT, Christ WJ, Gusovsky F. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. J Biol Chem. 1999; 274:10689–92. https://doi.org/10.1074/jbc.274.16.10689 PMID: 10196138

18. Horseman MA, Surani S, Bowman JD. Endotoxin, Toll-like Receptor-4, and Atherosclerotic Heart Disease. Curr Cardiol Rev. 2017; 13(2):86–93. https://doi.org/10.2174/1573403X12666160901145313 PMID: 27586023

19. Hernanz R, Martinez-Revelles S, Palacios R. Toll-like receptor 4 contributes to vascular remodelling and endothelial dysfunction in angiotensin II-induced hypertension. Br J Pharmacol. 2015; 172 (12):3159–76. https://doi.org/10.1111/bph.13117 PMID: 25712370

20. Jia SJ, Niu PP, Cong JZ, Zhang BK, Zhao M. TLR4 signaling: a potential therapeutic target in ischemic coronary artery disease. Int Immunopharmacol 2014; 23(1):54–9. https://doi.org/10.1016/j.intimp.2014.08.011 PMID: 25158302

21. Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation Circulation. 2002; 105:1158–61. PMID: 11889007

22. Pelham CJ, Agrawal DK. Emerging roles for triggering receptor expressed on myeloid cells receptor family signaling in inflammatory diseases. Expert Rev Clin Immunol. 2014; 10:243–56. https://doi.org/10.1586/1744666X.2014.866519 PMID: 24325404

23. Schröder NW, Schumann RR. Single nucleotide polymorphisms of Toll-like receptors and susceptibility to infectious disease. Lancet Infect Dis. 2005; 5(3):156–64. https://doi.org/10.1016/S1473-3099(05)01308-3 PMID: 15766650

24. Boekholdt SM, Agema WR, Peters RJ, Zwinderman AH, van der Wall EE, Reitsma PH, et al. Variants of toll-like receptor 4 modify the efficacy of statin therapy and the risk of cardiovascular events. Circulation. 2003; 107:2416–21. https://doi.org/10.1161/01.CIR.0000068311.40161.28 PMID: 12742999

25. Ameziane N, Beillat T, Verpillat P, Chollet-Martin S, Aumont MC, Seknadji P, et al. Association of the Toll-like receptor 4 gene Asp299Gly polymorphism with acute coronary events. Arterioscler Thromb Vasc Biol. 2003; 23:e61–e4 https://doi.org/10.1161/01.ATV.0000101191.92392.1D PMID: 14563652

26. Yang IA, Holloway JW, Ye S. TLR4 Asp299Gly polymorphism is not associated with coronary artery stenosis. Atherosclerosis. 2003; 70:187–90.

27. Netea MG, Hijmans A, van Wissen S, Smilde TJ, Trip MD, Kullberg BJ, et al. Toll-like receptor-4 Asp299Gly polymorphism does not influence progression of atherosclerosis in patients with familial hypercholesterolaemia. Eur J Clin Invest 2004; 34(2):94–9. https://doi.org/10.1111/j.1365-2362.2004.01303.x PMID: 14764071

28. Kutikhin AG, Ponasenko AV, Khutornaya MV, et al. Association of TLR and TREM-1 gene polymorphisms with atherosclerosis severity in a Russian population. Meta Gene. 2016; 9:76–89. https://doi.org/10.1016/j.mgene.2016.04.001 PMID: 27200266

29. Guven M, Ismailoglu Z, Batar B, Unal S, Onaran I, Karadag B, et al. The effect of genetic polymorphisms of TLR2 and TLR4 in Turkish patients with coronary artery disease. Gene 2015; 558(1):99–102. https://doi.org/10.1016/j.gene.2014.12.047 Epub Dec 24. PMID: 25542811

30. Zhang K, Zhang L, Zhou B, Wang Y, Song Y, Rao L. Lack of association between TLR4 Asp299Gly polymorphism and atherosclerosis: evidence from meta-analysis. Thromb Res. 2012; 130:e203–e8. https://doi.org/10.1016/j.thromres.2012.07.008 PMID: 22857799

31. Brenchley J, Price D, Schacker T, Asher T, Silvestri G, Rao S, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med. 2006; 12:1365–71. https://doi.org/10.1038/nm1511 PMID: 17115046

32. Nasiri H, Forouzandeh M, Rasaee MJ, Rahbarizadeh F. Modified salting-out method: high-yield, high-quality genomic DNA extraction from whole blood using laundry detergent. J Clin Lab Anal. 2005; 19 (6):229–32. https://doi.org/10.1002/jcla.20083 PMID: 16302208

33. Lorenz E, Frees KL, Schwartz DA. Determination of the TLR4 genotype using allele-specific PCR. Biotechniques. 2001; 31(1):22–4. https://doi.org/10.2144/01311bm01 PMID: 11464514

34. Stein J, Korcarz C, Hurst R, Lonn E, Kendall C, Mohler E, et al. Use of carotid ultrasound to identify subclinical vascular disease and evaluate cardiovascular disease risk: a consensus statement from the American Society of Echocardiography Carotid Intima-media Thickness Task Force. Endorsed by the Society for Vascular Medicine. J Am Soc Echocardiogr 2008; 21(2): 93–111. https://doi.org/10.1016/j.echo.2007.11.011 PMID: 18261694

35. Lepper P, Schumann C, Triantafilou K, Rasche F, Schuster T, Frank H, et al. Association of lipopolysaccharide-binding protein and coronary artery disease in men. J Am Coll Cardiol 2007; 50 (1):25–31. https://doi.org/10.1016/j.jacc.2007.02.070 PMID: 17601541

36. Trojova I, Kozarova M, Petrasova D, Malachovska Z, Paranicova I, Joppa P, et al. Circulating lipopolysaccharide-binding protein and carotid intima-media thickness in obstructive sleep apnea. Physiol Res 2018 67(1):69–78. Epub 2017 Nov 10.

37. Wiedermann CJ, Kiechl S, Schratzberger P, Dunzendorfer S, Weiss G, Willeit J. The role of immune activation in endotoxin-induced atherogenesis. Journal of Endotoxin Research 2001; 7: 322–6. PMID: 11717590

38. White SN, Taylor KH, Abbey CA, Gill CA, Womack JE. Haplotype variation in bovine Toll-like receptor 4 and computational prediction of a positively selected ligand-binding domain. Proc Natl Acad Sci USA 2003; 100:10364–9. https://doi.org/10.1073/pnas.1333957100 PMID: 12915733

39. Bell JK, Mullen GE, Leifer CA, Mazzoni A, Davies DR, Segal DM. Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol. 2003; 24:528–33. https://doi.org/10.1016/s1471-4906(03)00242-4 PMID: 14552836

40. Rallabhandi P, Bell J, Boukhvalova MS, Medvedev A, Lorenz E, Arditi M, et al. Analysis of TLR4 polymorphic variants: new insights into TLR4/MD-2/CD14 stoichiometry, structure, and signaling. J Immunol 2006; 177(1):322–32. https://doi.org/10.4049/jimmunol.177.1.322 PMID: 16785528

41. Baker AR, Qiu F, Randhawa AK, Horne DJ, Adams MD, et al. Genetic Variation in TLR Genes in Ugandan and South African Populations and Comparison with HapMap Data. PLoS ONE. 2012; 7(10): e47597. https://doi.org/10.1371/journal.pone.0047597 PMID: 23112821

42. May A, Hazelhurst S, Li Y, Norris SA, Govind N, Tikly M, et al. Genetic diversity in black South Africans from Soweto. BMC Genomics 2013; 14: 644. https://doi.org/10.1186/1471-2164-14-644 PMID: 24059264

43. Kresfelder TL, Janssen R, Bont L, Venter M. Confirmation of an Association Between Single Nucleotide Polymorphisms in the VDR Gene With Respiratory Syncytial Virus Related Disease in South African Children. J Med Virol 2011; 83:1834–40. https://doi.org/10.1002/jmv.22179 PMID: 21837802

44. Ferwerda B, McCall MBB, Alonso S, Giamarellos-Bourboulis EJ, Mouktaroudi M, Izagirre N, et al. TLR4 polymorphisms, infectious diseases, and evolutionary pressure during migration of modern humans. Proc Natl Acad Sci USA 2007; 104:16645–50. https://doi.org/10.1073/pnas.0704828104 PMID: 17925445