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Plasma advanced glycation end products (AGEs) and NF-κB activity are independent determinants of diastolic and pulse pressure

Abstract

Background: High levels of circulating advanced glycation end products (AGEs) can initiate chronic low-grade activation of the immune system (CLAIS) with each of these factors independently associated with cardiovascular (CV) morbidity and mortality. Therefore, our objective was to characterize the relationship between serum AGEs, CLAIS and other risk factors for CV disease in normotensive non-diabetic individuals.

Methods: We measured body mass index (BMI), waist-to-hip ratio (WHR), blood pressure, lipid and glucose profile in 44 non-diabetic volunteers (17 female, 27 males). Carboxymethyl-lysine (CML) was measured by ELISA as a marker for circulating AGEs and NF-κB p65 activity as an inflammatory marker by DNA-binding in peripheral blood mononuclear cells lysates (PBMC).

Results: Plasma CML concentrations were related to diastolic blood pressure ($r=-0.51$, $p<0.01$) independently of age, sex, BMI and WHR ($p<0.05$). Diastolic blood pressure was also related to NF-κB activity in PBMC ($r=0.47$, $p<0.01$) before and after adjustment for age, sex, BMI and WHR ($p<0.05$). Plasma CML concentrations were related to the pulse pressure before ($r=0.42$; $p<0.05$) and after adjustment for age, sex, BMI and waist ($p<0.05$). Neither CML nor NF-κB activity were related to systolic blood pressure (both p=ns). Plasma CML concentrations were not associated with plasma lipid or glucose concentrations (all p=ns).

Conclusions: Plasma AGE levels and NF-κB activity in PBMC were independent determinants of diastolic and pulse pressure in healthy normotensive individuals. This association suggests a role for AGEs in the etiology of hypertension, possibly via the initiation of CLAIS and aortic stiffening.

Keywords: advanced glycation end products; alkaline phosphatase; blood pressure; central obesity; chronic low-grade inflammation; NF-κB activity.

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Introduction

Cardiovascular disease (CVD) is one of the leading causes of death globally [1]. Hypertension is the major risk factor for CVD with a global prevalence of 26% and it is expected to increase by 60% by year 2025 [1]. Elevated blood pressure also increases the risk of stroke or myocardial infarction by four- or two-fold, respectively. Both the escalating prevalence of hypertension and its sequelae are contributing to a rising global economic burden [1].

Advanced glycation end products (AGEs) may play an important role in the development and/or progression of CVD and specifically hypertension [2–7]. AGEs are a heterogeneous group of compounds that are formed by non-enzymatic glycation of proteins, lipids and nucleic acids. AGEs are known to elicit their deleterious effects by binding to the pro-inflammatory, cell surface bound receptor for AGEs (RAGE). A number of soluble forms of RAGE have been identified in the circulation of humans. Soluble RAGE (sRAGE) is thought to exist as a result of protealytic cleavage of RAGE from the cell surface. By contrast, endogenous secretary RAGE (esRAGE) is c-terminally

truncated splice variant of RAGE which is released from the cell. The soluble forms of RAGE have been identified as decoy receptors for RAGE as they are capable of binding to RAGE yet lack the c-terminus which translate the deleterious signaling [8, 9]. It is for this reason, that esRAGE and sRAGE have been proposed as possible biomarkers for diabetic complications and CVD. Circulating levels of both esRAGE and sRAGE have been shown to be associated with arterial stiffness in diabetic and hypertensive patients [10, 11].

The two main sources of AGEs within the body are exogenous dietary AGEs mostly from food processing and endogenous AGEs that are generated in excess by abnormal glucose and lipid metabolism [2, 7, 12, 13]. We and others have demonstrated that the consumption of diets high in AGEs elicit deleterious effects throughout the body including: increased inflammation [14, 15] driving insulin resistance [16] and impairing renal function [14, 17, 18].

Experimental evidence has shown that AGE accumulation affords structural and functional changes in the cardiovascular system and results in myocardial relaxation abnormalities, atherosclerotic plaque formation, large artery stiffening and endothelial dysfunction [4, 19–22]. In the vessel wall, AGEs form covalent crosslinks with matrix proteins, which increases vessel rigidity, trap lipoproteins within the arterial wall and disrupts their clearance [23–25]. Increased circulating AGE levels and increased vascular tissue AGE deposition have been reported to be associated with impaired endothelium dependent and endothelium-independent vasodilatation and increased arterial stiffness in patients with diabetes [26, 27]. The latter action would be expected to elevate pulse pressure. Importantly, these effects have been reversed using AGE lowering therapies [26] suggesting that AGEs may play a role in the pathophysiology of hypertension. To our knowledge, there are no studies demonstrating the relationship between AGEs and blood pressure or development of hypertension in healthy non-diabetic individuals.

Chronic low-grade activation of the immune system (CLAIS) has been identified as an important determinant of a number of chronic diseases including atherosclerosis and CVD [28, 29]. Moreover, CLAIS has been associated with risk factors of CVD such as glucose intolerance/diabetes [30], dyslipidemia [31–33] and hypertension [34, 35]. The relationship between CLAIS and hypertension is likely due to both direct AGE deposition in the vasculature which contributes to arterial stiffening as well as impaired capacity of the endothelium to generate vasodilating factors [34, 36]. The nuclear factor-kappa B (NF-κB) pathway is considered to be a major intracellular inflammatory pathway which is also known to mediate many of the vascular inflammatory processes [34, 36, 37]. AGEs are thought to activate the NF-κB pathway primarily as a result of their ligation with their pro-inflammatory full-length cell surface receptor RAGE [34, 38–41]. The relationship between AGEs, NF-κB in peripheral blood mononuclear cells (PBMC) and blood pressure has not been previously investigated in healthy humans.

We hypothesized that plasma AGE levels, their receptors and NF-κB activity in PBMC will be associated with blood pressure and other cardiovascular risk factors in a group of healthy young normotensive non-diabetic individuals.

Materials and methods

Study participants and design

Forty-four non-diabetic, non-hypertensive volunteers (17 female, 27 males) between 18 and 50 years of age participated in the study. Participants were non-smokers at the time of the study, generally healthy according to a detailed physical examination and routine blood analyses. No participant had signs of acute or chronic infection, or took any medication or illicit drugs. All participants underwent a medical screening, which included a history, physical examination with anthropometric measurement and basic laboratory tests including fasting plasma lipid levels, liver function tests, urea, creatinine and electrolytes as well as a 75 g glucose load oral glucose tolerance test (OGTT).

The protocol was approved by the Alfred Hospital Ethics Committee and complied with the Declaration of Helsinki. All participants provided written informed consent prior to participation.

Anthropometric data

Height and weight were measured and body mass index (BMI) calculated. Waist and hip circumferences were measured and waist-to-hip ratio (WHR) calculated as an index of body fat distribution. Seated brachial blood pressure was measured three times, separated by 1 min using a Dinamap monitor (Model 1846SX, Critikon, Tampa, FL, USA) after 15 min of rest. The mean of the three measurements taken was calculated and reported. Pulse pressure was calculated as a difference between systolic and diastolic mean blood pressure. Mean arterial pressure (MAP) was estimated as diastolic blood pressure + pulse pressure/3.

Blood analyses

Various inflammatory markers, circulating AGE parameters, glucose (during OGTT) and lipid levels were measured in plasma collected following a 12-h overnight fast. Glucose tolerance status was determined by WHO 1999 criteria [42]. Prior to these measurements, par-

ticipants were asked to abstain from strenuous exercise and caffeine for 3 days. Blood samples were drawn using standard phlebotomy techniques into sodium citrate Vacutainers. The tubes were centrifuged immediately (1500×*g*, 15 min, 4°C), and the plasma stored at −80°C until analyses were performed. Lipids were measured by standard laboratory assays on the Abbott Archicentre ci162000 by Alfred Pathology laboratory. Plasma glucose concentrations were measured using the glucose oxidase method (ELM 105, Radiometer Copenhagen, Denmark). Plasma high sensitivity CRP was measured by an immunotubidimetric assay (Abbott Archicentre ci162000). White blood cell counts (WBC) were measured by Beckman Coulter LH 785 analyzer by Alfred Pathology Services. Circulating AGE (CML) concentrations were determined using an indirect ELISA as previously described [43, 44]. sRAGE was analyzed in serum samples using a human sRAGE sandwich ELISA (R&D Systems, Minneapolis, MN, USA). esRAGE was measured in plasma by ELISA (B-bridge International, USA). Interleukin-6 (IL-6) and Monocyte Chemotactic Protein-1 (MCP-1) were measured in serum samples with high sensitivity sandwich ELISA according to manufacturer's instructions (R&D Systems). PBMC were isolated from whole blood by Ficol-paque density centrifugation (Amersham Biosciences, Uppsala, Sweden) as previously described [44, 45]. Briefly, PBMC samples were washed in excess PBS to remove residual FBS and DMSO. The supernatant was discarded and the pellets resuspended in 100 µL of triple detergent lysis buffer (50 mM Tris HCl pH 8.0; 150 mM NaCl; 0.02% NaN_3; 0.1% SDS; 100 µg/mL PMSF; 1 µg/mL aprotinin; 1% nonidet P-40; 0.5% sodium deoxycholate, in milliQ H_2O). These were sonicated and then centrifuged. The resulting whole cell lysate was used in the NF-κB assay. The protein concentration of all protein isolates was determined using the Pierce Bicinchoninic Acid protein assay (Pierce, Rochford, IL, USA) performed according to manufacturer's instructions. The TransAM NF-κB DNA-binding activity assay (Active Motif, Carlsbad, CA, USA) was used to detect and quantify NF-κB factor activation, using a p65 NF-κB consensus sequence in labeled DNA in a 96-well plate format. Results are expressed as nanogram (ng) DNA bound p65 activity per µg protein.

Statistical analyses

Statistical analyses were performed using SAS Jump Statistics Software (Cary, NC, USA). Results are reported as mean±SD or median with interquartile range. Some of the variables were logarithmically transformed (Log10) to approximate normal distribution. Correlations were performed using the Pearson correlation coefficient with Bonferroni correction. Multiple regression was used to assess the determinants of blood pressure variables after adjusting for co-variates. Statistical significance was assumed when $p<0.05$.

Results

Participant characteristics

The anthropometric and metabolic characteristics of the study population are summarized in Table 1. All participants were healthy according to the physical examination. Specifically, they were normotensive, had normal full blood counts, liver function tests, plasma hsCRP and renal function and some of them were overweight or obese (Table 1, liver and renal function data not shown). Females had lower body weight, WHR, systolic blood

Table 1 Anthropometric, metabolic and inflammation parameters of study population.

	All, n=44	Female, n=17	Male, n=27
Age, years	30±10	30±10	31±10
Weight, kg	84±16	74±12	90±16[a]
BMI, kg/m²	28±5	27±4	29±5
Waist circumference, cm	93±13	87±12	97±14[a]
WHR	0.89±0.08	0.83±0.06	0.92±0.07[a]
Systolic blood pressure, mm Hg	120±2	114±11	124±11[a]
Diastolic blood pressure, mm Hg	74±10	70±9	76±9
Pulse pressure, mm Hg	47±8	45±6	49±9
MAP, mm Hg	89±10	85±10	91±9[a]
Fasting glucose, mmol/L	4.8±0.6	4.5±0.6	4.9±0.5
2 hour glucose, mmol/L	5.1±1.5	5.2±1.5	5.1±1.5
IL-6, pg/mL	0.5 (0.3–1.0)	0.6 (0.4–0.7)	0.6 (–0.3–1.40)
hsCRP, mg/L	1.3 (0.1–2.0)	1.4 (0.5–2.2)	1.1 (0.4–1.9)
White blood cells, 10^9/L	6.0±1.0	5.9±1.0	6.0±1.1
Total cholesterol, mmol/L	4.5±0.8	4.4±0.7	4.5±0.9
HDL cholesterol, mmol/L	1.2±0.3	1.4±0.3	1.0±0.3[a]
LDL cholesterol, mmol/L	2.7±0.7	2.6±0.6	2.8±0.8
Triglycerides, mmol/L	1.3±0.8	1.0±0.4	1.5±0.9[a]

Data are means ±SD, [a]$p<0.05$ male vs. female subjects. IL-6 and hsCRP are presented as median and interquartile range. BMI, body mass index; hsCRP, high sensitivity C-reactive protein; IL-6, interleukin 6; WHR, waist-hip ratio.

pressure and MAP compared with the males but no other gender differences were apparent (Table 1). There were no differences in plasma AGE, sRAGE, esRAGE and plasma inflammatory parameters between males and females (p>0.05).

Blood pressure, anthropometric measurements, lipid and inflammation parameters

Diastolic blood pressure was related to BMI (r=0.49, p<0.001) and WHR (r=0.46, p<0.01). Diastolic blood pressure was related to plasma triglyceride concentrations (r=0.36, p<0.05), but to none of the other plasma lipid parameters (data not shown).

Diastolic blood pressure was related to NF-κB activity in PBMC (Figure 1E, Table 2), WBC count (r=0.52, p<0.001), plasma IL-6 (r=0.36, p<0.05), MCP-1 (r=0.40, p<0.05) and hsCRP concentrations (r=0.31, p<0.05). The relationship between diastolic blood pressure and NF-κB activity and WBC count were independent of age, sex, BMI and WHR (both p<0.05). When both NF-κB activity in PBMC and WBC count were placed into the model including age, sex, BMI and WHR, neither were significantly associated with diastolic blood pressure (p>0.1).

Systolic blood pressure was not related to BMI or WHR (r=0.27, r=0.28 r=0.23, respectively, p=ns). Systolic blood pressure was not related to any of the plasma lipid parameters (data not shown). Systolic blood pressure was

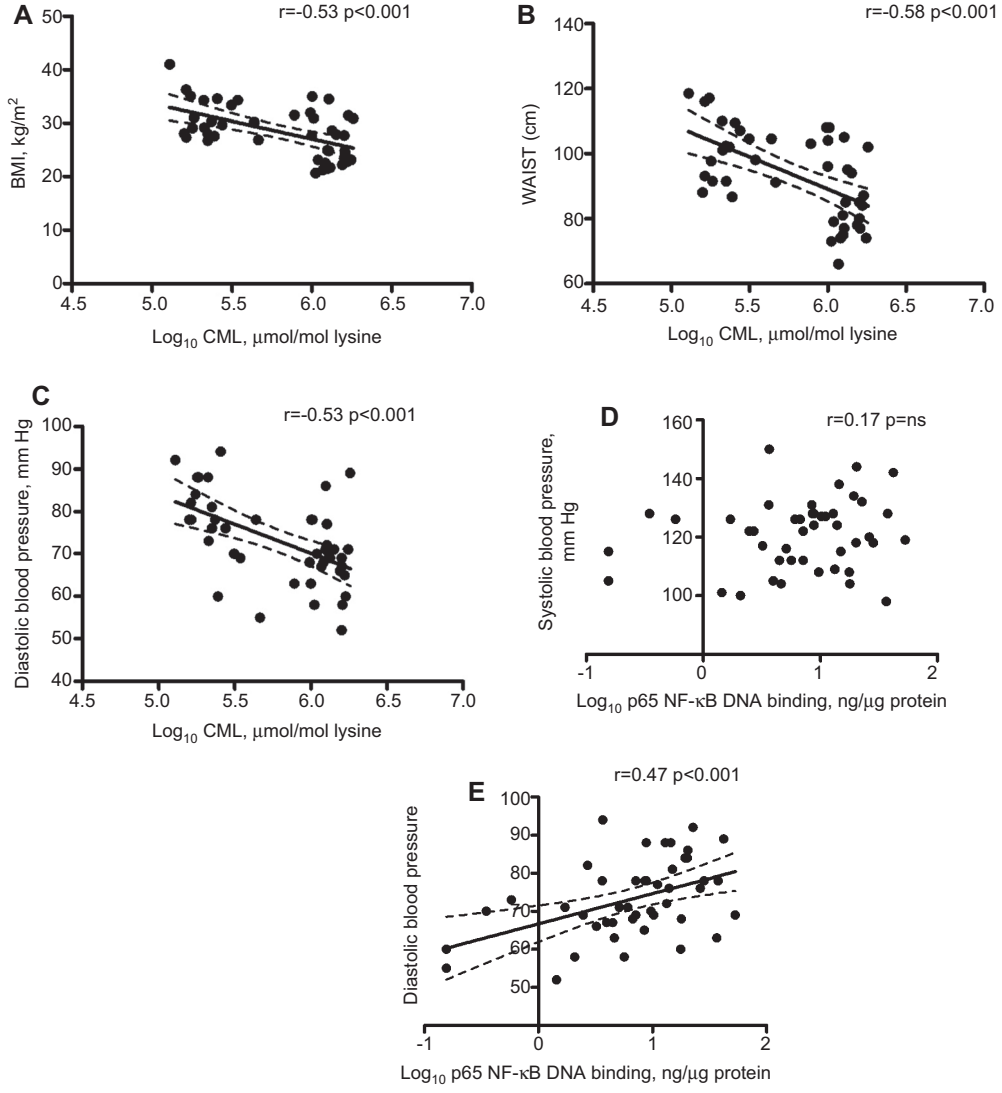

Figure 1 Interactions waist-hip ratio (WHR), between CML, NF-κB activity in PBMCs and blood pressure.
CML and BMI (A), CML and systolic blood pressure (B), CML and diastolic blood pressure (C). NF-κB activity in PBMCs and systolic blood pressure (D) and NF-κB activity in PBMCs and diastolic blood pressure (E).

Table 2 Pearson correlation coefficients between AGE parameters and selected cardiovascular risk factors.

	CML r; p	sRAGE r; p	esRAGE r; p	NF-κB r; p
BMI	−0.55; 0.002	−0.20; 0.2	−0.17; 0.3	0.24; 0.08
WHR	−0.34; 0.02	−0.22; 0.2	−0.15; 0.4	0.25; 0.1
Total cholesterol	−0.15; 0.2	−0.27; 0.09	−0.15; 0.4	0.07; 0.9
Triglycerides	−0.09; 0.5	−0.1; 0.6	−0.45; 0.003	0.14; 0.5
Systolic BP	−0.07; 0.5	0.03; 0.7	−0.05; 0.9	0.17; 0.4
Diastolic BP	−0.51; 0.001	−0.06; 0.9	−0.07; 0.9	0.47; 0.004
Pulse pressure	0.42; 0.004	0.18; 0.6	0.04; 0.6	−0.32; 0.2
MAP	−0.41; 0.002	0.004; 0.8	−0.13; 0.7	0.37; 0.008

BMI, body mass index; BP, blood pressure; MAP, mean arterial pressure.

related to WBC count (r=0.35, p<0.05) but was not related to any other inflammation parameters (Figure 1D, other data not shown).

NF-κB activity was related to the pulse pressure but not to M MAP (Table 2). After adjustment for age, sex, BMI and waist, NF-κB activity approached but did not reach statistical significance as a determinant of pulse pressure (p=0.06).

AGE parameters and anthropometric measures

Plasma AGEs (CML) (Figure 1A and B, Table 2) were related to BMI (r=−0.53, p<0.001) and WHR (r=0.36, p=0.02). Plasma sRAGE and esRAGE concentrations were not related anthropometric parameters (Table 2). None of the AGE parameters were related to age (p>0.1).

AGE parameters, blood pressure and lipid parameters

Plasma CML concentrations were inversely related to diastolic (r=−0.53, p<0.001) (Figure 1C) but not systolic blood pressure (r=−0.07, p=ns; Table 2). The inverse relationship between plasma CML concentrations and diastolic blood pressure persisted after adjustment for age, sex, BMI and WHR (p=0.02). Both sRAGE and esRAGE were not related to systolic or diastolic blood pressure (Table 2). Plasma CML concentrations were related to the pulse pressure before (Table 2) and after adjustment for age, sex, BMI and WHR (p=0.02). Plasma CML concentrations were not related to the MAP) (Table 2).

Plasma LDL cholesterol concentrations were related to plasma sRAGE concentrations before (r=−0.38, p<0.05) and adjustment for age and sex (p=0.02). LDL cholesterol concentrations were not related to plasma CML or esRAGE concentrations (data not shown). Plasma esRAGE concentrations were related to plasma triglycerides before (r=−0.45, p<0.01) and after adjustment for age, sex, BMI and wait/WHR (both p=0.005).

AGE parameters and plasma inflammation parameters

Plasma CML concentrations were related to plasma IL-6 (r=−0.53, p<0.001) and MCP-1 concentrations (r=−0.55, p<0.001) but not to hsCRP or WBC count (data not shown). sRAGE was related to plasma MCP-1 concentrations whereas esRAGE was not related to any parameters reflecting inflammation (data not shown). None of the AGE parameters were related to NF-κB activity in PBMCs (all p>0.1).

Multivariate analyses of CML and diastolic blood pressure

The plasma CML concentration was a significant determinant of diastolic blood pressure independently of age, sex, BMI and WHR (p<0.05). The relationship remained significant after adjustment for NF-κB activity in PBMC (p=0.01) and additional adjustment for WBC count (p=0.003). In the stepwise regression, plasma CML concentrations, WBC count and NF-κB activity in PBMC explained 52% of variance and were both significant determinants of diastolic blood pressure.

Both plasma CML concentrations (p=0.004) and NF-κB activity in PBMC (p=0.01) were significant determinants of pulse pressure after adjustment for age, sex, BMI and WHR.

Discussion

AGEs and the pro-inflammatory and pro-oxidant pathways, which they activate are likely to modulate the development and progression of many chronic disease processes including type 2 diabetes and CVD. In this present study, we investigated the relationship between circulating CML, soluble AGE-receptor concentrations, inflammation markers, including NF-κB activity with CVD risk factors in a healthy overwieight, normotensive cohort. Our findings demonstrate for the first time that serum CML and NF-κB activity in PBMCs are associated with diastolic blood pressure and pulse pressure in young healthy individuals.

Chronic low-grade inflammation has been related to the development of hypertension [46] independently of adiposity [47]. In our study, we extend this evidence by showing that NF-κB activity in PBMC was associated with diastolic blood pressure in healthy normotensive individuals in addition to plasma inflammation markers. This is consistent with results from an experimental rat model of hypertension, where administration of pyrrolidine dithiocarbamate reduced both NF-κB activity and blood pressure concurrently [48]. The NF-κB pathway is a central modulator of the immune system. Once activated it stimulates downstream pro-inflammatory molecules as well as adhesion molecules [34, 38], which then induce and maintain inflammation within the vascular wall, stimulate deposition of extracellular matrix and promote hypertrophy and/or hyperplasia of vascular smooth muscle cells [49, 50].

The accumulation of AGEs over the life span may contribute to the development of CVD by contributing to arterial stiffening, endothelial dysfunction and plaque formation [7, 26, 36, 51–54]. There is a paucity of evidence from humans in this area of research. Increased levels of circulating AGEs have been associated with elevations in systolic blood pressure in people with diabetes [55] and in those with chronic kidney disease on hemodialysis [56–58]. Furthermore, administration of AGE crosslink breaker (ALT-711) to patients with hypertension was associated with a concurrent reduction in AGEs, improved endothelial function and reduced arterial stiffness and pulse pressure [29]. These data bolster the evidence of the involvement of AGEs in hypertension as well as arterial stiffening [29, 59–61]. We showed for the first time that higher circulating levels of CML were associated with lower diastolic blood pressure and higher pulse pressure in young healthy normotensive individuals. This suggests that circulating AGE levels are associated with aortic stiffening in individuals without known cardiovascular risk factors. Crosslinking AGEs have been shown to induce aortic stiffening in an experimental model of diabetes [62]. Consistent with this, we have also previously shown that low CML levels are associated with abdominal aortic aneurysms in patients with type 2 diabetes [63]. As the aorta stiffens with aging, pulse pressure increases and diastolic pressure decreases. The decrease in diastolic blood pressure then compromises the coronary circulation and furthermore contributes to ischemic heart disease and cardiovascular events [64, 65]. As CML within the arterial extracellular matrix was not measured, it is not possible to establish whether this mechanism contributed to aortic stiffening via crosslinking.

The soluble receptors for AGEs are considered to act as decoy receptors to prevent the pro-inflammatory interaction of AGEs with their cell-surface full length receptor RAGE [13, 66, 67]. In older community dwelling women, both esRAGE and sRAGE were found to be predictive of CVD mortality [68]. In a cross-sectional analysis of hypertensive patients, serum sRAGE correlated with systolic blood pressure [69]. In addition, sRAGE and esRAGE were associated with carotid atherosclerosis in hemodialysis patients [70, 71]. In diabetic patients with coronary artery disease, sRAGE has been shown to correlate with diastolic blood pressure [72]. In the present study, we found no correlation between blood pressure and the levels of soluble AGE receptors, sRAGE and esRAGE. This could be due to differences in our study population, which was younger, overweight, normotensive, non-diabetic and had normal renal function. In our study, plasma LDL cholesterol and triglyceride levels were correlated with sRAGE and esRAGE, respectively, whilst no association was found with CML. This is consistent with data in patients with diabetes and coronary artery disease, where sRAGE correlated with LDL cholesterol and triglycerides [72].

We and others have shown that plasma AGEs are related to adiposity [14, 73–75]. In the present study, we confirm these findings by showing the negative association between circulating levels of CML and various measures of adiposity. The direction of this relationship is surprising given that it is likely that obese individuals would consume more dietary AGEs compared to lean individuals. In addition, a short-term low calorie diet intervention reduced serum AGEs in healthy overweight or obese adults [76]. It has been proposed that the inverse cross-sectional relationship between circulating AGEs and adiposity may be a direct result of AGEs preferentially depositing in the adipose tissue as opposed to the circulation. This could be due to the fact that adipocytes are known to express CD36, a scavenger receptor that binds AGEs and facilitates endocytosis and degradation of AGEs [75].

In the present study, we showed that plasma AGEs levels and NF-κB activity in PBMC were related to diastolic blood pressure and pulse pressure independent of obesity. This may relate in part to AGEs promoting stiffening of the aorta. Furthermore, AGEs in the vascular tissue may induce these deleterious vascular effects is by impairing nitric oxide synthase activity in endothelial cells as has been previously described [36, 77, 78]. As noted previously, AGEs are thought to activate the NF-κB pathway primarily as a result of their ligation with RAGE. This subsequently induces the activation of a number of NF-κB mediated pro-inflammatory pathways, generation of reactive oxygen species and increased expression of adhesion molecule which then promote atherosclerosis further contributing to arterial stiffening [38, 41, 79].

Limitations

First, the cross-sectional nature of the study cannot delineate the cause-and-effect relationships among plasma CML, NF-κB activity and diastolic blood pressure. Second, the sample size is relatively small, and hence, the results should be interpreted with caution. However, the strength of the correlations and their robustness in various adjusted models attest to the validity of the major findings.

In conclusion, we have demonstrated that plasma CML and NF-κB activity in PBMC are both important determinants of diastolic and pulse pressure in a healthy non-diabetic normotensive population. Further investigation is warranted to determine the mechanisms by which changes in AGEs and NF-κB may contribute to the development of aortic stiffening in humans.

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Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article.
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