Original Article

Inflammatory mediators in saliva associated with arterial stiffness and subclinical atherosclerosis

Carlos Labat[a], Mohamed Temmar[b], Edit Nagy[c,d], Kathy Bean[e], Charles Brink[f], Athanase Benetos[a], and Magnus Bäck[c,d]

Objective: Whereas circulating levels of C-reactive protein (CRP) have been associated with, for example, arterial stiffness, subclinical atherosclerosis and metabolic syndrome, other inflammatory biomarkers with potential interest for these conditions may not be measurable systemically. The predictive value of salivary biomarkers in these contexts has remained largely unexplored. The aim of the present study was to establish the association of different salivary biomarkers of inflammation with subclinical cardiovascular disease.

Methods: Two hundred and fifty-nine individuals were included in the study. Saliva and plasma samples were collected, and each individual underwent carotid ultrasound and measures of pulse wave velocity and blood pressure. Medical history of previous cardiovascular disease, current medications and smoking were collected by questionnaire.

Results: Salivary levels of CRP, leukotriene B_4 (LTB_4), prostaglandin E_2 (PGE_2), matrix metalloproteinase 9 (MMP-9), creatinine and lysozyme were measured. Salivary levels of CRP were significantly correlated with plasma levels ($r = 0.73$, $P < 0.0001$). In an age-adjusted and sex-adjusted analysis, salivary CRP was significantly and positively correlated with mean arterial blood pressure, pulse pressure, pulse wave velocity, BMI, metabolic syndrome, waist-to-hip ratio and intima–media thickness. Increasing age and sex-adjusted salivary CRP tertiles were in addition associated with carotid plaques. In a multivariate analysis, CRP and MMP-9 were associated with intima–media thickness, LTB_4 and PGE_2 with arterial stiffness, and lysozyme with hypertension.

Conclusion: Saliva may represent an alternative mean for evaluation of cardiovascular risk.

Keywords: C-reactive protein, inflammation, leukotriene, pulse wave velocity, saliva

Abbreviations: CRP, C-reactive protein; HR, heart rate; IMT, intima–media thickness; MAP, mean arterial blood pressure; MMP, matrix metalloproteinase; PP, pulse pressure; PWV, pulse wave velocity

INTRODUCTION

Over the past years, a plethora of information has established the diagnostic and prognostic value of various mediators of inflammation in cardiovascular disease [1–3]. The majority of these studies have evaluated agents measurable in blood samples. However, all mediators with potential interest as cardiovascular biomarkers on the basis of their role in the pathophysiology of atherosclerosis cannot be reliably detected in blood samples. For example, circulating levels may either not be detectable or limited by extensive ex-vivo formation during blood sampling. The latter is especially true for neutrophil-derived molecules such as lysozyme, myeloperoxidase, some of the matrix metalloproteinases (MMPs) and leukotrienes. For the latter two groups of mediators, low or undetectable levels in plasma samples are contrasted by high levels in serum samples [3–6], and it has been suggested that these measurable serum levels may be solely due to neutrophil secretion during the coagulation process in the tube [4,6]. Indeed, serum LTB_4 levels depend on the time from blood sampling to centrifugation [6]. To circumvent these limitations, several studies have isolated neutrophils for ex-vivo stimulation and established significant correlations, for example between PMA-stimulated MMP-9 release and stable coronary disease [4], and between calcium ionophore-induced LTB_4 release and subclinical atherosclerosis [7].

Several inflammatory mediators are present, and can be reproducibly measured, in saliva [8–11]. There are, however, only limited data available on salivary biomarkers for cardiovascular disease. Salivary lysozyme has been associated with prevalent hypertension and coronary artery disease [9,12]. In addition, salivary levels of MMP-9 are increased in acute coronary syndromes [13]. Furthermore, prostaglandins and leukotrienes are measurable in the saliva [8,11] and have been implicated as mediators of atherosclerosis [14–17], but these agents have not been fully explored for their value as biomarkers of

cardiovascular disease. Finally, an assay for the detection of salivary levels of CRP was recently validated [10], but the association of salivary CRP with measures of subclinical cardiovascular disease has not previously been explored.

The aim of the present study was to establish whether salivary levels of potential mediators of atherosclerosis could be used as biomarkers for subclinical cardiovascular disease. Salivary measures may not only represent an easy to use, noninvasive test for cardiovascular risk, but could potentially also expand the possibility to assess novel biomarkers, whose systemic levels may be of questionable value. To this end, the present study evaluated the relation between salivary levels of inflammatory mediators and subclinical cardiovascular disease. To further validate salivary measures, CRP (an established inflammatory biomarker of nonleukocyte origin) and creatinine were evaluated in both plasma and saliva.

MATERIALS AND METHODS

Study population

Individuals were participants in the ERA (Etude de la Rigidité Artérielle) Study, a prospective study of carotid-femoral pulse wave velocity (PWV), initiated in 1992–1993 as previously described [18,19]. All ERA Study participants who participated in the first follow-up visit in 1998–1999 were invited to participate in a second follow-up visit in 2008. In response to this invitation, 271 individuals were examined at the Centre d'Investigations Préventives et Cliniques (the IPC Center, Paris, France), and saliva samples were obtained from 259 of these individuals.

The study protocol was approved by the local ethics committee (Comité d'Ethique du Centre Hospitalier Universitaire de Cochin) and written informed consent was obtained from all study participants.

Clinical investigations

PWV was measured at a constant room temperature of 19–21°C and calculated using Complior (Colson, Garges les Genosse, France) as previously described [18]. Briefly, two pressure waves were recorded transcutaneously at the base of the neck for the right common carotid artery and over the right femoral artery. PWV was determined as the foot-to-foot velocity. Pulse transit time was determined as the average of 10 consecutive beats. The distance travelled by the pulse wave was measured over the body surface as the distance between the two recording sites.

Ultrasound examinations were performed by two trained ultrasonographers using the Aloka SSD-650 (Aloka, Zug, Switzerland), with a transducer frequency of 7.5 MHz. Acquisition, processing and storage of B-mode images were computer-assisted using the M'ATHS software (Metris, Paris, France). The protocol involved scanning of the common carotid arteries, the carotid bifurcations and the origin (first 2 cm) of the internal carotid arteries. At the time of the examination, the near and far walls of these arterial segments were scanned longitudinally and transversally to assess the presence of plaques. The presence of plaques was defined as localized echo-structures encroaching into the vessel lumen for which the distance between the media–adventitia interface and the internal

side of the lesion was 1 mm. For intima–media thickness and lumen diameter measurements, near and far walls of the right and the left common carotid arteries, 2–3 cm proximal to bifurcation, were imaged. In patients with carotid artery plaques, intima–media thickness measurements were realized in plaque-free segments of the common carotid arteries. Details of the methodology used have been previously described [19,20].

Supine blood pressure was measured using a manual sphygmomanometer. After a 10-min rest period, blood pressure was measured three times, and the average of the last two measurements was used for statistical analyses. According to the predefined ERA Study criteria, hypertension was defined as either the use of antihypertensive treatment or SBP greater than 140 mmHg and DBP greater than 90 mmHg. All participants were administered a standardized questionnaire that provided information related to occupation, medical history, past and current medications, and personal habits such as cigarette consumption. Individuals were classified as 'nonsmokers,' 'former smokers' or 'current smokers'. Total plasma cholesterol, high and low-density lipoprotein (HDL and LDL, respectively), cholesterol, triglycerides, creatinine, fasting plasma glucose and hsCRP were also measured.

Sample collection and preparation

Saliva and blood samples were obtained the same day at the time of the second follow-up visit in 2008. Unstimulated whole buccal saliva was collected from individuals as previously described [8]. Briefly, saliva was collected during 3 min after an overnight fast and without prior oral hygiene measures. Saliva samples were immediately frozen at −80°C and stored for less than 3 months before biochemical analysis. At thawing, the collected saliva volume was measured followed by centrifugation of the sample (4000 rpm/10 min/4°C) and prepared into aliquots for each analysis.

Biochemical measurements

Measurements of CRP were performed using enzyme immune assay kits developed for salivary measures from Salimetrics (Suffolk, UK). The levels of prostaglandin E_2 (PGE$_2$) and leukotriene B_4 (LTB$_4$) were determined using enzyme-immunoassays kits from Cayman Chemical Co (Ann Arbor, Michigan, USA). MMP-9 concentrations were measured using Quantikine Enzyme Immuno Assay kit from R&D Systems (Minneapolis, Minnesota, USA). Creatinine was measured by a colorimetric test from Cayman Chemical Co. Lysozyme activity was determined spectrophotometrically by measuring the lysis of a suspension of *Micrococcus lysodeikticus* (0.075%; Sigma) in phosphate/citrate buffer (0.1 mol/l; pH 5.8). Standard curves were constructed by incubation with serial dilutions of egg white lysozyme (20–0.3125 µg/ml; Sigma). Changes in turbidity were monitored at a wavelength of 450 nm.

Statistics

Clinical parameters are expressed as either percentage or mean ± SD. Plasma and salivary measures are expressed as median and ranges. Statistically significant differences were

determined using either a Student's *t*-test or a chi-square test for clinical parameters, and a Mann–Whitney *U*-test for plasma and saliva measures. Correlations between the salivary biomarker concentrations and the clinical parameters were established by Spearman correlation. A one-way analysis of variance (ANOVA) was used for analysis of data according to CRP tertiles. A multiple stepwise linear regression was performed to evaluate salivary biomarkers as predictors of the clinical parameters monitored. A *P* value of less than 0.05 was considered significant. All analyses were performed using the NCSS 2000 statistical software package (NCSS, LLC, Kaysville, Utah, USA).

RESULTS

The baseline characteristics for the 259 individuals in whom salivary measures were performed are reported in Table 1. As expected, several parameters were significantly different between hypertensive and nonhypertensive individuals (Table 1). Individuals with hypertension were significantly older, had higher BMI and waist-to-hip ratio, higher prevalence of metabolic syndrome, higher arterial stiffness, higher IMT and more subclinical atherosclerosis defined as the presence of a carotid plaque [21–23] (Table 1).

The levels of CRP were markedly and highly significantly correlated between saliva and plasma ($r = 0.73$, $P < 0.0001$), as indicated in Fig. 1. Interestingly, the linear equation generated from this correlation had a slope not different from one (slope = 1.08; 95% confidence interval 0.946–1.215). From this equation, there were 4000-fold higher CRP levels in plasma than in saliva, and a plasma CRP of 5 μg/ml corresponded to 1.2 ng/ml CRP in saliva. Thirty-two saliva samples in which the CRP measures yielded a result of 0 ng/ml were excluded in the analysis of correlation between saliva and plasma CRP (Fig. 1). All these individuals, however, had low plasma CRP (median 0.38 μg/ml; 5–95%: 0.15–0.94 μg/ml), and salivary
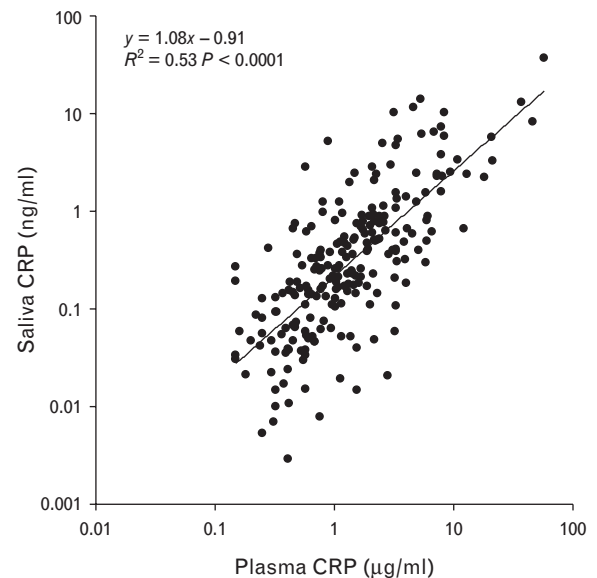

FIGURE 1 Comparison between salivary and plasma C-reactive protein: log regression for the correlation between salivary and plasma C-reactive protein.

CRP concentrations of 0 ng/ml were included in subsequent analyses. The significantly increased levels of CRP in plasma from hypertensive compared with normotensive individuals were also replicated in the saliva (Table 2).

Also, the salivary levels of creatinine were significantly correlated with plasma levels ($r = 0.21$; $P = 0.001$; slope 0.74), and neither plasma nor salivary levels of creatinine were significantly different between normotensive and hypertensive individuals (Table 2). For other salivary markers measured, lysozyme, MMP-9 and PGE$_2$ were significantly higher in hypertensive than in normotensive individuals, whereas salivary LTB$_4$ levels did not significantly differ between these groups (Table 2).

TABLE 1. Characteristics of the study participants

		Hypertension		
	All	**No**	**Yes**	**P**
N	259	178	81	
Age (years)	66 ± 10	61 ± 10	69 ± 9	<0.0001
Women	31%	32%	31%	0.64
SBP (mmHg)	140 ± 19	130 ± 16	148 ± 18	<0.0001
DBP (mmHg)	85 ± 9	83 ± 7	86 ± 10	0.03
HR (beats/min)	70 ± 11	68 ± 10	70 ± 11	0.26
MAP (mmHg)	103 ± 11	98 ± 9	106 ± 10	<0.0001
PP (mmHg)	56 ± 17	47 ± 12	62 ± 16	<0.0001
PWV (m/s)	12.9 ± 3.5	11.4 ± 2.8	13.9 ± 3.6	<0.0001
BMI (kg/m^2)	26 ± 4	25 ± 4	27 ± 4	<0.0001
Metabolic syndrome	22%	9%	31%	0.002
Waist-to-hip ratio	0.94 ± 0.09	0.92 ± 0.08	0.95 ± 0.10	0.01
Smoking (former or current)	51%	57%	45%	0.36
Diabetes	1.5%	0.0%	2.6%	0.10
Carotid ultrasound measures				
Carotid plaque	53%	35%	66%	<0.0001
Mean IMT (mm)	0.75 ± 0.11	0.70 ± 0.10	0.79 ± 0.11	<0.0001
Luminal diameter (mm)	5.90 ± 0.71	5.71 ± 0.57	6.03 ± 0.76	0.0002

Values are expressed as either mean ± SD or percentage. Statistical analyses were performed using either a Student's *t*-test or a chi-square test. IMT, intima–media thickness; MAP, mean arterial pressure; PP, pulse pressure; PWV, pulse wave velocity.

TABLE 2. Plasma and saliva measures

		Hypertension		
	All	**No**	**Yes**	**P**
Plasma measures				
Cholesterol (g/l)	2.2 (1.5–2.8)	2.2 (1.7–2.8)	2.2 (1.4–2.8)	0.45
HDL cholesterol (g/l)	0.49 (0.30–0.76)	0.52 (0.35–0.77)	0.47 (0.28–0.75)	0.006
Tryglycerides (g/l)	0.96 (0.57–2.10)	0.90 (0.57–1.70)	1.08 (0.56–2.19)	0.0003
Glucose (g/l)	0.99 (0.87–1.21)	0.97 (0.86–1.20)	1.01 (0.87–1.21)	0.009
Creatinine (mg/l)	10.0 (8.0–14.0)	10.0 (8.0–13.0)	10.0 (8.0–15.0)	0.23
CRP (μg/ml)	1.05 (0.17–8.18)	0.76 (0.22–5.65)	1.41 (0.17–12.14)	0.0002
Saliva measures				
Volume (ml)	3.0 (1.3–5.5)	3.2 (1.5–5.2)	2.8 (1.2–5.5)	0.07
Lysozyme (μg/ml)	5.0 (2.1–37.9)	4.1 (2.1–22.8)	5.6 (2.1–47.7)	0.008
MMP-9 (pg/ml)	202.2 (9.3–1105.9)	147 (4–1116)	263 (15–1081)	0.002
LTB$_4$ (pg/ml)	300 (58–2401)	299 (43–1357)	304 (78–2629)	0.09
PGE$_2$ (pg/ml)	118.5 (28.0–506.4)	101 (25–492)	127 (32–511)	0.047
Creatinine (mg/l)	0.24 (0.05–0.72)	0.23 (0.05–0.57)	0.26 (0.06–0.90)	0.14
CRP (ng/ml)	0.21 (0.0002–5.20)	0.16 (0.0002–1.56)	0.30 (0.0002–6.21)	0.002

Values are expressed as median and 5–95% confidence interval. Statistical analyses were performed using Mann–Whitney *U*-test. CRP, C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LTB$_4$, leukotriene B$_4$; MMP-9, matrix metalloproteinase 9; PGE$_2$, prostaglandin E$_2$.

The characteristics of the cohort stratified by sex are shown in Supplemental Tables 1 and 2, http://links.lww.com/HJH/A271. Women exhibited significantly lower levels of lysozyme and PGE$_2$ than men, whereas other salivary biomarkers were not significantly different between sexes (Supplemental Table 2, http://links.lww.com/HJH/A271).

In the unadjusted analyses, all salivary biomarkers evaluated, except lysozyme, were significantly correlated

TABLE 3. Spearman correlations (pairwise deletion), unadjusted and adjusted for age and sex

	Creatinine	**Lysozyme**	**MMP-9**	**LTB$_4$**	**PGE$_2$**	**CRP**
Age						
Unadjusted	0.14*	0.07	0.18**	0.18**	0.13*	0.13*
Sex						
Unadjusted	−0.14*	−0.13*	−0.08	0.05	−0.12*	0.10
MAP						
Unadjusted	0.20**	0.10	0.20**	0.05	0.11	0.17**
Adjusted	0.17**	0.09	0.17**	0.01	0.08	0.15*
PP						
Unadjusted	0.17**	0.12	0.21***	0.11	0.17**	0.18**
Adjusted	0.14*	0.12	0.14*	0.00	0.14*	0.13*
PWV						
Unadjusted	0.17**	0.07	0.22***	0.12*	0.14*	0.20**
Adjusted	0.07	−0.01	0.12	0.02	0.05	0.18**
BMI						
Unadjusted	−0.02	0.17**	0.09	0.14*	0.14*	0.19**
Adjusted	−0.06	0.15*	0.07	0.15*	0.11	0.22***
Metabolic syndrome						
Unadjusted	−0.03	0.06	0.07	0.07	0.13*	0.16**
Adjusted	−0.04	0.05	0.06	0.05	0.12*	0.15*
Waist-to-hip ratio						
Unadjusted	0.15*	0.07	0.09	0.08	0.15*	0.07
Adjusted	0.05	−0.04	0.03	0.14*	0.08	0.18**
Carotid plaque						
Unadjusted	0.04	0.00	0.06	0.01	0.00	0.13*
Adjusted	−0.05	−0.06	−0.03	−0.06	−0.09	0.10
IMT						
Unadjusted	0.12	0.08	0.20**	0.20**	0.13*	0.21***
Adjusted	0.04	0.04	0.12	0.13*	0.06	0.17**
Luminal diameter						
Unadjusted	0.21***	0.19**	0.16*	0.06	0.16*	0.10
Adjusted	0.13*	0.13*	0.08	0.02	0.08	0.11

CRP, C-reactive protein; IMT, intima–media thickness; LTB$_4$, leukotriene B$_4$; MAP, mean arterial pressure; MMP-9, matrix metalloproteinase 9; PGE$_2$, prostaglandin E$_2$; PP, pulse pressure; PWV, pulse wave velocity.
*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.

TABLE 4. Stepwise multivariate regressions, for log-transformed salivary biomarkers

Variable	CRP (ng/ml)			PGE$_2$ (pg/ml)			LTB$_4$ (pg/ml)			MMP-9 (pg/ml)			Lysozyme (µg/ml)		
	Regression ± SEM	R2 Adj for all	Prob level	Regression ± SEM	R2 Adj for all	Prob level	Regression ± SEM	R2 Adj for all	Prob level	Regression ± SEM	R2 Adj for all	Prob level	Regression ± SEM	R2 Adj for all	Prob level
Age (10 years)			0.94			0.27			0.29			0.21			0.97
Sex (women)			0.81	−69.2 ± 28.5	2.3%	0.016			0.94			0.21			0.16
PP (1 SD)			0.76	37.3 ± 13.4	3.0%	0.006			0.51			0.12			0.22
Hypertension			0.24			0.93			0.26			0.49	4.36 ± 1.64	2.8%	0.009
Carotid plaque			0.75			0.13			0.88			0.20			0.68
PWV (1 SD)			0.89			0.69	189 ± 75	2.4%	0.013			0.23			0.77
IMT (1 SD)	3.47 ± 1.65	1.7%	0.037			0.81			0.81	72 ± 25	3.2%	0.004			0.24
Metabolic syndrome	0.82 ± 0.45	1.3%	0.068			0.50			0.33			0.63			0.24

CRP, C-reactive protein; IMT, intima–media thickness; LTB$_4$, leukotriene B$_4$; MMP-9, matrix metalloproteinase 9; PGE$_2$, prostaglandin E$_2$; PP, pulse pressure; PWV, pulse wave velocity.

with age (Table 3). The correlation coefficients for salivary biomarkers in the unadjusted as well as in the age and sex-adjusted model are reported in Table 3. Measures of blood pressure and arterial stiffness [mean arterial pressure (MAP), pulse pressure (PP) and PWV] exhibited significant correlations with salivary concentrations of CRP, MMP-9, PGE$_2$ and creatinine as presented in detail in Table 3. In addition, there were significant correlations of echographic carotid artery measurements with CRP, LTB$_4$ and lysozyme (Table 3). Of the anthropometric measures, BMI was significantly correlated with LTB$_4$, lysozyme and CRP, and the waist-to-hip ratio was significantly correlated with LTB$_4$ and CRP (Table 3).

As a next step, a stepwise multiple regression analysis was performed to determine which of the parameters included in the univariate analysis could independently predict the salivary levels of each mediator. In this analysis, CRP and MMP-9 were associated with IMT, whereas LTB$_4$ and PGE$_2$ were associated with measures of arterial stiffness, and lysozyme with hypertension (Table 4). Furthermore, a stepwise multiple regression analysis was performed to evaluate several salivary biomarkers against the individual clinical parameters. In these analyses, CRP remained significantly correlated with PWV, IMT and metabolic syndrome, and PGE$_2$ remained significantly correlated with PP (Supplemental Table 3, http://links.lww.com/HJH/A271).

In order to further characterize the relation of salivary CRP with arterial stiffness, metabolic syndrome and subclinical atherosclerosis, salivary CRP was divided into tertiles (Supplemental Fig 1, http://links.lww.com/HJH/A271). Increasing tertiles of salivary CRP were significantly associated with increasing PP and PWV (Fig. 2a,b) with increased prevalence of metabolic syndrome (Fig. 2c) and carotid plaques (Fig. 2d), and with increasing IMT (Fig. 2e).

DISCUSSION

The results of the present study point to inflammatory markers, which are present and measurable in saliva, as potential predictors of cardiovascular disease. In particular, salivary CRP predicted measures of arterial stiffness, IMT, subclinical atherosclerosis and metabolic syndrome, providing a first indication that salivary testing may serve as a noninvasive and easy-to-use alternative in the assessment of subclinical cardiovascular disease and cardiovascular risk. In addition, the results of the present study indicate that salivary measures of LTB$_4$, PGE$_2$, MMP-9 and lysozyme were associated with vascular diseases.

Systemic low-grade inflammation measured through circulating levels of CRP is a well established biomarker for cardiovascular risk. In addition, plasma CRP levels have been associated with arterial stiffness [24], IMT [25], subclinical carotid atherosclerosis [26] and metabolic syndrome [27]. However, the predictive value of salivary CRP in the latter contexts has remained largely unexplored. A novel finding of the present study is the associations of salivary CRP with arterial stiffness, IMT and subclinical carotid atherosclerosis, as well as with metabolic syndrome, BMI and waist-to-hip ratio.
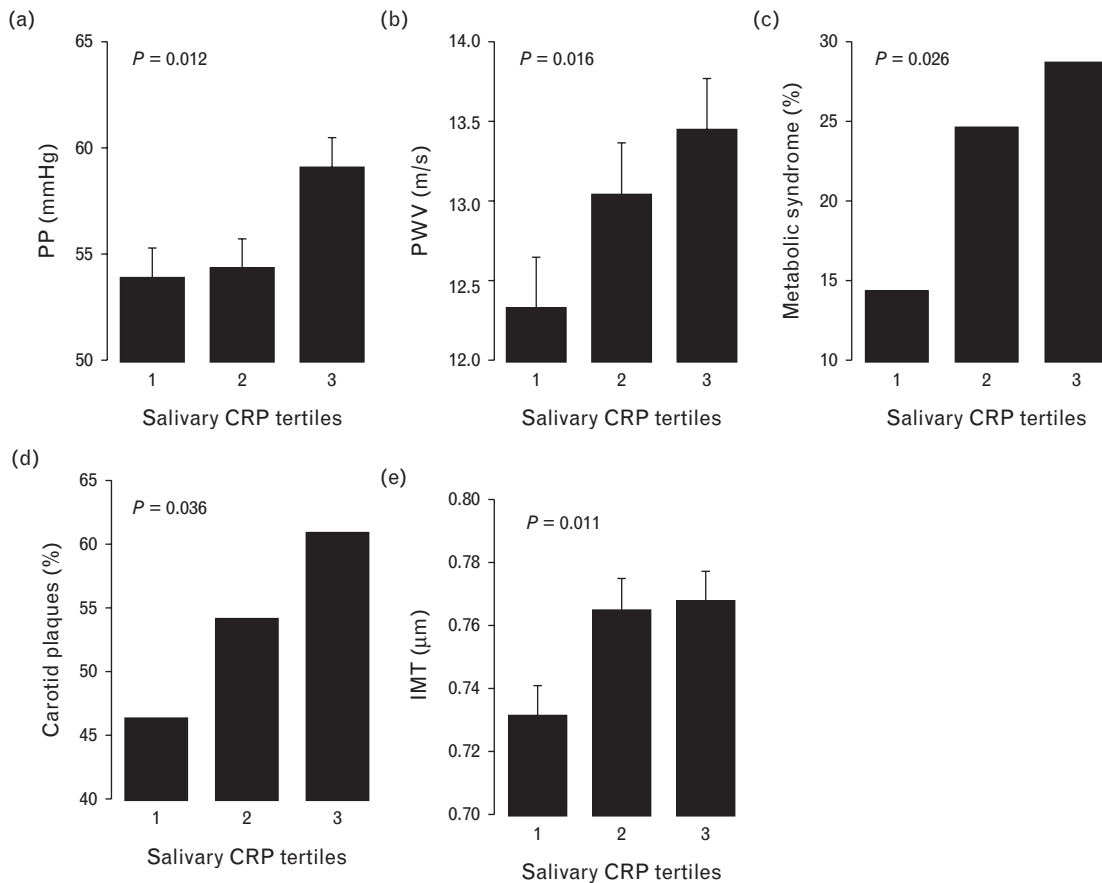
FIGURE 2 Clinical parameters according to age and sex-adjusted salivary C-reactive protein (CRP) tertiles. Increasing tertiles of salivary CRP were significantly associated with increasing pulse pressure (PP, a); pulse wave velocity (PWV, b), metabolic syndrome (c), carotid plaques (d) and intima–media thickness (IMT, e). *P* indicates ANOVA trend.

Previous studies with relatively small numbers of healthy individuals have generated contradictory results for the correlation between plasma and salivary levels of CRP. Whereas one study of 69 medical students revealed no significant association [28], another study of 61 healthy adult volunteers reported a moderate-to-strong association between CRP measured in saliva and in plasma [10]. In the present larger study sample exhibiting representative variations in terms of plasma CRP, there was a strong and highly significant association between plasma and salivary CRP. Importantly, the slope of the correlation did not differ from unity, hence supporting the notion that salivary CRP levels directly reflect systemic levels and may be used to assess systemic inflammation.

In addition to CRP, salivary concentrations of MMP-9 were associated with both PP and PWV in the present study. The MMP family of endopeptidases is involved in arterial wall extracellular matrix degradation [3] and MMPs are key mediators of the vascular remodelling associated with arterial stiffness [29]. Whereas studies of circulating MMP-9 as a biomarker of cardiovascular disease have generated contradictory results, salivary MMP-9 activity or concentrations have previously been associated with age [8] and acute myocardial infarction [13].

The lipid inflammatory mediators leukotrienes were initially associated with asthma [30], and have more recently been implicated also in cardiovascular diseases, such as atherosclerosis [14] and aortic stenosis [31]. A recent study [32] has in addition demonstrated beneficial effects of the antiasthmatic leukotriene receptor antagonist montelukast on cardiovascular outcomes. However, the use of leukotrienes as biomarkers is limited due to undetectable plasma levels and an extensive ex-vivo formation during serum preparation [5,6]. In contrast, oral samples have been shown to contain readily measurable LTB_4 concentrations [8,33], which are inhibited after oral treatment with an antiasthmatic leukotriene synthesis inhibitor [11]. The present study adds to those findings by demonstrating an association of salivary LTB_4 with IMT and anthropometric measures, such as BMI and the waist-to-hip ratio. The association of the leukotriene pathway with BMI was first reported in a study of patients with obstructive sleep apnoea [34], and experimental studies have supported a role of LTB_4 in adipose tissue inflammation [35]. Nevertheless, the multivariate analysis in the present study revealed PWV as the independent predictor of salivary-LTB_4, which is consistent with the direct effects of LTB_4 on the vascular wall [36,37].

In addition, salivary levels of another lipid-derived inflammatory and vasoactive mediator, PGE_2, were associated with PP in the present study and remained significant in multivariate analyses taking into account both other

clinical variables and other salivary biomarkers. Taken together, salivary levels of lipid-derived inflammatory mediators, such as prostaglandins and leukotrienes, may reflect the increased activation of these inflammatory pathways in arterial stiffness.

The salivary biomarkers assessed in the present study represent bioactive molecules of different structure and origin and with different actions. Concentrations of CRP and creatinine measured in the saliva reflected systemic levels, as suggested by the present and previous [10,38] studies. In contrast, the high salivary LTB$_4$ concentrations are in contrast to the previously established low circulating levels of LTB$_4$ [5,6] and permitted the association of this lipid mediator of inflammation with arterial stiffness.

The present study represents the first assessment of a panel of different salivary biomarkers, which allowed the association with cardiovascular disease and risk factors after noninvasive sampling. However, several limitations should be acknowledged. As the follow-up of these individuals has not yet been performed, the prognostic value of the measured salivary markers for cardiovascular outcome could not be evaluated. In addition, all salivary biomarkers evaluated, except lysozyme, were significantly correlated with age, which corroborates our previous findings in healthy individuals [8] and confirms that age is a significant confounder in studies of salivary biomarkers. In order to address this limitation, both unadjusted and adjusted analyses were performed for age and sex. Currently, the lack of comparable plasma levels for each mediator prevents a general conclusion concerning the potential differential predictive value for saliva and the systemic biomarkers measured. However, investigations involving this perspective may provide further evidence in support of the initial observations provided in the present study.

In summary, the present study identified inflammatory markers in the saliva, with the potential to predict cardiovascular disease and risk factors. The inflammatory states associated with atherosclerosis, arterial stiffness and metabolic syndrome were all associated with increased salivary CRP levels, which in turn correlated to CRP levels in plasma. Salivary measures of biomarkers correlating with circulating levels could represent an easy-to-use, noninvasive test to evaluate the systemic inflammation associated with cardiovascular risk. In addition, other markers or mediators of inflammatory, for which levels may be difficult to assess in blood samples, were also associated with arterial stiffness, atherosclerosis and metabolic disease in the present study, supporting that salivary testing may offer an additional value in cardiovascular risk evaluation. In conclusion, the results of the present study indicate that saliva may represent a useful and noninvasive diagnostic tool for cardiovascular disease.

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Conflicts of interest
There are no conflicts of interest.

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Reviewers' Summary Evaluations

Reviewer 1

The principal strength of this paper is that it opens the door to a new approach to assessing relationships between inflammation and cardiovascular disease. If some inflammatory mediators such as leukotrienes can be assessed in saliva but not in plasma, this may be important.

A weakness was the assessment of plaque only as presence/absence; it would have been preferable to have measured plaque burden. Continuous variables are approximately three times more powerful than categorical variables, and total plaque area can be measured by anyone who can measure IMT, without additional equipment.

Reviewer 2

This is a well presented and well designed study aiming to determine whether saliva could be a reliable mean to assess the levels of biomarkers of cardiovascular diseases. Using plasma and saliva samples from a population of 250 subjects with and without hypertension, the authors established a perfect correlation between plasma and salivary levels of the pro-inflammatory marker CRP. A similar correlation was obtained with creatinine.