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Association of salivary lysozyme and C-reactive protein with metabolic syndrome

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Abstract

Introduction: Salivary lysozyme (SLZ) is a proteolytic enzyme secreted by oral leucocytes and contains a domain that has an affinity to advanced glycation end products (AGE). Thus, we hypothesized that SLZ would be associated with metabolic syndrome (metS), a pro-inflammatory state.

Methods: Utilizing cross-sectional data from 250 coronary artery disease (CAD) and 250 non-CAD patients, the association of SLZ with metS was tested by logistic regression analyses controlling for age, sex, smoking, total cholesterol and C-reactive protein (CRP) levels. The analyses were stratified by CAD status to control for the possible effects of CAD.

Results: MetS was found in 122 persons. The adjusted odds ratio (OR) for metS associated with the highest quartile of SLZ was 1.95 with 95% confidence interval (CI) 1.20–3.12, p-value = 0.007, compared with the lower three quartiles combined. Among the 40 subjects with metS but without CAD, the OR was 1.63 (CI: 0.64–4.15, $p = 0.31$), whereas in the CAD group, SLZ was significantly associated with metS [OR = 1.96 (1.09–3.52), $p = 0.02$]. In both subgroups, CRP was not significantly associated with metS.

Conclusion: SLZ was significantly associated with metS (OR = 1.95) independent of CRP level. Future longitudinal research is warranted.

Abstract of this study has been presented at The 3rd International Metabolic Syndrome Congress in Nice, France in April 2009.

Key words: atherosclerosis; C-reactive protein; inflammation; metabolic syndrome; salivary lysozyme

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Conflict of interest and source of funding statement

Declaration of competing interests: Nothing to declare.

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Salivary lysozyme (SLZ) is a proteolytic enzyme expressed by neutrophil leucocytes in response to infection (Klempner & Malech 1998) and is capable of cleaving the glycosidic linkage on the bacterial cell wall resulting in an anti-infective action. It also contains a domain that has a strong affinity for advanced glycation end products (AGEs) (Vlassara et al. 2002, Karima et al. 2005) and has been used for the removal of AGEs in diabetic patients (Zheng et al. 2001).

Although oral infections, including periodontitis, have been presumed to contribute to systemic inflammation, hyperglycaemia is also known to be a powerful source of inflammatory response (Liu & Willett 2002, Pickup 2004). Because SLZ is closely related to both infection and impaired glucose metabolism, we postulated that SLZ might provide a measure of pro-inflammatory processes involved in metabolic syndrome (metS) and diabetes (Janket et al. 2008a). Previous studies evaluating the relationship of C-reactive protein (CRP) and metS (Ridker et al. 2003, Conen et al. 2009) were conducted in apparently healthy women and did not adjust for the multitude of other factors affecting CRP levels (Kushner et al. 2006). Although Drs. Ridker and Silvertown acknowledged the close relationship of oral infection/inflammation systemic inflammation, (Ridker & Silvertown 2008) the three-way relationship assessment (oral inflammation, systemic inflammation and vascular inflammation) has never been conducted. The objective of this study was to test the hypothesis that ''SLZ is associated with metS, independent of

systemic inflammation'' in a population with high prevalence of coronary artery disease (CAD).

The present cohort of the Kuopio Oral Health and Heart (KOHH) Study consists of 250 (50%) of subjects with diagnosed CAD and 250 (50%) without CAD. The aims of this study were: (1) to evaluate whether SLZ is associated with metS; (2) to assess if adjusting systemic inflammation changes the relationship of SLZ to metS; and (3) finally, if (2) is proven true, then, to determine which inflammatory factor (local or systemic) has a stronger association with metS.

Table 1a. Frequencies of metabolic syndrome components according to NCEP criteria

Metabolic syndrome component	Frequency
Trig, obese and low_HDL	36
Trig, obese and hypertension	23
Trig, low_HDL and hypertension	67
Hypertension, obese and low_HDL	19
Both metS and DM in the same person	23
Total metabolic syndrome (metS)	122

Trig, high triglyceride according to NCEP criterion ($\geqslant 1.69$ mmol/l); obese, body mass index $\geqslant 28$; HDL, high-density lipoprotein cholesterol (< 1.04 mmol/l in males, 1.29 mmol/l in females); metS, metabolic syndrome; DM, diabetes mellitus.

Table 1b. Salivary lysozyme levels related to each component of metabolic syndrome

	Mean SLZ (SD) (μg/ml)		*p*-value
	criterion absent	criterion present	
NCEP_obesity	31.5 (35.4)	33.0 (37.1)	0.76
NCEP_triglyceride	28.4 (32.7)	34.9 (37.1)	0.04
NCEP_HDL	27.6 (31.8)	37.2 (38.3)	0.001
Hypertension	28.3 (32.8)	39.4 (40.3)	0.0008
Diabetes	31.8 (35.9)	35.6 (36.0)	0.10

NCEP, national cholesterol education program; HDL, high-density lipoprotein.

Materials and methods

Ethical and human subjects' protection

This is a secondary data analysis of the Kuopio Oral Health and Heart (KOHH) Study. The Joint Ethical Committee of the Kuopio University Hospital and the University of Kuopio approved the study protocol. All participants signed a written informed consent and the KOHH Study adhered to the guidelines set forth by the Declaration of Helsinki and the Belmont Accord to assure the safety of human research subjects.

Study population

Kuopio Oral Health and Heart (KOHH) study was initiated in 1996 to investigate the relationship between oral health and CAD in Kuopio, Finland. We recruited 250 consecutive cardiac patients at Kuopio University Hospital who were referred for coronary angiography and confirmed as having CAD determined by the presence of at least 50% stenosis in one of the coronary arteries. Potential subjects were excluded if they took antibiotics during the previous 30 days or had chronic infection other than dental disease. Also recruited were 250 age- and gender-matched controls who were admitted to the general surgery or otorhinolaryngology (ORL) departments at the same hospital for an elective surgery. They were considered as not having heart disease based on the medical history and pre-admission electrocardiogram (ECG) and were the representative of the population of the same catchment area where the cases arose. The same exclusion and inclusion criteria were applied to non-cardiac patients. Additional exclusion criteria were: (1) those who needed emergency coronary bypass surgery or valvular replacement surgery; (2) those whose disease status was so grave that a dental examination or dental X-ray could not be performed safely; and (3) those who received antibiotic prophylaxis before periodontal probing. Further details regarding this cohort have been published elsewhere (Janket et al. 2004, 2006, Qvarnstrom et al. 2008).

Main exposure

Oral examinations were conducted by a single examiner (M. Q.) and the results were published previously (Meurman et al. 2003a, b, Janket et al. 2004). To avoid diurnal fluctuation, saliva samples were collected from the subjects between 07:00 and 09:00 hours. Subjects had been advised not to eat or smoke 1 h before saliva collection. Using the free flow method, saliva was collected into a 10 ml test tube for 5 min. after initial swallowing. Whenever possible, fresh saliva was centrifuged (10 min., 12,000 *g*) and analysed immediately. SLZ was quantified at the Kuopio University research laboratory by the modified lysoplate method utilizing *Micrococcus lysodeikticus* (Sigma Chemical Co., St. Louis, MO, USA) and human milk lysozyme (Sigma Chemical Co.) and bovine serum albumin (Sigma Chemical Co.) as standards according to the methods used previously(Rudney & Smith 1985). The reported coefficient of variation (CV) of lysozyme was between 4.2% and10% (Sato et al. 2001, Desai et al. 2006).

Other covariates assessed

Age in years, smoking in three categories; never-smokers, current smokers and past smokers were assessed. Body mass index (BMI) was calculated by weight in kg divided by squared height in metres. CRP was measured by high-sensitivity immuno-turbidometry assay utilizing the Hitachi 717 analyzer (Roche Chemical Analyzer, Basel, Switzerland). The reported CV for CRP assay was between 8.1% and 11.4% (Sung et al. 2002, Aziz et al. 2003). All blood samples were analysed immediately in the hospital laboratory. The analyses were performed in batches including both cases and controls to evenly distribute any potential environmental changes and measurement variability. Total cholesterol (TC), triglyceride (TG) and high-density lipoprotein cholesterol (HDL) were measured by the automated enzymatic technique. Hypertension (HTN) and diabetes were ascertained by medical record review by one of the authors (M. Q.). Subjects were categorized as hypertensive or diabetic if their medical records documented these diagnoses or their treatments.

Ascertainment of metS

From the laboratory measures, if a person satisfied three out of five National

Table 2. General characteristics of the cohort

	Without CAD ($N = 250$)			With CAD ($N = 250$)		
	without metS $N = 212$	with metS $N = 38$	*p*-value	without metS $N = 152$	with metS $N = 81$	*p*-value
Mean age (SD)	58.9 (9.8)	61.0 (9.2)	0.29	59.6 (9.3)	60.1 (8.8)	0.49
Sex, *N* (%)						
Men	137 (65.2%)	22 (55.0%)	0.46	97 (63.8%)	50 (61.7%)	0.78
Women	75 (34.8%)	16 (45.0%)		55 (36.2%)	31 (38.3%)	
Body mass index (BMI)						
Mean (SD)	25.4 (3.4)	28.9 (4.1)	<0.0001	23.3 (2.8)	25.6 (3.6)	<0.0001
Salivary lysozyme (mg/l)*						
Median	11.5	18.8	0.08	26.9	36.2	0.09
Inter-quartile range	(4.94–30.7)	(8.3–36.2)		(10.1–54.8)	(12.0–56.7)	
Proportion with >12 years of education, *N* (%)	85 (40.5%)	13 (32.5%)	0.59	37 (24.3%)	17 (21.0%)	0.62
Edentulism (*N*, %)	27 (12.9%)	9 (22.5%)	0.08	47 (30.9%)	35 (43.2%)	0.06
Total cholesterol (mmol/l)						
Mean (SD)	5.17 (0.97)	5.77 (1.03)	0.03	5.60 (1.01)	5.70 (1.03)	0.51
Triglyceride (mmol/l)						
Mean (SD)	1.67 (0.96)	2.21 (0.84)	0.0003	1.81 (0.89)	2.69 (1.16)	<0.0001
HDL cholesterol (mmol/l)						
Mean (SD)	1.34 (0.32)	0.95 (0.27)	<0.0001	1.18 (0.30)	1.01 (0.24)	<0.0001
	Without CAD ($N = 250$)			With CAD ($N = 233$)		
	without metS $N = 212$	with metS $N = 38$	*p*-value	without metS $N = 152$	with metS $N = 81$	*p*-value
Hypertension, *N* (%)	29 (13.9%)	23 (57.5%)	0.0001	50 (32.9%)	66 (81.5%)	<0.0001
CRP (mg/l)†						
Median (inter-quartile range)	4.0 (2.0–5.0)	4.0 (2.0–5.0)	0.57	9.0 (8.0–20.0)	10.0 (9.0–21)	0.54
Smoking, *N* (%)						
Never smoker	172 (81.9%)	31 (77.5%)	0.67	80 (52.6%)	41 (50.6%)	0.63
Current smoker	19 (9.1 %)	5 (12.5%)		18 (11.8%)	7 (8.6%)	
Past smoker	19 (9.1%)	4 (10.0%)		54 (35.5%)	33 (40.7%)	

*SLZ and †CRP were compared using the non-parametric, median test due non-normal distribution.
Owing to missing values in dependent, explanatory and confounding variables, the actual analyses included <500 observations.

Cholesterol Education Program (NCEP) criteria for metS (Grundy et al. 2004), s/he was deemed to have metS.

NCEP criteria include (Grundy et al. 2004):

1. Central obesity waist circumference >102 cm (male) or 88 cm (female).
2. Fasting blood glucose >110 mg/dl (6.1 mmol/l) or having diabetes.
3. Systolic blood pressure ⩾130 mm Hg and diastolic blood pressure ⩾85 mmHg.
4. TG ⩾150 mg/dL (1.69 mmol/l).
5. HDL <40 mg/dL (1.04 mmol/l in male) <50 mg/dL (1.29 mmol/l in female).

We modified NCEP criteria by substituting waist circumference with BMI⩾28, a more stringent criterion than the International Diabetes Federation (IDF) criteria following the precedence (Ridker et al. 2003).

Statistical analysis

Using Statistical Analysis System (SAS) version 9.1, the basic characteristics such as mean age, sex, smoking status, body mass index, number of teeth, SLZ and cholesterol levels were compared between those with and without metS in univariate analyses. We used *t*-tests, χ^2-tests or Wilcoxon's non-parametric tests depending on the normality of the variable distributions. In multivariate analyses, the relationship between the probability of having metS who belong in the top 25% of SLZ levels was examined utilizing logistic regression methods. All *p*-values were calculated as two-tailed, and all confidence intervals (CIs) were computed at the 95% level. Initially, we compared the odds of having metS in quartiles of SLZ levels. Because there appeared to be a threshold at the top quartile, we dichotomized the data at the top 25% to better capture this trend. To control for the effect of systemic inflammation, we dichotomized CRP at 2 or 3 mg/l as they were considered a marker for moderate risk for cardiovascular disease (CVD; Yeh & Willerson 2003). However, in this cohort with high vascular inflammation, these cutoffs did not generate a valid maximum likelihood estimates suggesting that both compared groups have CRP levels above these cutoffs. Therefore, we used log-transformed CRP. To compare the robustness in the relationship to metS, we fitted three separate models: one SLZ as the predictor, CRP as the predictor and the third with both SLZ and CRP as the predictors.

Results

There were 122 subjects with metS or diabetes. High TG and low HDL levels were prominent features in the patients with metS. The frequencies of each component of metS subgroups are presented in Table 1a. SLZ levels appear to be highly

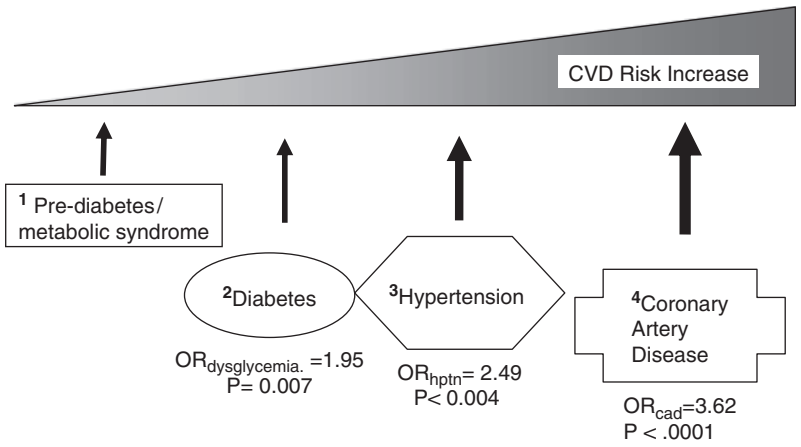

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Fig. 1. Cardiovascular disease (CVD) risk increase associated with salivary lysozyme.

Table 3. Multivariate models to assess the association of salivary lysozyme and metabolic syndrome

	Main predictors	Odds ratio (OR) (95% confidence interval)	*p*-value
Model 1	Lysozyme quartiles (mg/l)		
Whole cohort ($N = 500$)	Q1 (0–7.70)	1.00 (reference)	
	Q2 (7.71–20.43)	2.12 (1.12–4.03)	0.007**
	Q3 (20.44–44.56)	1.49 (0.77–2.89)	(For trend)
	Q4 (>44.56)	2.75 (1.45–5.20)	
	Dichotomy of SLZ		
Model 2	Lower 3 quartiles of SLZ	1.00 (reference)	
Whole cohort ($N = 500$)	Fourth quartile of SLZ	1.95 (1.20–3.12)	0.007**

**Significant at the α-level of 0.05.
All models adjusted for age, sex, smoking (in three categories), education, total cholesterol (log-transformed) and CRP (log-transformed).

correlated to cholesterol subtypes and to a lesser degree with diabetes. However, SLZ did not appear to be associated with BMI (Table 1b), which was significantly associated with periodontitis in another study (Offenbacher et al. 2009). In Table 2, the baseline characteristics, age and sex, were not statistically different between the groups. CRP levels appeared to differ according to the CAD status rather than to the metS status. In other words, CRP levels were distinctly higher in the CAD group than in the non-CAD group regardless of metS status. On the contrary, SLZ displayed a dose–response relationship consistent with the hypothesis with the increasing atherogenicity beginning from metS/diabetes, HTN to CAD. This hypothesis is illustrated in Fig. 1.

In a multivariate analysis where age, sex, education, TC and logCRP were controlled for, the odds ratios (ORs) for each quartile of SLZ were 1.00, 2.12, 1.49 and 2.75 (p-value for trend = 0.007). It appeared that the odds for having metS spiked at the top quartile. To capture this trend better, we dichotomized SLZ at the top 25% and lower three quartiles combined. Because adjusting smoking by categorical variable or linear trend did not materially change the main parameter estimate, we used smoking as a linear trend to save the degree of freedom. Adjusting TC did not change the parameter estimate of SLZ (1.93 *versus* 1.92) and for the reason of parsimony, we removed it from the model in some analyses. As shown in Table 3, being in the highest quartile of SLZ was associated with a nearly twofold increase in the OR of having metS with OR = 1.95, (95% CI: 1.20–3.12), $p = 0.007$.

To control for the effect modification by CAD, we conducted stratified analyses. Among 250 persons without CAD, both SLZ and CRP were not significantly associated with metS. Considering the small number of individual with metS (N = 38), non-significant p-values were expected. Among 250 persons with CAD, high SLZ was significantly associated with having metS ($N = 81$), OR = 1.96 (1.09–3.52), p-value = 0.02. However, CRP was not significantly associated with metS. These results are presented in Table 4.

To make a comparison, we fitted either top 25% of SLZ or the same of CRP or both in the model (models 5, 6 and 7) as given in Table 5. The 4th quartile of CRP was not significantly associated with metS while the 4th quartile of SLZ was. When SLZ was added to the CRP model, CRP became significant suggesting contribution of SLZ to CRP. It should be noted, however, that adding CRP in the model did not change the point estimate or the p-value of SLZ.

Discussion

In this cohort with a high prevalence of inflammatory vascular disease, SLZ was significantly associated with metS, independent of CRP. This finding is consistent with our previous observation that approximately 26% of CRP can be explained by oral infection (Janket et al. 2010) and further confirms that SLZ has been significantly associated with every step in the continuum of the inflammatory CVD risk, called the ''common soil'' of atherogenesis (Stern 1995) as illustrated in Fig. 1. An increasing level of SLZ coincided with an increasing intensity of postulated atherogenicity (in Fig. 1 and Table 1a) while CRP showed distinct threshold effect (Table 1a). As shown in Table 2, SLZ was nearly significantly associated with metS in both subgroups ($p = \sim 0.08$) with or without CAD. On the contrary, CRP was not significantly associated with metS in either group ($p = \sim 0.54$). These subtle differences may not be obvious in large cohorts because a significant p-value is a function of sample size (Gardner & Altman 1986, Panagiotakos 2008).

Table 4. Multivariate adjusted association of SLZ with metabolic syndrome stratified by CAD

	Main predictors	Odds ratio (OR) (95% confidence interval)	*p*-value
Model 3			
Subgroup without CAD ($N = 250$)	Lower three quartiles of SLZ	1.00 (reference)	
	Fourth quartile of SLZ	1.63 (0.64–4.15)	0.31
Model 4			
Subgroup with CAD ($N = 250$)	Lower three quartiles of SLZ	1.00 (reference)	
	Fourth quartile of SLZ	1.97 (1.09–3.56)	0.02

Model 3 is adjusted for age, sex, smoking (in three categories), education and log-transformed CRP. Model 4 is adjusted for age, sex, smoking (in three categories), education, total cholesterol (log-transformed) and CRP (log-transformed).
CAD, coronary artery disease; SLZ, salivary lysozyme; CRP, C-reactive protein.

Table 5. Comparison of explanatory ability of SLZ and CRP

	Main predictor (s)	Odds ratio (OR) (95% confidence interval)	*p*-value
Model 5			
Subgroup with CAD ($N = 250$)	Fourth quartile of SLZ level ($\geqslant 44.6$ mg/l)	1.81 (1.02–3.21)	
	Reference	1.00	0.04***
Model 6			
Subgroup with CAD ($N = 250$)	Fourth quartile of CRP level (> 10 mg/l)	1.68 (0.96–2.93)	0.07 (NS)
	Reference	1.00	
Model 7			
Subgroup with CAD ($N = 250$)	Both SLZ and CRP In the same model		
	Fourth quartile of SLZ	2.00 (1.10–3.61)	0.02***
	Fourth quartile of CRP	1.84 (1.03–3.26)	0.04***

Models 5, 6 and 7 were adjusted for age, sex, smoking (in three categories), education, total cholesterol (log-transformed).
***Significant at the α-level of 0.05.
CRP, C-reactive protein; CAD, coronary artery disease; SLZ, salivary lysozyme; NS, not significant.

After the JUPITER result publication, dispensation of rosuvastatin has been approved by the US Food and Drug Administration (FDA) in individuals *without* clinically evident coronary heart disease but with an increased risk of CVD based on age (men $\geqslant 50$ and women $\geqslant 60$), CRP $\geqslant 2$ mg/l, and the presence of at least one additional CVD risk factor (FDA 2010). The median CRP levels and mean age of our cohort are similar to those of the JUPITER cohort (Ridker et al. 2009). CRP alone was not a significant predictor of metS in the group with CAD in the current study. Only when SLZ was added to the model, CRP became significant. This suggests that SLZ and CRP are confounders (Table 5). As reported in the previous publication, CRP appeared to be more strongly associated with the BMI and diabetes components (Ridker et al. 2003) while SLZ was more closely associated with the HTN, TG and HDL cholesterol components of metS (Table 1b in the current study). This is in agreement with the latest report that HTN and cholesterol levels were more powerful markers of atherosclerosis than dysglycaemia (Sarwar et al. 2010) and supports our hypothesis illustrating the echelon of dysglycaemia and HTN on the continuum of the atherosclerosis pathway (Fig. 1). Furthermore, a newly published report demonstrated that arterial lysozyme was a more powerful predictor of atherosclerosis than CRP and indirectly supports our observation presented herein (Abdul-Salam et al. 2010). Considering our results together with the fact that high proportions of current smokers (16%) and of individuals with metS (41%) in the JUPITER cohort (Ridker et al. 2008), we find that remediating the underlying factors contributing to elevated CRP such as oral inflammation, smoking or metS may be a more prudent approach than statin administration.

Although lysozymes are expressed in various mucosal surfaces through out the human body, e.g., in the ophthalmic, respiratory and digestive mucosae, its local production is not affected by the expression in other locations (Wagner & Wagnerova 1989). The variability of SLZ and CRP assays being similar (at approximately 10%), the differences we observed in SLZ and CRP in relation to metS could be measurement errors. It, however, could also be due to a non-specific nature of CRP, which has been noted by several researchers (Levinson & Elin 2002, Kushner et al. 2006).

Poor oral health is a restricting factor for a healthy diet. Edentulous persons have been shown to have lower intake of fruits and vegetables (Nowjack-Raymer & Sheiham 2003), which is a significant predictor for future diabetes (Liu et al. 2004). The mastication difficulties force the edentulous persons to favour soft, easily chewable carbohydrates with high glycaemic index (Liu & Willett 2002). This impact of oral health on diet has been largely ignored by nutrition research and is often attributed to dietary effects. Nonetheless, reverse causation cannot be completely ruled out due to our cross-sectional study design.

A major strength of the present study is that we explicitly adjusted for CRP to control for the systemic inflammation. Many reports involving CRP have not adjusted for the multitude of other potential contributors to systemic inflammation (Kushner et al. 2006) including oral infection.

Several limitations are also noted. The cross-sectional study design is one of them. Therefore, our results may not be interpreted in a causal context. However, the significant association of SLZ with every early stage on the pathway to CAD suggests that the relationship of SLZ and atherosclerosis may be longitudinal (Fig. 1). However, whether the relationship between SLZ and metS is causal or not must be determined from future studies. Also it should be noted that not all significant risk factors identified in longitudinal studies have causal relationship (Janket et al. 2008b, Wang 2008).

Another limitation is that we did not have information regarding alcohol consumption or fasting glucose levels. Thus, some participants in non-metS group actually might have metS, and our results might have under-estimated the true association of SLZ and metS.

In summary, our results suggest an association of SLZ with metS above and beyond CRP. Future longitudinal studies may establish this novel marker as a risk factor for metS.

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Clinical Relevance

Scientific rationale for the study: Unlike previous studies, we examined the relationship between oral inflammation measured by SLZ and metS in a population with high prevalence of inflammatory vascular disease.

Principal findings: SLZ was significantly associated with metS controlling for CRP and other risk factors.

Practical implications: The positive results of the JUPITER trial brought greater public attention to CRP, a non-specific inflammatory marker. Additionally, US FDA approved the administration of rosuvastatin based on CRP levels along with one additional risk factor in men aged 50 and older, and women aged 60 and older. However, considering the pleiotropic nature of statins and the non-specific characteristic of CRP, rosuvastatin dispensation based on CRP level and one unspecified risk factor leaves many unanswered questions. The present study is consistent with our previous observation that oral infection explains approximately 26% of CRP and reducing oral inflammation before statin administration may be prudent.