

# Salivary IgA to MAA-LDL and oral pathogens are linked to coronary disease

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## Abstract

A large body of literature has established the link between periodontal disease and cardiovascular disease. Oxidized low-density lipoproteins (OxLDL) have a crucial role in atherosclerosis progression through initiation of immunological response. Monoclonal IgM antibodies to malondialdehyde-modified LDL (MDA-LDL) and to malondialdehyde acetaldehyde-modified LDL (MAA-LDL) have been shown to cross-react with the key virulence factors of periodontal pathogens *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*. We have previously shown that salivary IgA antibodies to MAA-LDL cross-react with *P. gingivalis* in healthy humans. In this study, we aim to assess whether oral mucosal immune response represented by salivary IgA to MAA-LDL and oral pathogens is associated with coronary artery disease (CAD). Also, the molecular mimicry through antibody cross-reaction between salivary IgA to MAA-LDL and oral pathogens was evaluated. The study subjects consisted of 451 patients who underwent a coronary angiography with no-CAD (n=133), stable-CAD (n=169) and acute coronary syndrome (ACS, n=149). Elevated salivary IgA antibody levels to MAA-LDL, Rgp44 (gingipain A hemagglutinin domain of *P. gingivalis*), and Aa-HSP60 (heat shock protein 60 of *A. actinomycetemcomitans*) were discovered in stable-CAD and ACS patients when compared to no-CAD patients. In a multinomial regression model adjusted for known cardiovascular risk factors, stable-CAD and ACS were associated with IgA to MAA-LDL (p=0.016, p=0.043), Rgp44 (p=0.012, p=0.004), Aa-HSP60 (p=0.032, p=0.030), *Tannerella forsythia* (p=0.002, p=0.004), *P. endodontalis* (p=0.016, p=0.020), *Prevotella intermedia* (p=0.038, p=0.005), and with total IgA antibody concentration (p=0.002, p=0.016). Salivary IgA to MAA-LDL showed cross-reactivity with the oral pathogens tested in the study patients. The study highlights an association between salivary IgA to MAA-LDL and atherosclerosis. However, whether salivary IgA to MAA-LDL and the related oral humoral responses play a causal role in the development in the coronary artery disease should be elucidated in future.

## Introduction

Oral mucosal immunity plays a crucial role in maintaining homeostasis in oral microbiome communities, and one way of achieving this is by secreting immunoglobulins (Brandtzaeg 2013). Dysbiosis in the oral microbiome leads to the development of diseases such as periodontitis (Kinane et al. 2017). The association of atherosclerosis with oral pathogens and periodontal disease has been established (Beck et al. 1996). Atherosclerosis is a chronic inflammatory disease; the disease starts by accumulation of low-density lipoproteins (LDL) beneath the intima of the artery wall (Gistera and Hansson 2017). Subsequently, retained lipoproteins go through oxidative modification leading to the formation of oxidized LDL (OxLDL) (Gistera and Hansson 2017). OxLDL plays a key role in the inflammation of arterial wall and atherogenesis. Oxidized LDL are also present in saliva fluid (De Giuseppe et al. 2015) and recently, Bright *et al.* reported that malondialdehyde acetaldehyde (MAA) adducts are increased in gingivitis and periodontitis lesions (Bright et al. 2018).

Oxidized epitopes include oxidized phospholipids (OxPLs) and malondialdehyde (MDA)-modified amino groups (Binder et al. 2016). They are presented not only in OxLDL but also in several biological structures, such as apoptotic cells and damaged proteins, representing danger (or damage)-associated molecular patterns (DAMPs). Oxidized epitopes are recognized by pattern recognition receptors and the proteins of the innate immune system, leading to removal and prevention of inflammatory effect (Binder et al. 2016). MAA epitopes are the final product of reaction between MDA and acetaldehyde. It has been proposed that the MAA adducts have potent immunogenicity, unique stability, and dose-dependent direct cellular toxicity among aldehyde adducts (Antoniak et al. 2015). Previously, we have shown that newborn babies possess natural IgM antibodies to MAA epitopes, and those antibodies may regulate apoptotic cell clearance during fetal development (Wang et al. 2013). In addition, we and others have proposed that MAA adducts contribute to pathogenic mechanisms in which classical risk factors of cardiovascular disease, such as hyperlipidemia and diabetes, may initiate inflammation and lead to development of atherosclerosis (Antoniak et al. 2015; Veneskoski et al. 2011). Anderson and colleagues have shown that coronary artery disease (CAD) patients possess higher levels of plasma IgA and IgG antibodies to MAA-LDL than controls (Anderson et al. 2014). In contrast, IgM antibody to MDA-LDL have an inverse association with carotid atherosclerosis (Karvonen et al. 2003).

*Porphyromonas gingivalis* (*P.gingivalis*) is a gram-negative bacterium that is considered crucial in periodontitis development due to its central role in the orchestration of microbial dysbiosis (Hajishengallis 2015). Several studies have shown high levels of serum IgG antibodies to *P.gingivalis* in atherosclerotic vascular diseases (Pietiäinen et al. 2018). Animal models infected by oral *P.*

*gingivalis* have also shown enhanced development of atherosclerosis comparing to controls (Lalla et al. 2003). Previously our group has shown that the immunization of atherosclerotic animal models with heat-killed *P. gingivalis* increased plasma IgM to MDA-LDL, and monoclonal IgM antibodies to MDA-LDL recognized *P. gingivalis* virulence factor gingipain (Rgp44) as an antigen (Turunen et al. 2012). The data reveal the existence of cross-reactive epitopes or molecular mimicry between MDA and the virulence factor. We have also shown that antibodies to a virulence factor of another key microbe in periodontitis, *Aggregatibacter actinomycetemcomitans* heat shock protein 60 (Aa-HSP60), cross-react with MAA-LDL (Wang et al. 2013). HSP60 is the most relevant and well-studied member of the heat shock protein family with regards to autoimmunity and development of atherosclerosis due to molecular mimicry between bacterial HSP60 and human HSP60 (Kilic and Mandal 2012).

We have recently shown that healthy subjects have salivary IgA antibodies to oxidized LDL (Akhi et al. 2017). In the present study we investigated whether levels of salivary antibodies to MAA-LDL and common oral bacteria are associated with coronary artery disease. Also, the molecular mimicry through antibodies cross-reaction between salivary IgA to MAA-LDL and oral pathogens was assessed.

## Materials and methods

### Study subjects and examinations

The current study consists of 451 patients originating from the Corogene prospective study which was constituted by 5,295 subjects who were assigned to coronary angiography in the region of southern Finland (Vaara et al. 2012). A subset (about 10%) of participants in the Corogene study was randomly invited to an extensive oral examination (Buhlin et al. 2011). Exclusion criteria were previous heart transplantation, low hemoglobin or blood transfusion during the same hospitalization period (Vaara et al. 2012). The medical history was obtained from hospital records. Patients were asked to complete a questionnaire reviewing their smoking status and oral hygiene habits, and were classified according to medications (Vaara et al. 2012). Patients were considered smokers if they smoked at the time or had quit smoking less than six months ago. Coronary artery disease was diagnosed according to coronary artery angiography, symptoms and clinical examination. “No-CAD” was categorized if coronary arteries had non-significant stenosis ( $\leq 50\%$ ) and “stable-CAD” if the stenosis was  $> 50\%$  in at least one coronary artery. Acute coronary syndrome (ACS) was defined as  $> 50\%$  of stenosis in at least one coronary artery and an episode of typical ischemic chest pain (Vaara et al. 2012). The oral examination was performed by two calibrated periodontists and previously published (Buhlin et al. 2011). Periodontal disease was categorized as: “healthy” = no alveolar bone loss (ABL) and bleeding on probing (BOP)  $< 25\%$ , “gingivitis” = no ABL and BOP  $> 25\%$ , periodontitis = mild to severe ABL. Saliva samples were collected and stored at  $-80^{\circ}\text{C}$ . Pooled subgingival bacterial samples were collected from the deepest pathological periodontal pocket ( $\geq 4$  mm) of each dentate quadrant, and analyzed by checkerboard DNA-DNA-hybridization (Mäntylä et al. 2013). This investigation conformed to STROBE guidelines for investigational studies.

### Preparation of antigens and chemiluminescence immunoassay

Low-density lipoproteins (LDL, density 1.019–1.063 g/ml) isolation from human plasma was carried out by sequential density ultracentrifugation (Hörkkö et al. 1999). Oxidization of freshly isolated LDL with 5  $\mu\text{M}$  copper sulfate (CuOx-LDL) was done as previous described (Hörkkö et al. 1999). Malondialdehyde acetaldehyde modification of LDL (MAA-LDL) was performed by mixing 20% acetaldehyde, LDL, and freshly prepared MDA. The pH was calibrated to 4.8 and the mixture was incubated at  $+ 37^{\circ}\text{C}$  for 2 h. Excessive aldehydes were extracted by overnight dialysis against 0.27 mM EDTA in PBS at  $+ 4^{\circ}\text{C}$ . The bacterial antigens are listed in supplemental file. The antigens from *P. gingivalis* and *A. actinomycetemcomitans* were a mixture of various serotypes. Bacteria were

cultured on fastidious anaerobe agar supplemented with 5%–10% blood. Heat-killed *bacteria* were prepared by incubation at 60°C for 1 hour in PBS. Recombinant *P. gingivalis* virulence factor, gingipain Rgp44, was prepared as previously described. (Turunen et al. 2012). The 60-kDa *A. actinomycetemcomitans* heat shock protein (Aa-HSP60) was prepared as previously described (Wang et al. 2016). Levels of salivary IgA and IgG antibodies to oxidized LDL and bacterial epitopes were determined by chemiluminescence immunoassay as previously described (Karvonen et al. 2003). Please see to detailed description of the methods and the intra-assay CV-variations in the materials and methods supplemental file.

## Statistical analysis

All statistical analyses were performed using SPSS statistics software (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.). Parameters with skewed distributions were logarithmically transformed and geometric means were calculated. The differences between characteristics of the CAD groups were analyzed with Kruskal-Wallis test and Pearson Chi-Square test. The difference in the antibody levels between the groups was analyzed by one-way ANOVA and post-hoc Dunnett test. The association of salivary IgA and IgG antibodies to studied antigens with stable-CAD and ACS was analysed by multinomial logistic regression model adjusted by clinical con-founders: age, gender, smoking, diabetes (yes/no), hypertension (yes/no), dyslipidemia (yes/no), total DNA probe and body mass index (BMI).. For analysis of competition assay Wilcoxon test pairwise statistics for two related samples was used. P-value below 0.05 was considered as significant. The correlation of salivary IgA antibody to MAA-LDL and oral pathogens were examined with bivariate Pearson analysis. The association of salivary IgA, IgG antibody to OxLDL with oral pathogens level in periodontal pocket were analyzed by Kruskal Wallis test. The error bars represent the standard error (SE). The pathogen DNA levels were shown in medians with interquartile ranges (IQR).The numbers of missing variables in statistical tests are presented in supplemental Table 1.

# Results

## Baseline characteristics

The basic characteristics and cardiovascular risk factors are presented in Table 1. The subjects' age varied between 33 and 82 years, with a mean of 63.3 years, and age variation between groups was significant ( $p<0.001$ ). On average, the population was overweight (mean BMI: 27.8); 67% of the male participants were overweight. The majority of the patients had medications for cardiometabolic disorders and the variation between groups were statistically significant: hypertension ( $p=0.03$ ), dyslipidemia ( $p<0.001$ ) and diabetes mellitus ( $p=0.001$ ). A significant variation of *A. actinomycetemcomitans* ( $p=0.046$ ) subgingival levels were observed between patients groups. *variation betwwn* bacterial Increased level of saliva total IgA was detected in patients with stable-CAD and ACS ( $p=0.001$ ) in comparison to no-CAD patients, whereas total salivary IgG concentrations did not differ between the groups.

## Increased level of salivary IgA to MAA-LDL, Rgp44 and Aa-HSP60 in Patients stable CAD and ACS

The mean levels of salivary IgA and IgG antibodies to oxidized LDL epitopes, MAA-LDL and CuOx-LDL are presented in Figure 1. Patients with stable-CAD ( $P=0.003$ ) and ACS ( $P=0.044$ ) had significantly higher levels of IgA antibodies to MAA-LDL than no-CAD patients. The levels of IgA and IgG antibodies to CuOx-LDL did not differ between the groups (Figure 1A, 1B). Patients with stable-CAD and ACS had significant higher IgA antibody levels of saliva to *P. gingivalis* gingipain Rgp44 ( $p=0.017$  and  $p=0.005$ , respectively) and to *A. actinomycetemcomitans* HSP60 ( $p=0.014$  and  $p=0.018$ , respectively) (Figure 1A) in comparison with no-CAD patients. Salivary IgG levels to Rgp44 and Aa-HSP60 did not differ between the groups (Figure 1B).

## Salivary IgA antibodies to oral pathogens in patients with angiography verified CAD

The salivary IgA and IgG antibody levels to periodontal pathogens are presented in Figure 2. Patients with stable-CAD had significantly higher salivary IgA antibodies levels to *P. gingivalis* ( $p=0.039$ ), *P. intermedia* ( $p=0.019$ ), *P. endodontalis* ( $p=0.001$ ), *T. forsythia* ( $p=0.002$ ) and *A. actinomycetemcomitans* ( $p=0.014$ ) than the no-CAD group (Figure 2A). Patients with ACS had increased salivary IgA levels to *P. intermedia* ( $p=0.005$ ), *P. endodontalis* ( $p=0.007$ ) and *T. forsythia* ( $p=0.003$ ) (Figure 2A) in comparison with the no-CAD group. Salivary IgG levels to periodontal pathogens did not differ between the groups (Figure 2B).

### **Salivary IgA antibodies to MAA-LDL epitopes and periodontal pathogens associate with CAD**

The association of salivary IgA antibody levels with the CAD status was analyzed by a multinomial regression model (Figure 3). After adjusting for established cardiovascular risk factors, stable-CAD associated significantly with IgA to MAA-LDL ( $p=0.016$ ), Rgp44 ( $p=0.012$ ), Aa-HSP60 ( $p=0.032$ ), *T. forsythia* ( $p=0.002$ ), *P. endodontalis* ( $p=0.016$ ), *P. intermedia* ( $p=0.038$ ), and with total IgA ( $p=0.002$ ) antibody levels (Figure 3A). ACS associated with IgA levels to MAA-LDL ( $p=0.043$ ), Rgp44 ( $p=0.004$ ), Aa-HSP60 ( $p=0.030$ ), *T. forsythia* ( $p=0.004$ ), *P. endodontalis* ( $p=0.020$ ), *P. intermedia* ( $p=0.005$ ), and with total IgA ( $p=0.016$ ) antibody levels (Figure 3B). These findings were independent of periodontal disease (data not shown).

### **Salivary IgA antibodies to OxLDL and periodontal pathogens.**

Appendix figure 1 shows the correlation of salivary IgA antibody to MAA-LDL with IgA antibody to Rgp44 ( $r = 0.628$ ,  $p<0.001$ ), *A. actinomycetemcomitans* ( $r = 0.481$ ,  $p<0.001$ ), Aa-HSP60 ( $r = 0.697$ ,  $p<0.001$ ), *P. endodontalis* ( $r = 0.648$ ,  $p<0.001$ ), *P. intermedia* ( $r = 0.580$ ,  $p<0.001$ ), *T. forsythia* ( $r = 0.735$ ,  $p<0.001$ ) and *P. gingivalis* ( $r = 0.476$ ,  $p<0.001$ ). Appendix table 2 shows the association of levels of bacteria, *A. actinomycetemcomitans* with the salivary IgA to CuOx-LDL ( $p=0.016$ ), IgA to MAA-LDL ( $p=0.001$ ) and IgG to MAA-LDL ( $p=0.001$ ). Also, levels of *P. endodontalis* associate with salivary IgG to Cuox-LDL ( $p=0.033$ ) and MAA-LDL ( $p=0.003$ ).

### **Salivary IgA binding specificity to MAA-LDL and oral pathogens**

The competitive binding of salivary IgA antibodies to MAA-LDL with oral pathogens and their virulence factors was investigated (Figure 4). The IgA binding to MAA-LDL in the presence and absence of competitors was significantly different ( $p<0.001$ ). *P. gingivalis*, Rgp44, Aa-HSP60, and *A. actinomycetemcomitans* showed a different extent of competitive binding with salivary IgA to MAA-LDL (Figure 4). The virulence factors competed more strongly than the whole bacteria in respect of salivary IgA binding to MAA-LDL.



## Discussion

In this study, we showed that salivary IgA antibodies to MAA-LDL and periodontal pathogens were associated with angiographically verified coronary artery disease. In addition, salivary IgA to MAA-LDL showed cross-reactivity with the oral bacteria *P. gingivalis* and *A. actinomycetemcomitans* and their respective virulence factors. Molecular mimicry between two structurally similar epitopes may have a role in the course of atherosclerosis by activation of cross-reactive immune response (Tsiantoulas et al. 2014), the results of the current study reveal an association between oral humoral response and coronary artery disease.

IgA is an arm of humoral immunity. In mucosal area, the dimeric IgA are secreted by plasma cells and provide defense against pathogenic and commensal bacteria (Pabst 2012). In serum, monomeric IgA antibodies are produced by plasma cells in the bone marrow, B1 cells and marginal zone B cells (Leong and Ding 2014). Recently, we have shown that the levels of salivary IgA, IgG antibodies to OxLDL epitopes do not correlate with the levels in plasma (Akhi et al. 2017). Limited information is available about the origin and the role of IgA antibody to OxLDL. Our previous data have suggested that plasma IgA to OxLDL is linked to glucose metabolism, and high level of IgA antibody could be an independent risk factor for type 2 diabetes mellitus (Sämpi et al. 2010). We have recently reported that plasma IgA antibodies to phosphocholine (PCho) and *Streptococcus pneumoniae* cell wall polysaccharide predict independently long-term risk of cardiovascular event (Kankaanpää et al. 2018). Furthermore, saliva from healthy human subjects has been shown to contain IgA and IgG antibodies to MAA-LDL that cross-react with the periodontal pathogen *P. gingivalis* (Akhi et al. 2017). However, the possible role of salivary IgA to MAA-LDL in atherogenesis remains elusive. In this study, a multinomial logistic regression model was built by adjusting with major cardiovascular risk factors. Stable-CAD and ACS were found to be associated with highly increased cross-reactive salivary IgA antibody to MAA-LDL compared to No-CAD controls, implying that salivary IgA to MAA-LDL has a role in the pro-atherogenic processes.

Witztum and colleagues (Miller et al. 2011) have discussed how oxidized epitopes act as danger-associated molecular patterns (DAMPs) and are subsequently recognized by the pattern recognition receptor (PRR). In studies of endogenous antigens considered in atherosclerosis, most attention has been paid to HSP60 and OxLDL epitopes. HSP60 shares high homology with mycobacterial HSP65 and has been demonstrated to express in endothelial cells in response to high cholesterol diet (Miller et al. 2011). This finding suggests that the original immune response against microbial HSP65 antigen

has developed to the cross-reactive immune response against endothelial HSP60 (Tsiantoulas et al. 2014). Monoclonal antibodies are antibodies specific for an epitope. Our group has previously shown that a mouse natural monoclonal IgM antibody to MAA-LDL cross-reacts with *A. actinomycetemcomitans* virulence factor HSP60 (Aa-HSP60) (Wang et al. 2016). Cross-reactivity has also been documented between MAA-LDL and *P. gingivalis* gingipain A hemagglutinin domain (Rgp44) (Kyrklund et al. 2018), a key etiologic agent of periodontitis (Hajishengallis 2015). In this study salivary IgA antibody to MAA-LDL correlated and cross-reacted with periodontal pathogens, Rgp44, and Aa-HSP60. Also salivary IgA to Rgp44 and Aa-HSP60 cross-reacted more specifically and had stronger correlation with MAA-LDL than the whole bacteria of *P. gingivalis* and *A. actinomycetemcomitans*. Furthermore, stable-CAD and ACS were found to be associated with cross-reactive IgA antibody to Rgp44 and Aa-HSP60, but the association was non-significant for IgA antibodies to *P. gingivalis* and *A. actinomycetemcomitans*. The exact mechanism behind this phenomenon is not known. Obtained results provide further evidence that MAA epitopes mimic the structure of oral pathogens and their virulence factors, suggesting that the virulence factors, rather than the whole bacteria may be directly involved in the humoral immune responses to oxidation-specific epitopes. This mechanism may have a crucial role in the initiation and progression of atherosclerosis.

Previously, animal studies have shown that OxLDL or heat killed *P. gingivalis* immunization have atheroprotective effect on mice models of atherosclerosis (Binder et al. 2016; Turunen et al. 2012). On the other hand live *P. gingivalis* immunization enhanced atherosclerosis development (Lalla et al. 2003). Formerly, we showed that LDLR<sup>-/-</sup> mice immunized with MDA-LDL possess reduced aortic lipid depositions after challenge with live *P. gingivalis* compared with mice receiving only *P. gingivalis* challenge (Turunen et al. 2015). In the present study, we have shown the cross-reaction of salivary IgA to MAA-LDL and *P. gingivalis*. It can be speculated that cross reactivity of salivary IgA to OxLDL and *P. gingivalis*, may contribute to atheroprotective effect of OxLDL and heat killed *P. gingivalis* immunization through epitopes molecular mimicry.

A number of seroepidemiological studies has demonstrated that antibody response against bacterial biomarkers of periodontitis associates both with subclinical atherosclerosis, prevalent CAD, and incident CVD events (Pietiäinen et al. 2018). Recently, we showed in present population that combined serum IgA and IgG antibody levels to *A. actinomycetemcomitans*, *P. gingivalis*, *P. endodontalis*, *P. intermedia*, *T. forsythia*, *Campylobacter rectus*, and *Fusobacterium nucleatum* associate with the ACS, while the corresponding subgingival bacterial levels do not (Liljestrand et al.

2018). We showed in this study that salivary IgA antibodies to *T. forsythia*, *P. endodontalis*, and *P. intermedia* associated with stable-CAD and ACS after adjusting for atherosclerosis risk factors. Previously, more attention has been paid to the oral bacteria *P. gingivalis* and *A. actinomycetemcomitans* in research the association between periodontitis and atherosclerosis. Our current findings indicate the involvement of other oral pathogens in coronary artery disease. Further investigations are needed to clarify the role played by those oral pathogenic bacteria in atherogenesis.

Janket and colleagues (Janket et al. 2015) have discussed the role of oral infections in development of metainflammation associates diseases. Coronary artery diseases are secondary complication of diabetes and patients with poorly controlled diabetes shows increase incidence of chronic periodontitis (Lalla and Papapanou 2011). Formerly we have shown that plasma IgA antibody levels to MAA-LDL are associated with inflammatory mediators, obesity, and type 2 diabetes (Vehkala et al. 2013). Currently, we do not know whether IgA to MAA-LDL plays a functional role in atherosclerosis or diabetes. Future studies should address these questions.

Strengths and limitations of the study: relatively large population size of current study (n=451) and participants verified health data are the major strengths of the study. The main limitation of current study is cross sectional setup and patient recruitments on basis of existing cohort. Other limitations of current study is aged population and the gender, with majority of male participant. In current study, the issue whether groups are significantly different, or the differences are just due to random variation, we used inferential error bar (Cumming et al. 2007).

Taken together, our study showed increased level of salivary IgA to MAA-LDL, Rgp44, Aa-HSP60, and several oral pathogens in stable-CAD and ACS patients. Salivary IgA antibody to MAA-LDL was associated significantly with coronary artery disease and cross-reaction with Rgp44, Aa-HSP60, and periodontal pathogens. Oral mucosal immunity is constantly challenged by exogenous and endogenous antigens. This study highlights the role that mucosal humoral immune response may have on atherosclerosis development.

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Table 1. Baseline clinical characteristics of subjects according to cardiovascular groups.

	No-CAD n=133 (29.5%)	Stable CAD n=169 (37.5%)	ACS n=149 (33.0%)	
	Mean (SD)			p-value <sup>1</sup>
Age, years	61.0 (8.8)	65.8 (8.0)	62.6 (9.9)	<b>&lt;0.001</b>
BMI, kg/m <sup>2</sup>	27.5 (5.2)	27.8 (4.6)	28.0 (5.2)	0.612
Saliva IgA µg/mL	323.3 (149.6)	391.4 (164.1)	371.0 (151.0)	<b>0.001</b>
Saliva IgG µg/mL	46.6 (15.7)	49.6 (17.8)	49.2 (19.5)	0.319
	Median (IQR)			
Total DNA probe <sup>2</sup>	62.65 (109.74)	73.16 (105.63)	63.75 (103.05)	0.284
<i>P. gingivalis</i> <sup>2</sup>	0.53 (3.33)	0.58 (4.27)	0.58 (3.46)	0.893
<i>P. intermedia</i> <sup>2</sup>	0.72 (2.23)	0.79 (2.4)	0.79 (2.66)	0.637
<i>P. endodontalis</i> <sup>2</sup>	0.31 (2.09)	0.26 (1.80)	0.24 (2.05)	0.723
<i>T. forsythia</i> <sup>2</sup>	4.63 (13.90)	6.04 (20.63)	4.85 (21.76)	0.607
<i>A. actinomycetemcomitans</i> <sup>2</sup>	0.98 (4.65)	2.13 (5.31)	1.14 (3.83)	<b>0.046</b>
	N (%) <sup>3</sup>			p-value <sup>4</sup>
Gender(men)	64 (14.2)	129 (28.6)	108 (23.9)	<b>&lt;0.001</b>
Hypertension	75 (16.7)	119 (26.4)	92 (20.4)	<b>0.030</b>
Dyslipidemia	96 (21.4)	158 (35.2)	108 (24.1)	<b>&lt;0.001</b>
Diabetes mellitus	19 (4.3)	52 (11.6)	29 (6.5)	<b>0.001</b>
Stenosed arteries				
0	133 (29.5)	0	0	<b>&lt;0.001</b>
1	0	43 (9.53)	71 (15.7)	<b>&lt;0.001</b>
2	0	51 (11.3)	39 (8.6)	<b>&lt;0.001</b>
3	0	75 (16.6)	39 (8.6)	<b>&lt;0.001</b>
Periodontal disease				
Healthy	16 (3.5)	12 (2.7)	13 (2.9)	0.463
Gingivitis	26 (5.8)	14 (3.1)	21 (4.7)	<b>0.022</b>
Periodontitis	85 (18.8)	129 (28.6)	105 (23.3)	<b>0.008</b>
Edentate	5 (1.1)	12 (2.7)	10 (2.2)	0.431
Smoking				
No	72 (16.0)	76 (16.9)	64 (14.2)	0.142
Former	42 (9.3)	73 (16.2)	67 (14.9)	<b>0.044</b>
Current	19 (4.2)	19 (4.2)	18 (4.0)	0.730

<sup>1</sup> Kruskal-Wallis test<sup>2</sup> Unit, count × 10<sup>5</sup><sup>3</sup> Percentages were calculated from the total population (n=451)<sup>4</sup> Pearson Chi-square test

No-CAD: no coronary artery disease, Stable-CAD: stable coronary artery disease, ACS: acute coronary syndrome. Statistically significant p-values (p &lt; 0.05) are bolded.



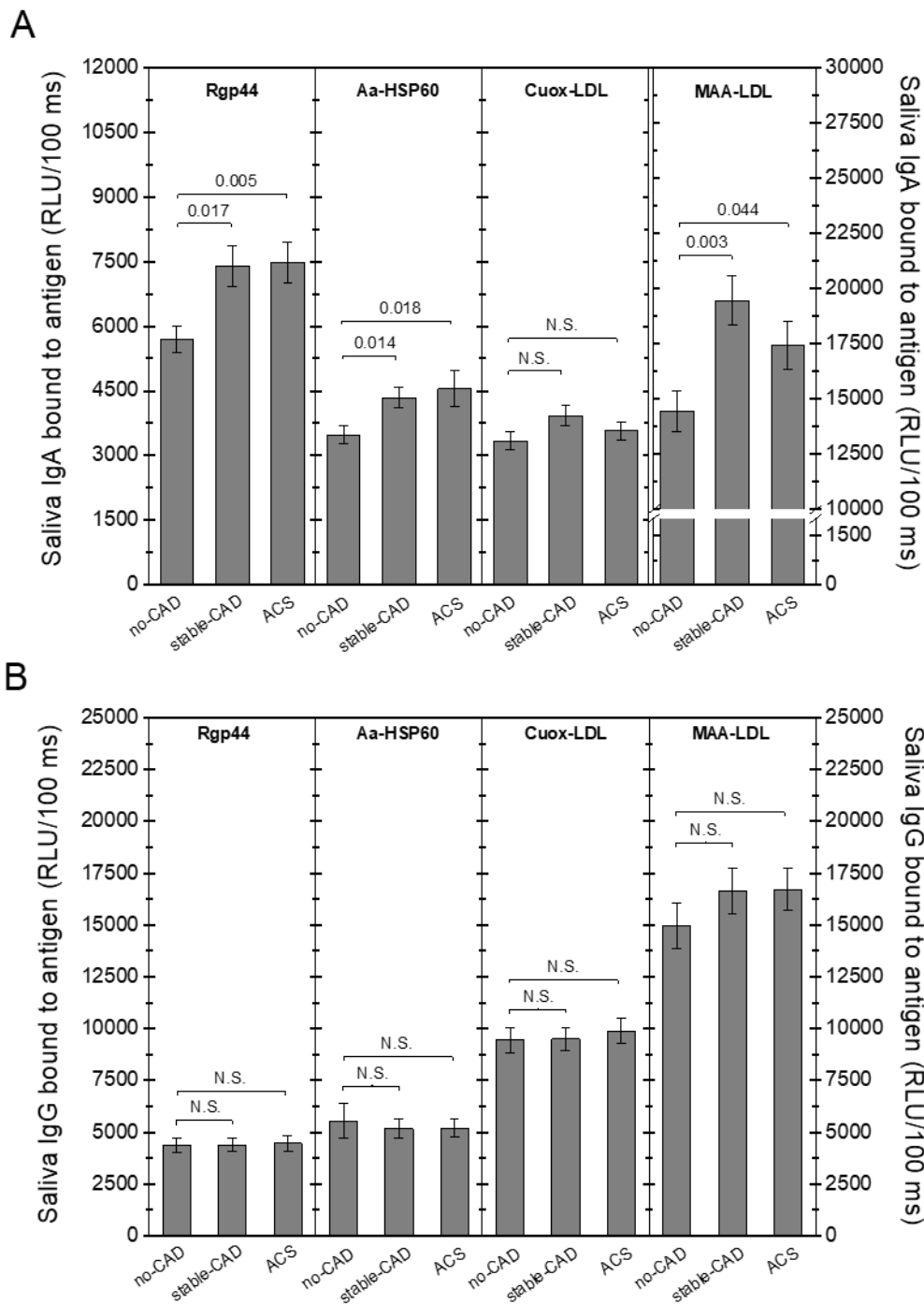


Figure 1: Antibody levels of salivary IgA (A) and IgG (B) according to cardiovascular status. The differences in antibody levels of stable coronary artery disease (stable-CAD) and acute coronary syndrome (ACS) groups with no-coronary artery (no-CAD) group were analyzed by one-way analysis of variance using the post hoc Dunnett test. Due to skewed data distributions, statistical tests were performed with logarithm-transformed values. The error bar represents standard error mean (SEM). Rgp44: *Porphyromonas gingivalis* A hemagglutinin domain, Aa-HSP60: *Aggregatibacter actinomycetemcomitans* heat shock protein 60, CuOx-LDL: copper oxidized low-density lipoprotein, MAA-LDL: malondialdehyde acetaldehyde-modified low-density lipoprotein, relative light unit (RLU), milliseconds (ms), not significant (N.S) with  $p < 0.05$ .

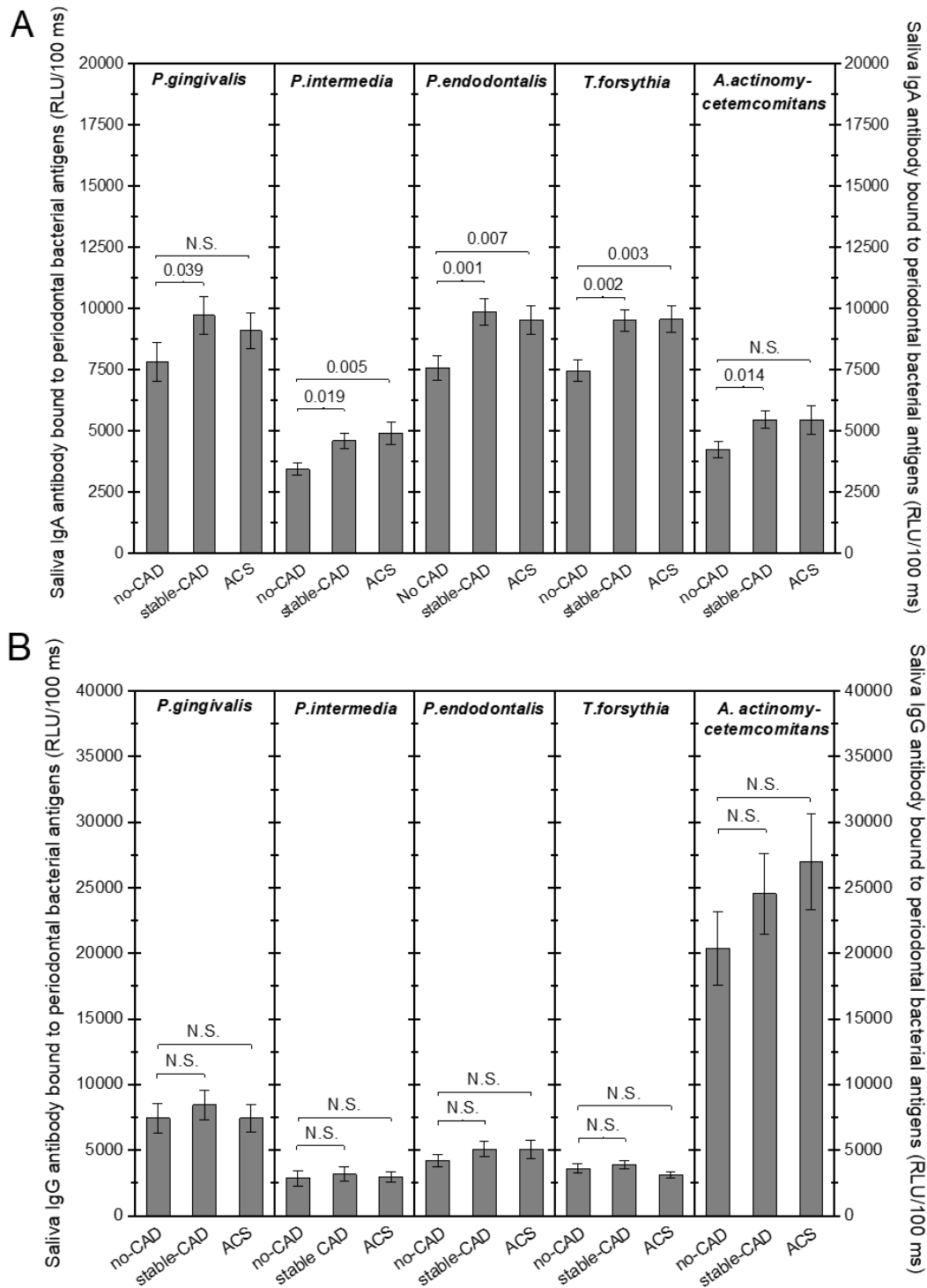
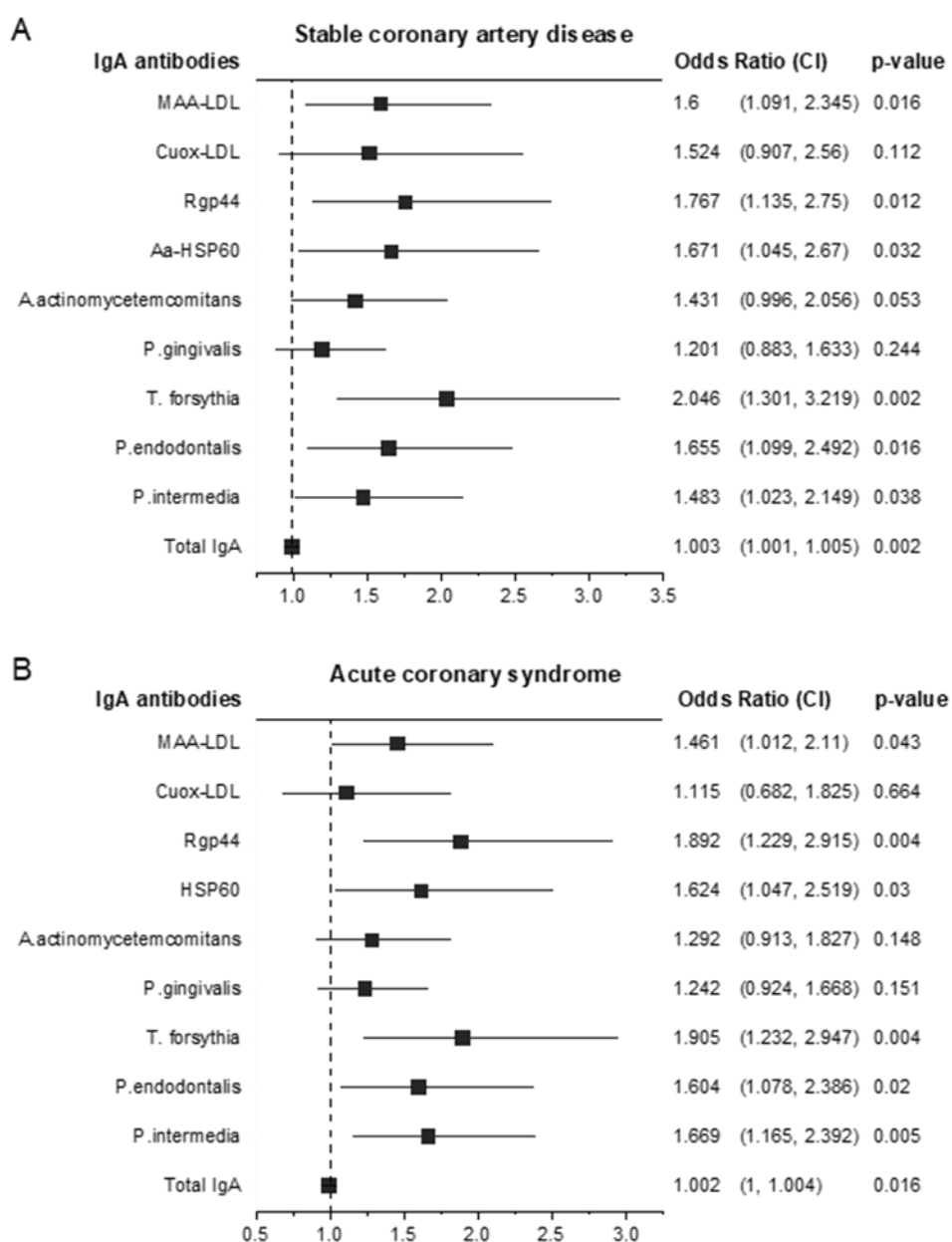


Figure 2: Antibody levels of salivary IgA (A) and IgG (B) to periodontal pathogens according to CAD status. The differences in antibody levels of stable coronary artery disease (stable-CAD) and acute coronary syndrome (ACS) groups with no-coronary artery disease (no-CAD) group were analyzed by one-way analysis of variance post hoc Dunnett test. Due to data skewed distributions, statistical tests were performed with logarithm-transformed values. The error bar represents standard error. Relative light unit (RLU), milliseconds (ms), not significant (N.S) with  $p < 0.05$ .



**Figure 3:** Levels of salivary IgA antibodies to different antigens in stable coronary artery disease (A) and acute coronary syndrome (B) were analyzed by multinomial logistic regression. Salivary IgA levels to different antigens in no coronary artery disease (no-CAD) were used as reference. Model was adjusted with age, gender, smoking (never/ex/current), body mass index, diabetes, dyslipidemia, hypertension, and total DNA probe. All data except “Total IgA” were logarithm-transformed due to skewed distributions and respectively, odds ratio represents the difference in logarithmic scale.

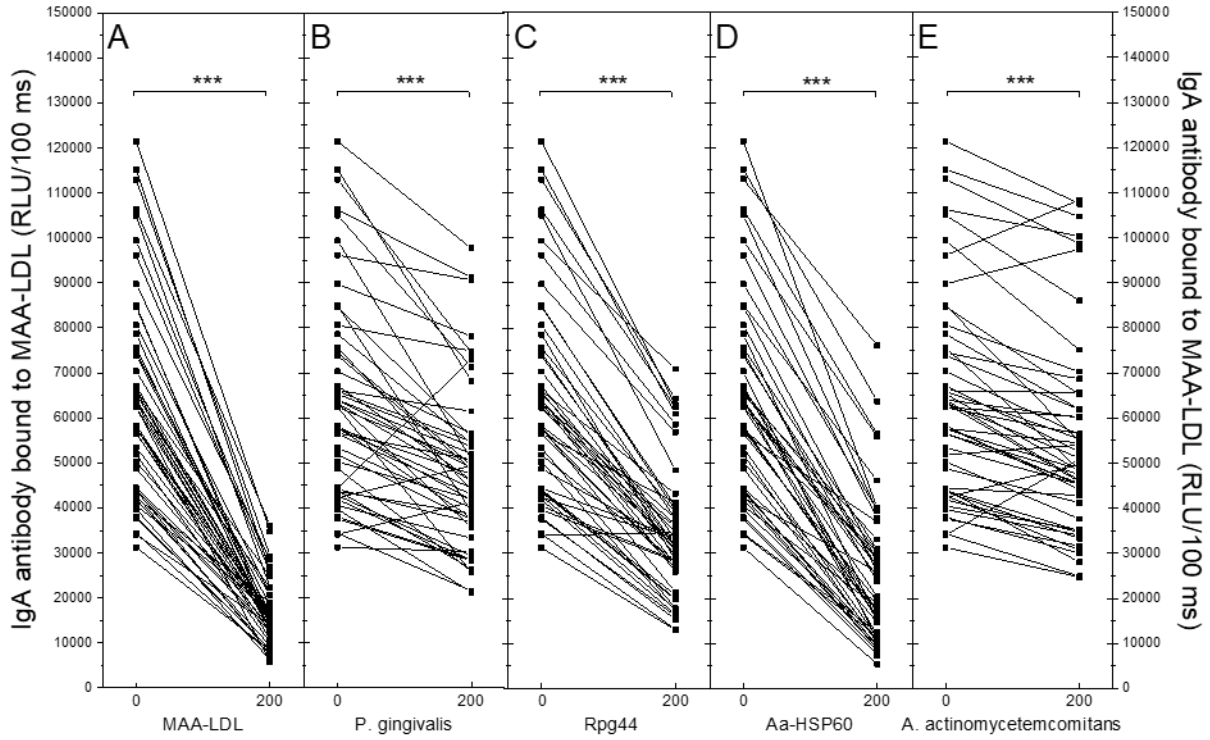


Figure 4

Comparative binding specificity of salivary IgA antibody to MAA-LDL in the absence (0  $\mu\text{g/mL}$ ) and presence (200  $\mu\text{g/mL}$ ) of soluble competitors. A total of 50 patients were chosen for the competitive immunoassay. Periodontal pathogens and their virulence factors were as competitors: A) MAA-LDL: Malondialdehyde acetaldehyde-modified LDL, B) *P. gingivalis* C) Rgp44: *P. gingivalis* A hemagglutinin domain, D) Aa-HSP60: *A. actinomycetemcomitans* heat shock protein 60, E) *A. actinomycetemcomitans*. For all panels, MAA-LDL was used as a solid-phase antigen (5 $\mu\text{g/mL}$ ). The difference between groups was analyzed with Wilcoxon test for two related samples. \*\*\* $p < 0.001$ .

## Supplemental materials and methods

### Bacterial antigens

The bacterial antigens were as follows: *P. gingivalis* serotype a (ATCC 33277), serotype b (W50), serotype c (OMGS 434), *Porphyromonas endodontalis* (ATCC 35406), *Prevotella intermedia* (ATCC 25611), *Tannerella forsythia* (ATCC 43037) and *Aggregatibacter actinomycetemcomitans* serotypes a, b, c, d, e, f and one nonserotypeable strain x (ATCC 29523, ATCC 43718, ATCC 33384, IDH 781, IDH 1705, CU 1000, C59A) (Hyvärinen et al. 2009; Pussinen et al. 2002).

### Chemiluminescence immunoassay

Briefly, plates coated with 5µg/mL of antigen (25 µL/well) in PBS/0.27mM EDTA were incubated overnight at +4°C. The next day, plates were incubated with 0.5% fish gelatin (50 µL/well) for 1 hour at room temperature (RT). Saliva samples (25µL/well) were incubated for 1 hour at RT. The antibody detection was carried out with alkaline phosphatase-labeled secondary antibodies: anti-human IgA (Sigma-Aldrich, St. Louis, MO, USA) and anti-human IgG (Sigma-Aldrich). The chemiluminescence was detected using Lumi-Phos 530 substrate (Lumigen, Southfield, MI, USA) and a Wallac Victor multilabel counter (Perkin-Elmer, Waltham, MA, USA). In the immunoassay, the saliva was diluted as following: 1:250 for total IgA and IgG, 1:50 for IgA to oxidized antigens, 1:20 for Aa-HSP60, and 1:10 for bacterial antigens. For measurement of IgG, saliva samples were diluted 1:10 for all antigens. Triplicate measurements of each saliva sample were made. A standard curve of purified human IgA, IgG and a control serum sample was added to each plate to correct variations between the assays. Immunoassay results were presented as relative light units (RLU) per 100 milliseconds (ms). The intra-assay coefficients of variation were as follows: IgA antibodies, total 10.7%, CuOx-LDL 13.1%, MAA-LDL 11.4%, *A. actinomycetemcomitans* 9.1%, *P. gingivalis* 10.2%, Rgp44 10.0%, Aa-HSP60 8.8%, *P. intermedia* 11.1%, *P. endodontalis* 11.8%, *T. forsythia* 8.9% and IgG antibodies, total 8.9%, CuOx-LDL 10.6%, MAA-LDL 7.9, *A. actinomycetemcomitans* 11.2%, *P. gingivalis* 11.4%, Rgp44 11.2%, Aa-HSP60 10.0%, *P. intermedia* 5.12%, *P. endodontalis* 10.8%, *T. forsythia* 11.4%.

### Competition Immunoassay

The specificity of antibodies to periodontal pathogens and oxidized LDL was tested by liquid-phase competition immunoassay. Fifty saliva samples with high IgA levels to MAA-LDL were selected from the cohort. Saliva samples were incubated overnight at +4°C in the presence and absence of MAA-LDL, *P. gingivalis*-mix, *A. actinomycetemcomitans*-mix, Rgp44 and Aa-HSP60 competitors

(0–200 µg/mL). The immuno-complexes were pelleted by centrifugation at  $16,000 \times g$  (+4°C) for 30 min, and antibodies remaining in the liquid phase were analyzed using chemiluminescence immunoassay as previously described (Akhi et al. 2017).

**Supplemental table 1.** Missing case data of variables from 451 subjects.

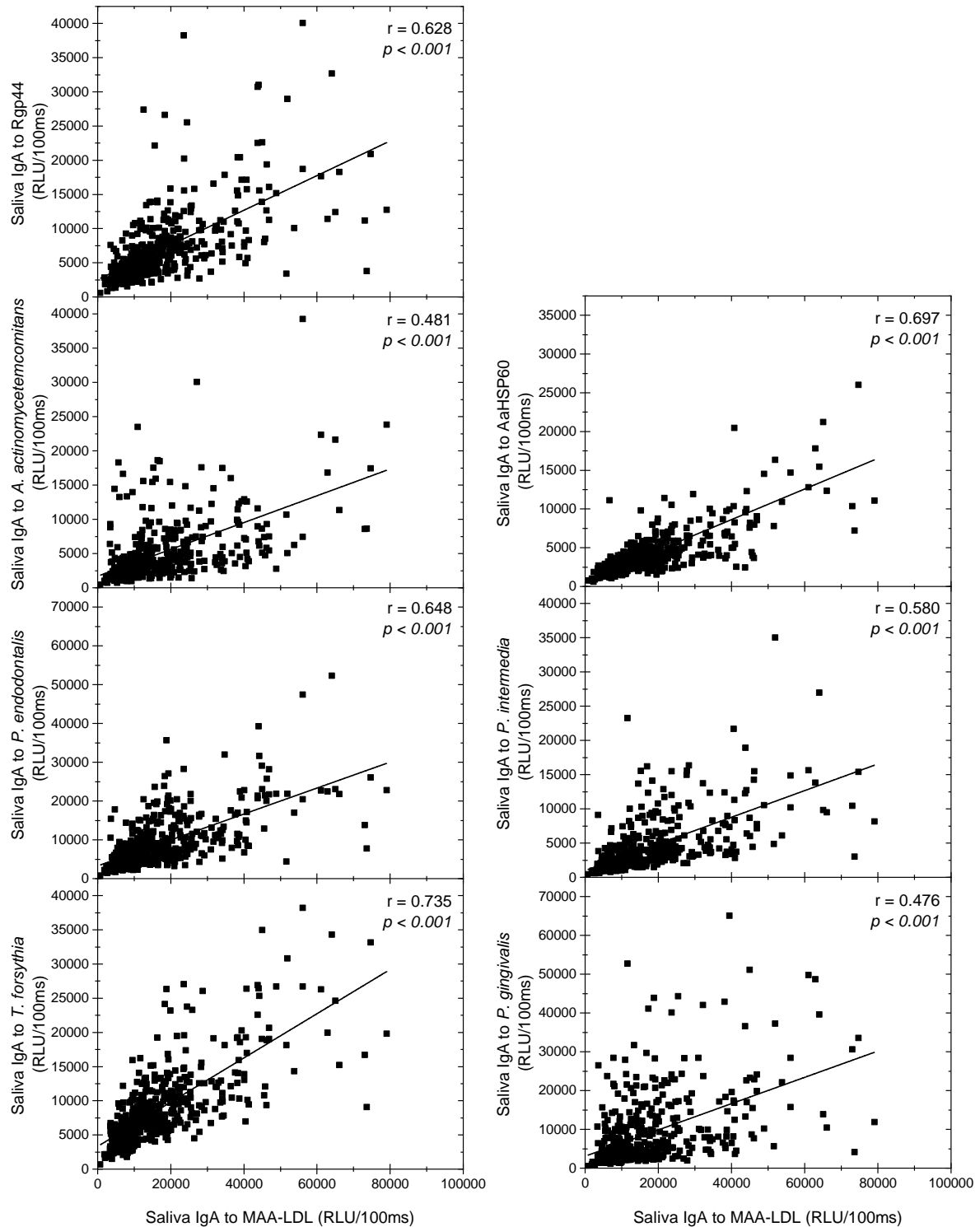
Missing case data according to variable(s)	Cases (n)
Hypertension	1
Dyslipidemia	2
Diabetes	4
Smoking	1
Periodontal disease	1
Edentate	2

**Supplemental table 2.** Association of saliva IgA, IgG antibodies to OxLDL with the subgingival levels of oral pathogens. The associations were analyzed by Kruskal Wallis test. Not significant (n.s) with  $p < 0.05$ .

Oral bacteria, quantity of DNA	IgA		IgG	
	CuOx-LDL*	MAA-LDL*	CuOx-LDL*	MAA-LDL*
<i>Aggregatibacter actinomycetemcomitans</i> **	<b><math>p=0.016</math></b>	<b><math>p=0.001</math></b>	n.s.	<b><math>p=0.001</math></b>
<i>Porphyromonas gingivalis</i> **	n.s.	n.s.	n.s.	n.s.
<i>Prevotella intermedia</i> **	n.s.	n.s.	<b><math>P=0.033</math></b>	<b><math>P=0.003</math></b>
<i>Porphyromonas endodontalis</i> **	n.s.	n.s.	n.s.	n.s.
<i>Tannerella forsythia</i> **	n.s.	n.s.	n.s.	n.s.

\*Relative light unit/ milliseconds (RLU/ ms)

\*\* Unit, count  $\times 10^5$



### Supplemental figure 1.

Correlation of salivary IgA antibody to MAA-LDL with salivary IgA antibody to oral pathogens. MAA-LDL: malondialdehyde acetaldehyde-modified low-density lipoprotein, Rgp44: *Porphyromonas gingivalis* A hemagglutinin domain, Aa-HSP60: *Aggregatibacter actinomycetemcomitans* heat shock protein 60. Correlations were analyzed using Pearson bivariate analysis. The data represent chemiluminescence relative light unit/ milliseconds (RLU/ ms).

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