Cross-reactive saliva IgA antibodies to oxidized LDL and periodontal pathogens in humans

Saliva antibodies to oxidized LDL

Keyword: Antibody, Oxidized low-density lipoprotein, Atherosclerosis, Bacteria, Periodontitis, Salivary diagnostics

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Abstract

Aim: Oxidized Low-Density Lipoproteins (oxLDL) are formed as a result of lipid peroxidation and are highly immunogenic and proatherogenic. In this study, saliva antibodies binding to oxLDL, *Porphyromonas gingivalis (Pg)* and *Aggregatibacter actinomycetemcomitans (Aa)*, were characterized and their cross reactivity was evaluated.

Material & Methods: Resting and stimulated saliva samples were collected from 36 healthy adults (mean age 26 years). Saliva IgA, IgG and IgM autoantibody levels to copper oxidized LDL (CuOx-LDL) and malondialdehyde acetaldehyde modified LDL (MAA-LDL), were determined with chemiluminescence immunoassay.

Results: Saliva IgA and IgG antibodies binding to MAA-LDL and CuOx-LDL were detected in all samples and they were associated with the saliva levels of IgA and IgG to *P.gingivalis* and *A.actinomycetemcomitans*. Competitive immunoassay showed that saliva antibodies to MAA-LDL cross-reacted specifically with *P.gingivalis*. The autoantibody levels to oxLDL in saliva were not associated with the autoantibody levels to oxLDL in plasma or with saliva apolipoprotein B 100 levels.

Conclusions: Saliva contains IgA and IgG binding to oxLDL, which showed cross-reactive properties with the periodontal pathogens *Porphyromonas gingivalis* (*P.g*). The data suggests that secretory IgA to *P.g* may participate in immune reactions involved in LDL oxidation through molecular mimicry.

Clinical Relevance:

Scientific rationale for the study: Periodontal pathogens may contribute to atherosclerosis through molecular mimicry in autoantibody cross-reaction.

Principal finding: Autoantibodies binding to oxLDL and malondialdehyde adducts were detected in healthy human saliva with a low salivary burden of periodontal bacteria. These antibodies showed cross-reactive binding to *P.gingivalis.*

Practical implications:

- The findings may elucidate the association of immune response to periodontal pathogens and LDL-oxidation through epitope mimicry.

Introduction

Human saliva is reported to contain over 2,000 proteins (Bandhakavi et al. 2009), including oxidized low-density lipoprotein (oxLDL) (De Giuseppe et al. 2015) and immunoglobulins (Brandtzaeg2013). Secretory IgA is one of the most abundant salivary protein, and has a role e.g. in preventing bacteria from adhering to the mucosa (Svanborg-Eden & Svennerholm1978). In addition, the oral cavity has a rich microbiome comprising over 600 phylotypes (Dewhirst et al. 2010). Poor oral health has been linked to atherosclerotic cardiovascular diseases, and several mechanisms have been proposed for how oral bacteria may initiate or accelerate the atherosclerotic processes in the artery intima (Gibson et al. 2006, Lockhart et al. 2012). The presence of gram negative rod bacteria such as *Porphyromonas gingivalis (Pg)* and *Aggregatibacter actinomycetemcomitans (Aa)* in the oral cavity is recognized as a risk factor for cardiovascular diseases via invasion of periodontal tissue, entering the circulation and evasion of host defense mechanisms (Hajishengallis2015). Circulating plasma oxLDL has also been of interest in periodontal diseases: the levels have been reported to be elevated in patients with chronic periodontitis (Schenkein & Loos2013), and plasma levels of oxLDL have been shown to be reduced by effective periodontal treatment (Tamaki et al. 2011).

Oxidative reactions on low-density lipoproteins are complex, and numerous oxidative end-products have not yet been fully characterized (Parthasarathy et al. 2010). LDL oxidation plays a major role in the development of atherosclerosis, in which trapped LDL particles are oxidized in the artery wall intima by reactive oxygen species or by enzymatic modifications (Glass & Witztum2001). OxLDL is highly immunogenic and circulating autoantibodies against various oxidized neo-epitopes have been documented in human plasma, as well as in the plasma of animal models of atherosclerosis (Hörkkö et al. 2000, Binder et al. 2005). The exact role of these autoantibodies in atherosclerosis has not been resolved. Natural IgM antibodies to *in vitro* produced models of oxLDL, such as copper oxidized LDL (CuOx-LDL) and malondialdehyde modified LDL (MDA-LDL) have been suggested to have atheroprotective properties (Tsiantoulas et al. 2014). Malondialdehyde is a small naturally occurring lipid peroxidation product and highly reactive with amino groups on various proteins, lipids and other biomolecules to form a variety of adducts (Wang et al. 2013, Esterbauer et al. 1991); in the presence of acetaldehyde, e.g. produced by microbial oxidation of the mucosal microbiome (Kurkivuori et al. 2007), generates malondialdehyde acetaldehyde adducts (MAA-adducts). In humans, circulating plasma IgA autoantibodies to malondialdehyde acetaldehyde modified LDL (MAA-LDL) are shown to associate with obesity and

type 2 diabetes (Vehkala et al. 2013), whereas plasma IgM antibodies to MAA-LDL are suggested to be germline-derived natural antibodies targeting oxidized epitopes, apoptotic cells and bacteria (Wang et al. 2013, Silverman2011). One of the antigenic epitopes in copper oxidized LDL is the phosphocholine (PCho) head group of oxidized phospholipids exposed on LDL surface (Binder et al. 2003).

By using mouse monoclonal IgM antibody clones we have previously identified cross-reactive epitopes in oxLDL, *P.gingivalis* (Turunen et al. 2012) and *A. actinomycetemcomitans* (Wang et al. 2016). The oxidized epitopes recognized were malondialdehyde adducts of proteins and epitopes in *P.gingivalis* gingipain proteases (Turunen et al. 2012). Our hypothesis was that healthy human saliva contains antibodies binding to oxLDL and that those antibodies cross-react with antibodies to *P.gingivalis* and *A. actinomycetemcomitans*.

Materials and Methods

Study design and human samples

The study population consisted of 36 dental students (27 female and 9 male) at the University of Oulu, Finland; a written informed consent was obtained. The mean age was 26 years (range 21-40 years). Resting and stimulated saliva samples were collected as follows: the resting saliva sample was collected during a 5-min collection period and the stimulated saliva sample was collected during 5-min mastication on a small paraffin block. The subjects were instructed not to eat and drink for at least 1 hour prior to the saliva collection. Blood samples were collected after an overnight fast. Plasma was separated by centrifugation at 1260 x g at +4C for 15 min. The saliva and plasma samples were stored at -70°C for analysis. The study had been approved by the ethical committee of Northbothnia District University Hospital and followed the Declaration of Helsinki.

Preparation of antigens for immunoassays

CuOx-LDL, MAA-LDL and MAA-BSA were prepared as previously described (Hörkkö et al. 1999, Kummu et al. 2014). Phosphocholine modified BSA (PC-BSA) was purchased from Biosearch Technologies Inc. (Novato, CA, USA). Heat-killed *Pg* strain ATCC 33277 and recombinant domains of *Pg* gingipain: Rgp44 were prepared as described in previous publications (Turunen et al. 2012, Turunen et al. 2015). *Aggregatibacter actinomycetemcomitans (Aa)* strains representing mixed serotypes of a, b, c, d, e, f and one nonserotypeable strain x (ATCC 29523, ATCC 43718, ATCC 33384, IDH 781, IDH 1705, CU 1000, C59A) were used in the study and cultured on fastidious anaerobe agar supplemented with 5-10% blood. Heat-killed *Aa* was prepared by incubation at 60 °C for one hour in PBS. The 60-kDa Aa chaperonin (HSP60) were prepared as previously described (Wang et al. 2016).

Chemiluminescence immunoassay

The levels of saliva IgA, IgG and IgM antibodies binding to various models of oxidized LDL and modified BSA were determined by chemiluminescence immunoassay as previously described (Karvonen et al. 2003). Saliva samples were diluted 1:10 for IgA, 1:10 for IgG, and 1:5 for IgM measurements. Binding of saliva immunoglobulins was detected with alkaline phosphatase-labeled goat secondary antibodies: anti-human IgA (Sigma-Aldrich, St. Louis, MO, USA), anti-human IgG (Sigma-Aldrich), anti-human IgM (Sigma-Aldrich). The chemiluminescence was detected using Lumi-Phos530 substrate (Lumigen, Southfield, MI, USA) and a Wallac Victor^3 multilabel counter (Perkin-Elmer, Waltham, MA, USA). Triplicate measurements of each saliva sample were

performed. A standard curve of purified human IgA, IgG, IgM and a control serum sample was added to each plate to correct variations between the assays. The total amounts of IgA, IgG and IgM immunoglobulins in saliva were determined as above (dilutions: IgA 1:250, IgG 1:50, IgM 1:10). Saliva IgA and IgG binding to *Pg*, *Aa*, *E.coli*, *Pg* hemagglutinin/adhesion domain of gingipain Rgp44 and heat shock proteins HSP60 of *Aa* were also determined using chemiluminescent immunoassay. Plates were first incubated with 0.5% fish gelatin (50 µL/well) for one hour at RT, followed by overnight incubation of heat inactivated bacteria (50 µL/well) 5 µg/mL in PBS-EDTA at 4°C. 1:10 dilution of saliva was used. Results are shown as relative light units (RLU) per 100 ms.

Competitive immunoassay for antibody specificity

The specificity of human saliva antibodies was tested using liquid-phase competition immunoassay. Saliva samples were incubated overnight at + 4°C in the presence or absence of MAA-LDL, CuOx-LDL, *Pg*, *Aa* and native-LDL competitors (0-200 µg/mL). The immunocomplexes were pelleted by centrifugation 16000 x g at +4°C for 30 min, and antibodies remaining in the liquid phase were analyzed using the chemiluminescence immunoassay described previously (Kummu et al. 2014).

Measurement of apoB100 containing particles in saliva

The apolipoprotein B-100 concentration in saliva was determined using anti-apoB48/100 (Median Life Sciences, Fullerton, CA, USA) as capture antibody and biotinylated anti apoB48/100 as detection antibody. NeutrAvidin alkaline phosphatase (1.3 mg/mL) (Thermo Scientific, Rockford, IL, USA) was used for detection. Saliva samples were diluted at 1:2. Duplicate measurements of each saliva sample were performed. A standard curve of purified human native-LDL and a control serum sample was added to each plate.

Measurement of bacterial DNA using real-time quantitative PCR

Bacterial DNA was extracted from stimulated saliva samples by ZR Fungal/Bacterial DNA kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions. The QPCR reaction mixture (20 µL) included 2 µL of template DNA, 10 µL Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent Technologies, Santa Clara, CA, USA), 30 nM ROX reference dye (Agilent Technologies), and specific primers (Thermo Scientific). The primer sequences and bacterial genome equivalent quantification method have been published previously (Hyvärinen et al. 2009).

Plasma cholesterol measurement

Plasma total cholesterol (TC), total triglycerides and high-density lipoprotein cholesterol were determined by enzymatic methods using commercial kits (Roche Diagnostics Mannheim, Germany).

Statistics

Data analysis was performed by IBM SPSS Statistics 21.0. For comparison of variables Wilcoxon-Mann-Whitney non-parametric test was used. The associations were tested by Spearman's correlation analysis. For the competition assay pairwise statistics, Wilcoxon test for 2 related samples were used. P-values less than 0.05 were considered significant.

Results

Saliva antibody binding to oxidized LDL

Both resting and stimulated saliva samples were investigated and the data compared. All saliva samples contained IgA antibodies binding to copper-oxidized LDL (CuOx-LDL) and malondialdehyde acetaldehyde modified LDL (MAA-LDL) (Fig 1A), and the binding was significantly higher in resting saliva samples compared to stimulated saliva samples. To investigate whether the LDL molecule was essential as a carrier antigen for oxidized modifications, saliva IgA binding to modified BSA antigens was tested: all saliva samples contained IgA binding to malondialdehyde acetaldehyde modified BSA (MAA-BSA) and phosphocholine modified BSA (PC-BSA) (Fig 1A).

In serum, immunoglobulin G is the most abundant isotype and accounts for the binding to oxLDL. Saliva samples also contained IgG to MAA-LDL and CuOx-LDL (Fig 1B). The saliva IgM antibody levels to oxLDL were minimal (Fig 1C). In contrast to saliva IgA, IgG antibody binding to oxLDL was significantly lower in resting saliva compared to stimulated saliva samples (Fig 1B).

Antibody binding to *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis* and association with antibody binding to oxLDL

IgA and IgG binding in stimulated saliva to *Aa*, *Pg*, *E.coli*, *Pg* hemagglutinin/adhesion domain of gingipain and heat shock proteins HSP60 of *Aa* was measured (Figure2). IgA and IgG binding to *Aa*, HSP60 and Rgp44 was observed, and the antibody levels measured were significantly higher compared to the levels binding to E.coli (Fig 2). Saliva IgA antibody binding to MAA-LDL was associated with IgA binding to *Pg* ($\rho = 0.61$, $p < 0.001$) and *Aa* ($\rho = 0.68$, $P < 0.001$) (Fig 3A). Saliva IgG binding to MAA-LDL was also associated with IgG binding to *Pg* ($\rho = 0.76$, $P < 0.001$) and *Aa* ($\rho = 0.67$, $P < 0.001$) (Fig 3B), but IgM binding to MAA-LDL was not associated with IgM binding to the oral pathogens (Fig 3C). Similarly, IgA and IgG antibody binding to CuOx-LDL was associated with binding to *Pg* and *Aa* (Fig 3D-E). The levels of saliva IgM to CuOx-LDL were low, but showed association with saliva levels of IgM binding to *Pg* ($\rho = 0.39$, $P < 0.02$, Fig 3F).

Binding specificity of saliva antibodies to oxLDL

To examine the binding specificity of saliva antibody binding to oxLDL a liquid phase competition assay was performed (Fig 4). First, a full-scale competition assay was performed on four different saliva samples; then, all saliva samples were tested with a fixed concentration based on the data

obtained. Figure 4A shows saliva antibody binding to oxidized LDL with increasing amounts of competitors (0-200 µg/mL). Saliva IgA, IgG and IgM binding to immobilized MAA-LDL was specifically competed by increasing amounts of soluble MAA-LDL, whereas native-LDL, E.coli and CuOx-LDL showed no or small competition (Fig 4A). Saliva IgA and IgG binding to MAA-LDL was also specifically competed by *P.gingivalis* (Fig 4A). Saliva antibody binding to immobilized CuOx-LDL was competed equally by all competitors, including native-LDL, suggesting that the binding was nonspecific (data not shown). The binding of saliva IgA to MAA-LDL showed significant cross-reactive competition with *P.gingivalis* bacteria when all saliva samples were tested (P <0.001, n= 36, paired sample t-test) (Fig 4B).

Saliva total immunoglobulin levels and normalized antigen specific binding

The saliva total immunoglobulin levels were measured (Suppl Fig 1) to estimate the normalized antigen-specific antibody binding. Resting saliva total IgA levels were significantly higher than stimulated saliva total IgA levels, but the normalized antigen-specific IgA binding to oxLDL also remained higher in the resting saliva samples (Suppl Fig 2A). The saliva total IgG levels were significantly lower in the resting samples compared to stimulated samples (Suppl Fig 1), and the normalized antigen-specific IgG to *A.actinomycetemcomitans* and *P.gingivalis* was significantly higher in resting saliva compared to stimulated saliva samples (Suppl Fig 2B). There were no differences in saliva total IgM levels between resting and stimulated samples (Suppl Fig 2C).

Measurement of bacterial DNA in saliva samples

Quantitative PCR was performed to examine the amount of *Pg* or *Aa* bacterial DNA in the saliva samples (n=36). Two saliva samples had detectable amounts of bacterial DNA, one for *P.gingivalis* and the other for *A.actinomycetemcomitans* (data not shown).

Saliva apolipoprotein B100 and plasma cholesterol levels

Previous studies have proposed that plasma antibody titers to oxLDL associate with plasma LDL cholesterol levels (Shoji et al. 2000).To investigate these associations in saliva, we measured apolipoprotein B100 (ApoB100) containing lipoprotein particles using sandwich immunoassay. Apolipoprotein B100 immunoassay measurement was selected, as the saliva cholesterol levels were below the detection limit of a standard detection system (see Methods). One copy of ApoB100 protein is present in each intermediate density lipoprotein (IDL), very low density lipoprotein (VLDL) and low-density lipoprotein (LDL) particle, but it is the primary apolipoprotein of LDL particles. The resting saliva ApoB100 concentration ranged from 3.3 to 372.9 ng/mL (mean 116.8

ng/mL), and the stimulated saliva concentration from 0.4 to 841.2 ng/mL (mean 209.5 ng/mL) (Suppl Fig 1). There was no significant difference between resting and stimulated saliva ApoB100 concentrations. The saliva ApoB100 levels did not correlate with plasma ApoB100 levels, plasma total cholesterol, plasma LDL, plasma HDL or plasma triglyceride levels (data not shown). Furthermore, the saliva ApoB100 levels were not associated with saliva or plasma antibody titers to various models of oxidized LDL (data not shown). However, plasma total cholesterol and LDL cholesterol levels were associated with resting saliva IgA levels (Fig 5). This serendipitous finding suggests a link between the mucosal immune system and cholesterol metabolism, and needs to be validated in a larger study cohort.

Plasma autoantibody binding to oxLDL, *A.actinomycetemcomitans and P.gingivalis*

Plasma antibody binding to various models of oxLDL, *Aa* and *Pg* (Suppl Fig 3) was investigated to evaluate the association to saliva antibody levels. The subjects had high plasma IgG and IgM antibody levels to oxidized epitopes, particularly to MAA-LDL. The plasma levels of IgA antibodies binding to oxidized epitopes and oral pathogens were low. No significant associations were observed between plasma and saliva antibody levels to oxLDL, *Aa* or *Pg* (data not shown).

Discussion

Mucosal membranes, including the oral cavity, are important sites of antigenic encounter, and mucosal IgA antibodies are recognized to participate in the regulation of commensal bacteria (Brandtzaeg2013). Here, we found significant levels of saliva IgA autoantibodies binding to oxidized LDL in healthy human subjects. Circulating autoantibodies to oxLDL have been linked to cardiovascular diseases, characterized by retention oxLDL in the arterial wall and activation an inflammatory response and development of atherosclerotic lesions.

In vivo oxidative modification of LDL was discovered several decades ago (Palinski et al. 1989), and subsequently a broad spectrum of oxidized epitopes conjugated to ApoB100 have been reported and documented to induce immune response. Oxidized LDL arise in lipid peroxidation of polyunsaturated fatty acids (PUFAs) of phospholipids within LDL particles, and several different antigenic epitopes are likely to be created (Hörkkö et al. 2000). For example, malondialdehyde (MDA), malondialdehyde acetaldehyde (MAA), 4-hydroxynonenal (4HNE-LDL), 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) are present in oxidatively modified low density lipoprotein and have also been found in atherosclerotic lesions (Palinski et al. 1989, Hörkkö et al. 1999). These lipid peroxidation end-products also closely modify proteins other than LDL ApoB100. For example, in the present study the immunogenic epitopes for saliva IgA were also MAA- and PC-adducts in the BSA protein. This suggests that saliva IgA specifically recognizes oxidized lipid adducts, malondialdehyde acetaldehyde or phosphocholine adducts, and the binding is not exclusively dependent on the carrier molecule.

Circulating plasma autoantibodies to oxidative conjugates of LDL were already documented in the early 1990s (Salonen et al. 1992, Virella et al. 1993). Since then, numerous studies have been published, but the role of autoantibodies to oxLDL in cardiovascular disease has yielded varying results (Karvonen et al. 2003, Boullier et al. 1995, Tornvall et al. 2003). Despite this, increasing evidence shows that high levels of IgM antibodies to oxLDL are inversely associated with cardiovascular disease, suggesting a protective role for these antibodies (Tsimikas et al. 2012, Karvonen et al. 2003). Many of the IgM-type autoantibodies binding to oxLDL are thought to be natural antibodies with high homology to germ-line encoded variable regions (Tsiantoulas et al. 2014). Currently, it is not fully known what mechanisms are responsible for the natural IgM antibodies to confer atheroprotection, and whether there are other target antigens involved. One of

the suggested mechanisms for atheroprotection has been the ability to bind to oxLDL and inhibit its uptake by macrophage scavenger receptors (Hörkkö et al. 1999). The data of the present study revealed that human saliva contained very minimal amounts of IgM, and also anti-oxLDL-IgM, suggesting that natural IgM-type autoantibodies may not be dominant contributing players on mucosal surfaces and oral immunity linked to atherosclerosis and periodontal disease.

Human atherosclerotic lesions contain bacterial DNA from more than 50 bacterial species with high bacterial diversity (Ott et al. 2006). As noted above, human atherosclerotic lesions are also well documented to contain oxLDL and antibodies binding to oxLDL (Ylä-Herttuala et al. 1989, Tsiantoulas et al. 2014). Our previous studies have investigated the antigenic epitopic molecular mimicry between *P.gingivalis* and oxLDL using monoclonal antibodies cloned against oxLDL, and we showed cross-reactive epitopes on the hemagglutinin/ adhesin domain of *Pg* gingipain protease and MDA-LDL (Turunen et al. 2012). Recently, we discovered *A. actinomycetemcomitans* chaperonin 60 or HSP60 as another antigenic epitope cross-reactive with oxLDL (Wang et al. 2016). Both of these mouse monoclonal antibodies were close to germ-line encoded natural IgM antibodies. It is believed, however, that very little germ-line encoded natural IgA would be present in humans colonized with a normal microflora, as most intestinal plasma cells in humans and aged mice have highly mutated antibody genes (Lindner et al. 2012, Barone et al. 2011). Our competitive immunoassays showed that a large portion of the polyclonal saliva IgA binding to MAA-LDL was also competed by *P.gingivalis* suggesting the presence of some mucosal saliva IgA antibodies to oxLDL having antigenic epitopes cross-reactive with the periodontal pathogenic bacteria *P.gingivalis*.

The link between atherosclerosis and periodontal disease has been shown by several large-scale epidemiological studies (Schenkein & Loos2013, Lockhart et al. 2012). Various hypotheses for the observed association have been proposed, including systemic inflammation, bacteremia caused by periodontal pathogens and molecular mimicry through cross-reaction of autoantibodies (Lockhart et al. 2012, Chun et al. 2005). Elevated levels of serum antibodies to *A.actinomycetemcomitans* or *P.gingivalis* associate with an increased risk for incident CAD and stroke (Pussinen et al. 2005). The link between periodontal disease and the development of atherosclerosis and whether it includes molecular mimicry has not yet been fully resolved. In principle, when the structural properties of two antigens are sufficiently similar to allow them to mimic one another, the antibodies generated may cross-react. Molecular mimicry due to sequence similarities between e.g. foreign and self-peptides may cause disease (Kohm et al. 2003). Cross-reactivity of autoantibodies

between periodontal bacterial lipopolysaccharides and heat shock proteins has been previously reported (Schenkein & Loos2013). In atherosclerosis, vaccination with *S. pneumoniae* has been shown to be atheroprotective in mice and suggested to act via antibody cross-reaction and molecular mimicry with oxLDL antigens (Binder et al. 2003). Similarly, vaccination with MDA-LDL was shown to ameliorate atherosclerosis in $LDLR^{-/-}$ mice challenged with live *P.gingivalis* (Turunen et al. 2015). The data of the present study demonstrated that there may exist cross-reactive epitopes in oxidized proteins and periodontal bacteria, and it can be further hypothesized that molecular mimicry of these epitopes induces mucosal immune responses linking cardiovascular and periodontal diseases.

Immunoglobulin A is the isotype dominating on mucosal surfaces, and also the second most abundant immunoglobulin found in plasma. Mucosal secretory IgA has dimeric structure, complexed with the ectodomain of the polymeric immunoglobulin receptor (pIgR) when transcytosed through the epithelial layer into mucosal secretions. T cells have been shown not to be an absolute requirement for IgA generation (Pabst2012). Both monomeric and dimeric IgA bind to FcαRI, triggering receptor clustering and downstream signaling that activates e.g. phagocytosis and antibody-dependent cell-mediated cytotoxicity. Human circulation contains different classes of autoantibodies to oxLDL, including IgA isotype of autoantibodies (Wu et al. 2003). Little is known about the origin and role of plasma anti-oxLDL-IgA, but our previous data suggested that circulating plasma IgA to oxLDL is linked to glucose metabolism, and high IgA was shown to be an independent risk factor for type 2 diabetes mellitus (Sämpi et al. 2010). The plasma levels of IgA antibodies to oxLDL have also been associated with plasma IgA antibodies to heat shock proteins HSP 60 and HPS65, and also with plasma IgA to *A. actinomycetemcomitans* (Buhlin et al. 2015). The potential role(s) of anti-oxLDL-IgA in saliva calls for further studies.

In summary, significant levels of saliva IgA autoantibodies binding to oxLDL and malondialdehyde adducts were found in the saliva of healthy humans with a low salivary burden of periodontal bacteria. These antibodies showed cross-reactive binding to *P.gingivalis,* and may participate in immune reactions involved in atherosclerosis and periodontal disease.

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Figure legends:

Figure 1

Resting (R) and stimulated (S) saliva antibody binding to oxidized LDL. Panel A, immunoglobulin A; Panel B, immunoglobulin G; Panel C, immunoglobulin M. Copper oxidized, CuOx-LDL; malondialdehyde acetaldehyde modified LDL, MAA-LDL, malondialdehyde acetaldehyde MAA modified bovine serum albumin, MAA-BSA; phosphocholine modified BSA, PC-BSA. Duplicate measurements were performed. The box plots represent 25%, 50% and 75%, and the whiskers represent 5% and 95% distribution of the values. The solid squares represent the mean values.* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 2

Stimulated saliva antibody binding to bacteria antigens. Panel A, immunoglobulin A; Panel B, immunoglobulin G, *Aggregatibacter actinomycetemcomitans* (*Aa*); *Porphyromonas gingivalis* (*Pg*). , *Escherichia coli* (*E.coli*), *Pg* hemagglutinin/adhesion domain of gingipain Rgp44 and heat shock proteins HSP60 Duplicate measurements were performed. The box plots represent 25%, 50% and 75%, and the whiskers represent 5% and 95% distribution of the values. The solid squares represent the mean values. ** $p < 0.01$, *** $p < 0.001$.

Figure 3

Association of stimulated saliva immunoglobulin binding to oxidized-LDL, *Aggregatibacter actinomycetemcomitans* (*Aa*) and *Porphyromonas gingivalis* (*Pg*). Panels A-C, malondialdehyde acetaldehyde LDL, MAA-LDL; Panels D-F, copper oxidized LDL, CuOx-LDL. Associations were determined using Spearman correlation coefficient. The data represent chemiluminescence relative light unit (RLU) values.

Figure 4

Competitive immunoassay to test the specificity of saliva antibody binding to oxidized LDL and periodontal bacteria. Panel A, stimulated saliva antibody binding to oxidized LDL in the absence (B_0) or presence (B) of increasing amounts of soluble *Porphyromonas gingivalis* (*Pg*), *Aggregatibacter actinomycetemcomitans* (*Aa*), Malondialdehyde acetaldehyde LDL (MAA-LDL), copper oxidized LDL (CuOx-LDL) or native-LDL (nLDL) as competitors. Malondialdehyde acetaldehyde LDL, MAA-LDL, was used as solid-phase antigen. Panel B, stimulated saliva IgA binding to MAA-LDL in all samples (n=36) in the presence of *P.gingivalis* as competitor (200

ug/mL). Duplicate measurements of one saliva sample for each competition concentration are shown. In panel A each competitor was compered with nLDL using Wilcoxon test for 2 related samples. * $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$.

Figure 5

Association between plasma total cholesterol levels, LDL cholesterol levels and resting saliva IgA levels. Associations were determined using Spearman correlation coefficient.

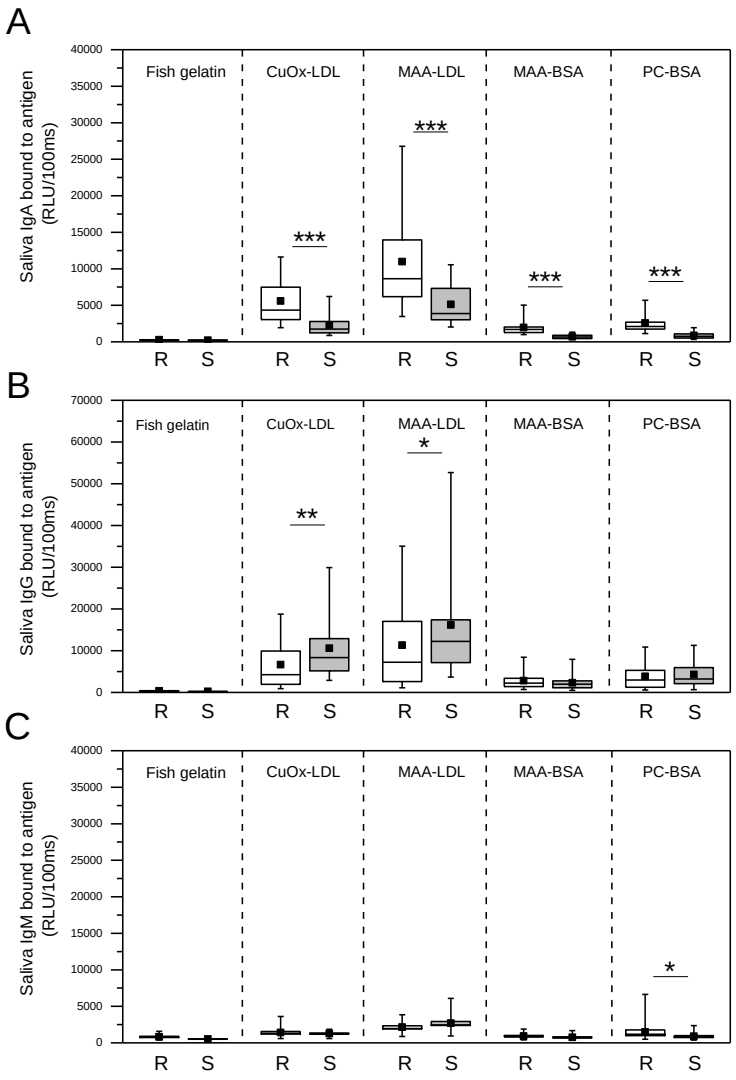

A
Saliva IgA bound to antigen (RLU/100ms)
Fish gelatin
CuOx-LDL
MAA-LDL
MAA-BSA
PC-BSA
***
***
***
***
R S R S R S R S R S
B
Saliva IgG bound to antigen (RLU/100ms)
Fish gelatin
CuOx-LDL
MAA-LDL
MAA-BSA
PC-BSA
**
*
R S R S R S R S R S
C
Saliva IgM bound to antigen (RLU/100ms)
Fish gelatin
CuOx-LDL
MAA-LDL
MAA-BSA
PC-BSA
*
R S R S R S R S R S

Figure 1

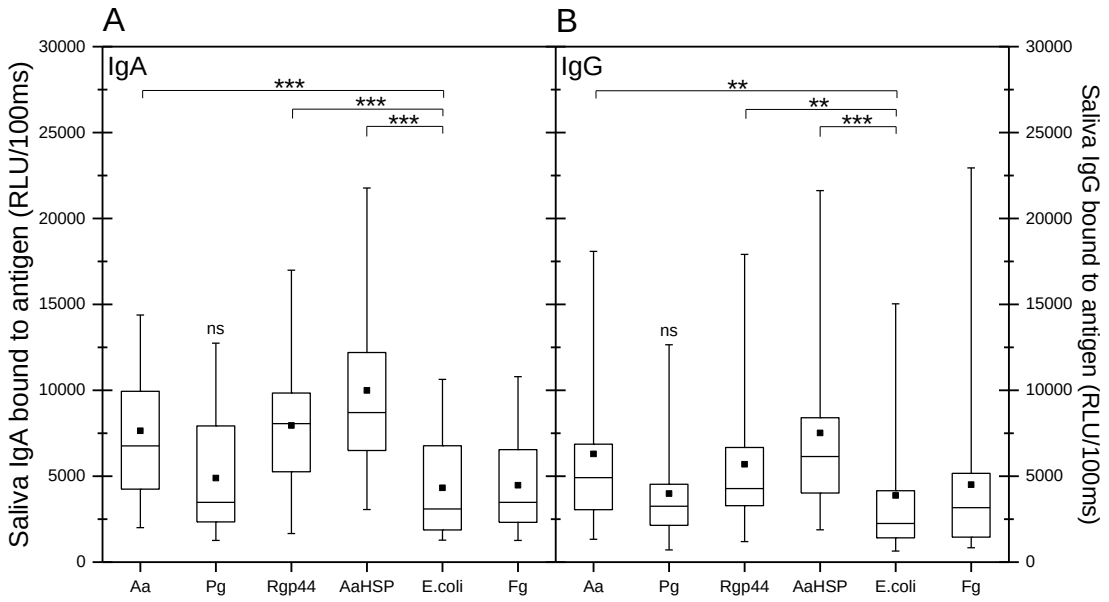

A
B
IgA
IgG
Saliva IgA bound to antigen (RLU/100ms)
Saliva IgG bound to antigen (RLU/100ms)
30000
25000
20000
15000
10000
5000
0
***
***
***
**
**
***
ns
ns
Aa
Pg
Rgp44
AaHSP
E.coli
Fg

Figure2

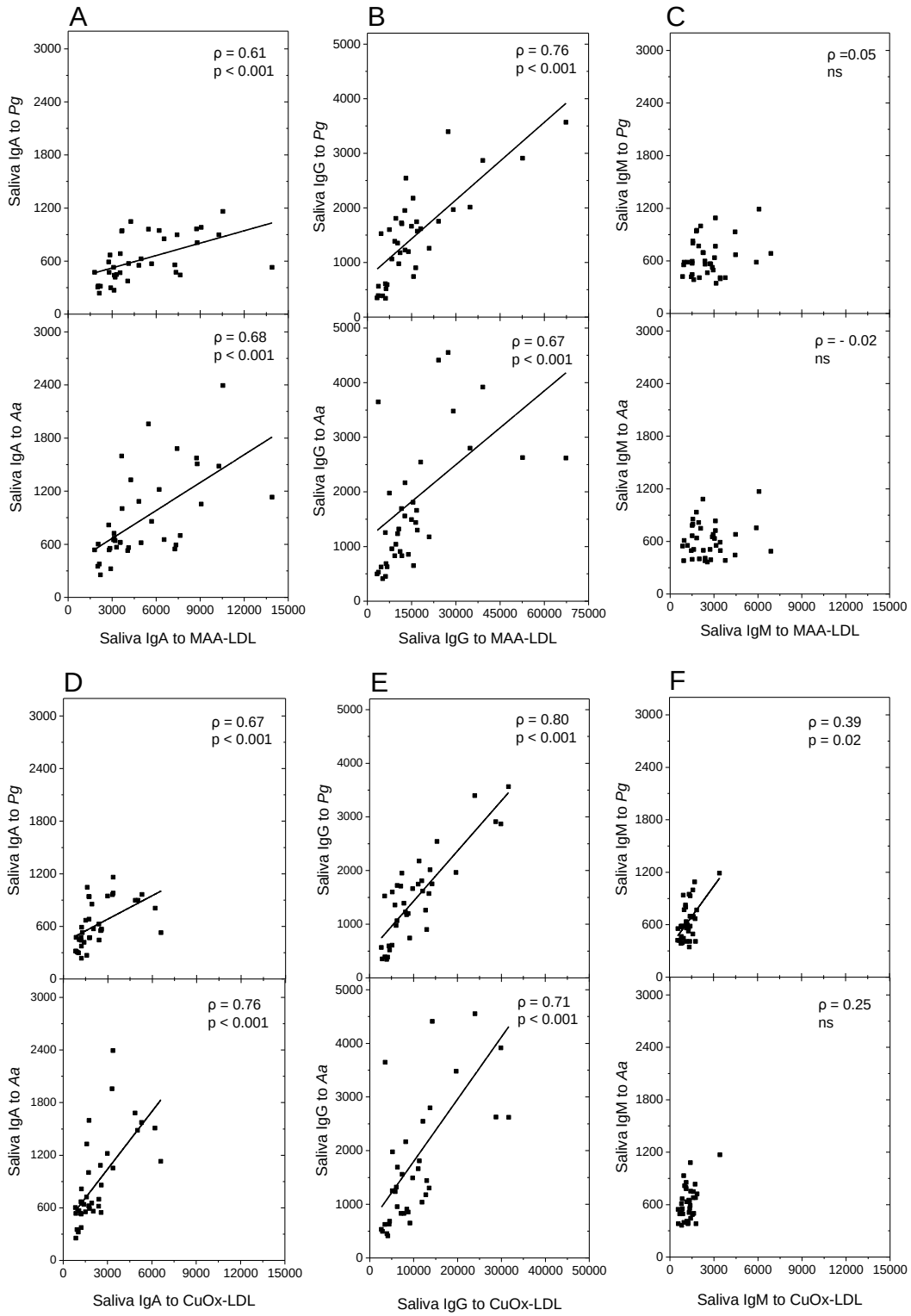

A
B
C
D
E
F
Saliva IgA to Pg
Saliva IgA to Aa
Saliva IgA to MAA-LDL
ρ = 0.61
p < 0.001
ρ = 0.68
p < 0.001
Saliva IgG to Pg
Saliva IgG to Aa
Saliva IgG to MAA-LDL
ρ = 0.76
p < 0.001
ρ = 0.67
p < 0.001
Saliva IgM to Pg
Saliva IgM to Aa
Saliva IgM to MAA-LDL
ρ =0.05
ns
ρ = - 0.02
ns
Saliva IgA to CuOx-LDL
ρ = 0.67
p < 0.001
ρ = 0.76
p < 0.001
Saliva IgG to CuOx-LDL
ρ = 0.80
p < 0.001
ρ = 0.71
p < 0.001
Saliva IgM to CuOx-LDL
ρ = 0.39
p = 0.02
ρ = 0.25
ns

Figure3

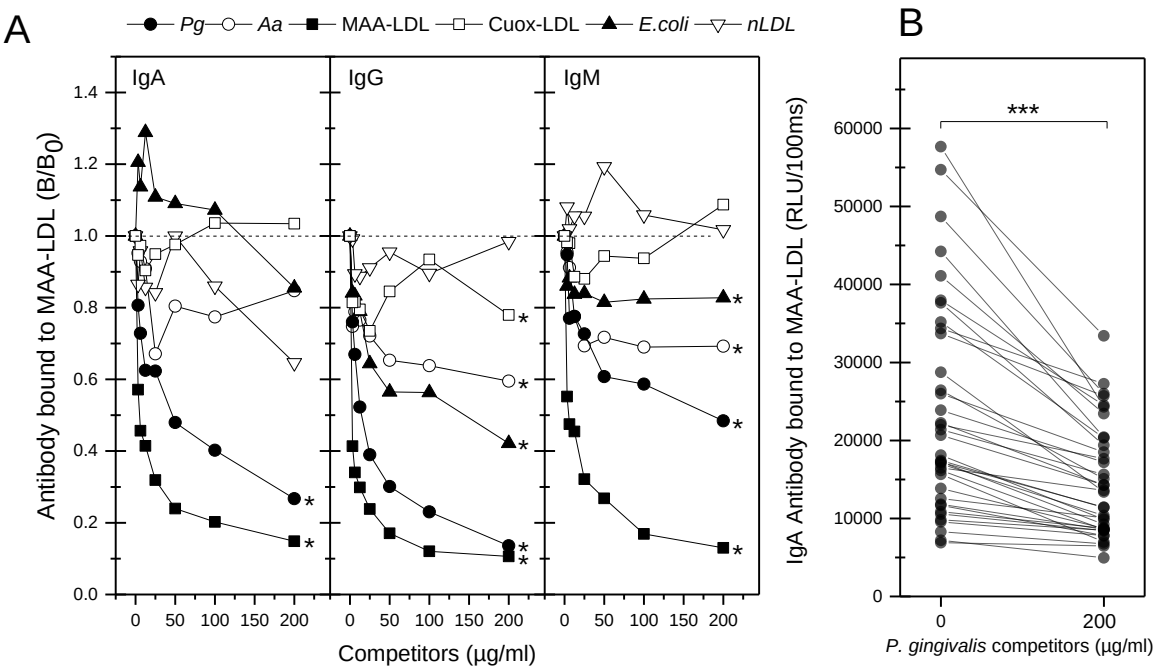


Figure4

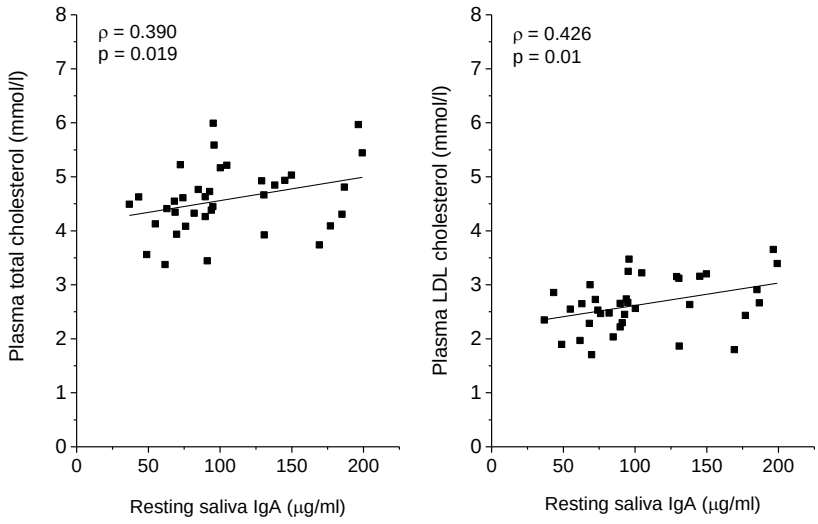
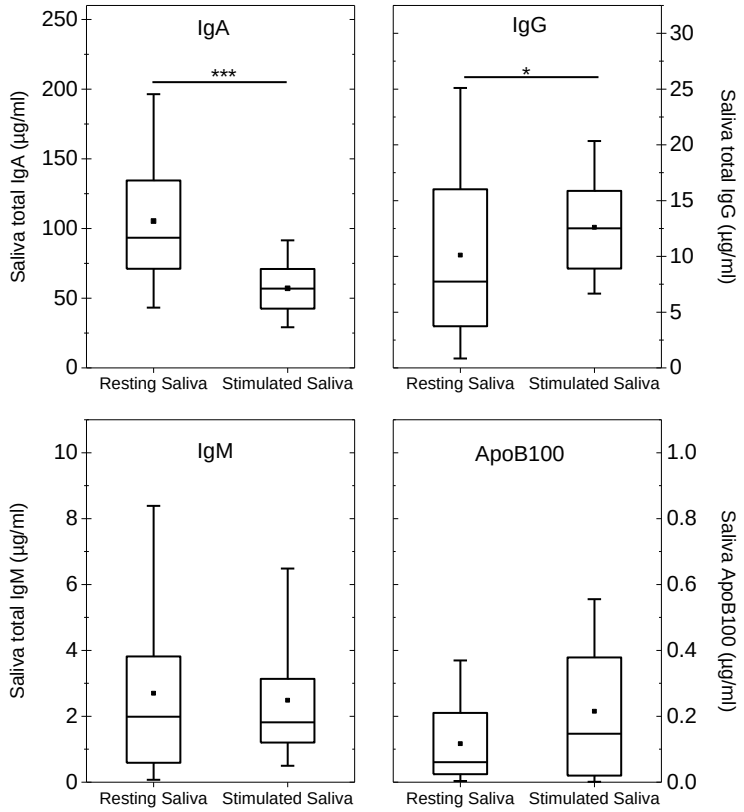
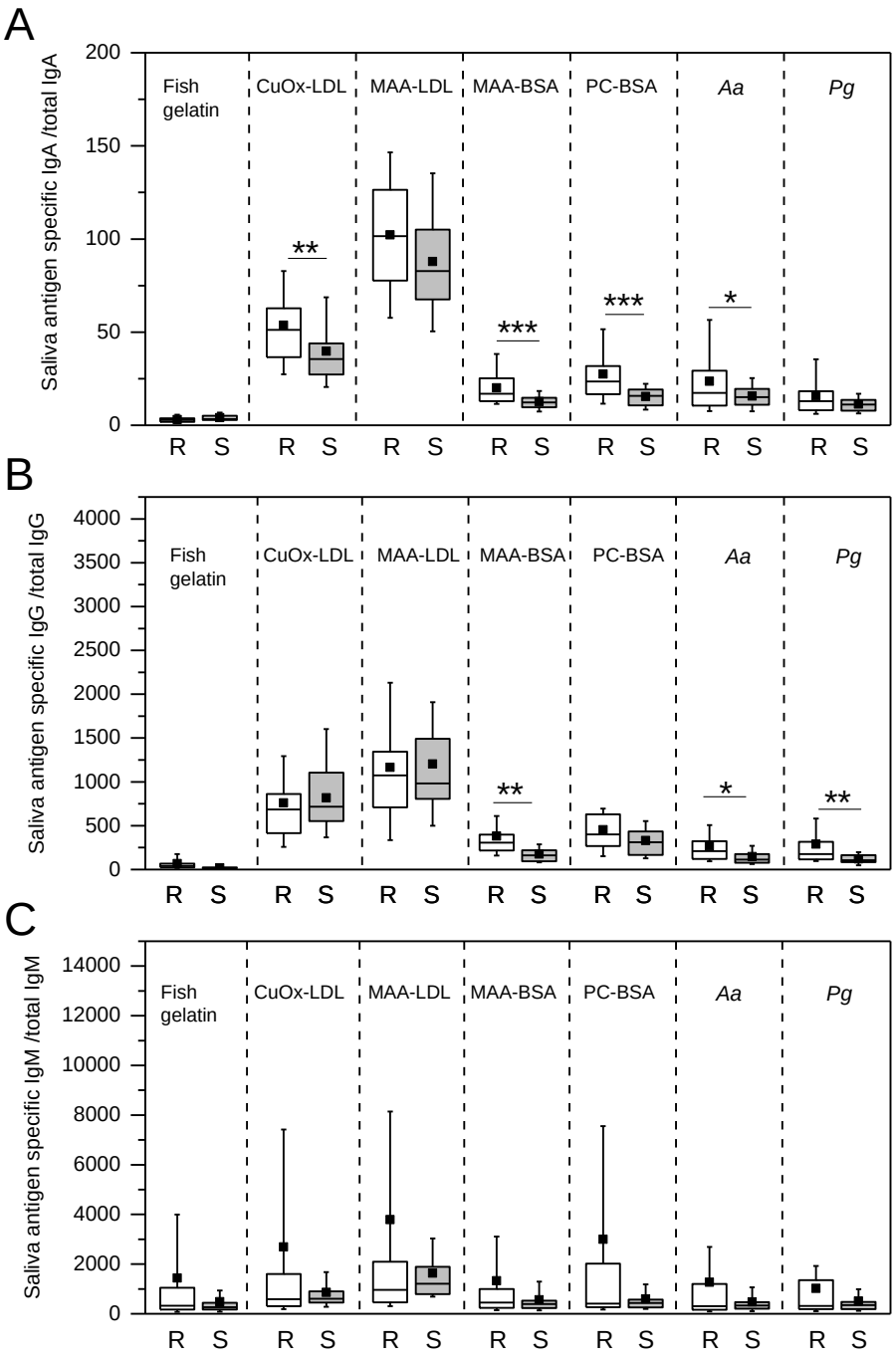


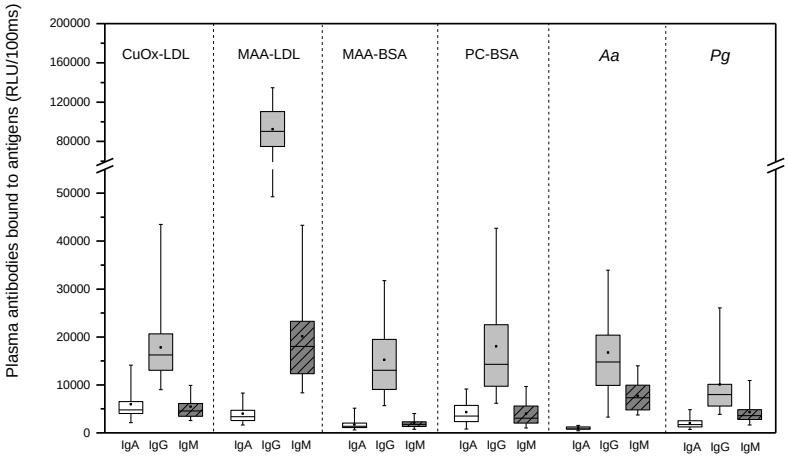
Figure 5



Supl. Figure 1



Supl. Figure 2



Supl. Figure 3