Exploring seropositive rheumatoid arthritis: from immunological depths to clinical course

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Evidence of site-specific mucosal autoantibody secretion in rheumatoid arthritis

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Abstract

Objectives Anti-citrullinated protein antibodies (ACPA) have been detected in sputum and saliva, indicating that anti-modified protein antibodies (AMPA) can be produced at mucosal sites in rheumatoid arthritis (RA) patients. However, the body's largest mucosal compartment, the gut, has not yet been examined. We therefore investigated the presence of several AMPA (antibodies against citrullinated (ACPA), carbamylated (anti-CarP) and acetylated (AAPA) proteins) at different mucosal sites, including the intestinal tract.

Methods Paired faecal/ileal wash, saliva and serum samples of RA patients and healthy volunteers were collected in two independent cohorts. Data involving faeces was replicated in a third cohort. In these secretions AMPA were analysed using in-house ELISA with unmodified peptides as control. In faecal samples total IgA and anti-E. coli IgA were measured.

Results ACPA, anti-CarP and AAPA IgA were measurable in saliva of seropositive RA patients (prevalence 9-40%). No AMPA could be detected in faeces. IgA was present since total IgA and anti-E. coli IgA was detectable in faeces of ACPA-positive RA patients and healthy donors. Results were confirmed in another cohort using colonoscopically collected ileal wash samples.

Conclusions Our study shows the presence of ACPA, anti-CarP and AAPA IgA in saliva of ACPA-seropositive RA patients. However, no AMPA could be detected in faeces/ileal wash samples of these patients, although our assays were able to measure other antigen-specific antibodies. These data suggest that mucosal autoantibody secretion may occur in the oral mucosa of RA patients, while no evidence could be found for this process in the lower gastro-intestinal tract.

Introduction

The presence of anti-modified protein antibodies (AMPA) directed against post-translational modified proteins (PTMs) is a hallmark of seropositive rheumatoid arthritis (RA). The most well-known and clinically important AMPA are anti-citrullinated protein antibodies (ACPA), while other AMPA recognize carbamylated proteins (anti-CarP) or acetylated proteins (AAPA). The processes leading to the break of tolerance against PTMs, to the maturation of the AMPA response, and eventually to the development of seropositive RA are not fully understood. One of the hypotheses gaining increased attention is that mucosal surfaces play a role in AMPA formation (1-3). Inflammation at mucosal surfaces, triggered by environmental factors and microbiome-host interactions in combination with the local presence of PTMs, might create conditions in which tolerance to PTMs is broken (1, 2, 4, 5).

There is accumulating evidence that the airway mucosa is involved in seropositive RA. Smoking, along with exposure to silica dust and other inhalants, are a major risk factor for the development of seropositive RA and the concurrent presence of multiple RA-associated antibodies (6-8). It is hypothesized that these environmental factors induce airway inflammation, which can contribute to autoantibody formation (9). However, the lungs are not the only mucosal sites which may be involved in RA development. The epidemiologic association between RA and periodontitis was already reported years ago (10). Oral and intestinal microbiome disturbances have been described in RA patients and people at risk of developing RA (11-14). Novel research shows that RA patients with ongoing periodontitis experience repeated bacteraemia with oral bacteria. Bacteria can be citrullinated and citrullinated bacterial epitopes can be recognized by ACPA. These findings provide an interesting potential link between a physiological anti-bacterial responses and autoimmunity in RA (15). The association between the lung and oral mucosa and RA is further substantiated by the discovery that ACPA can be present in both sputum, bronchoalveolar fluid and saliva of RA patients (16-18). Rheumatoid factor can also be present in saliva of those patients (19).

However, the largest mucosal site in the body, the intestine, has received less attention over the years compared to the lung and mouth. Lately, this has changed with the finding that monoclonals derived from circulating plasmablasts in individuals at risk of RA can bind both RA-associated citrullinated autoantigens and bacteria in faeces (20). These findings suggests that also the intestinal mucosa might be involved in the pathophysiology of RA. The presence of other AMPA (besides ACPA) at mucosal sites has not yet been investigated, though this could provide new information on the development of the AMPA response in RA, especially in the gut. AAPA might provide an interesting angle when it comes to

mucosal microbial exposures as potential trigger for autoimmunity, since various bacterial species use acetylation of self-proteins to regulate cell processes (21, 22). Anti-carbamylated protein responses may also have an intestinal origin, since carbamylation has been shown to occur in the human gastrointestinal tract (23). It therefore appears plausible that anti-CarP and AAPA could be produced at mucosal sites and that local availability of specific post-translational modifications, as a product of microbiome, food constituents and host cell interactions, might diversify and broaden the AMPA response in RA. Nevertheless, whether AMPA are secreted in the intestinal tract is currently unknown.

Hence, we investigated whether ACPA, anti-CarP and AAPA can all be detected in mucosal secretions, with emphasis on material derived from the intestinal tract of RA patients. To this end, we collected paired serum, saliva and faeces of RA patients and healthy donors and tested these samples for the presence of ACPA, anti-CarP and AAPA. Two other independent cohorts were used to replicate our findings.

Patients and Methods

Cohorts

In the Dutch MUCOSA (MUCosal Origin of Serum Autoantibodies in rheumatoid arthritis) study, paired serum, saliva, and faeces samples were collected cross-sectionally from 47 RA patients visiting the outpatient clinic (of whom 36 were ACPA-seropositive) and from 21 healthy controls. Saliva was collected by passive drooling and faeces was collected by participants themselves and immediately frozen. One patient suffered from severe hyposalivation precluding the collection of saliva. Details are provided in supplementary data 1.

To substantiate our findings, we made use of samples from another independent study. The Swedish IntestRA study included 20 ACPA-seropositive RA patients, 10 healthy donors and 9 patients with inflammatory bowel disease (IBD) as additional controls. Serum and saliva samples were collected in a similar fashion. However, to investigate the presence of autoantibodies in the gut, ileal wash fluid was collected via colonoscopy instead. Details are provided in supplementary data 2.

Faeces samples of a third cohort, the Dutch Plants For Joints trial (PFJ) (24, 25), were examined to corroborate the results regarding AMPA in faeces. Baseline faeces samples of 42 ACPA-seropositive RA patients and 10 osteoarthritis patients (OA) as control were investigated. Faeces was collected by participants themselves at home and sent by mail, after where samples were stored at -80°C.

In all three studies, all patients fulfilled the 2010 ACR/EULAR criteria for RA (26) and most had long-standing disease. Throughout this manuscript, ACPA-seropositive refers to ACPA IgG seropositivity. All studies were performed in concordance with the declaration of Helsinki, approved by the relevant local medical ethical committees and all participants provided written informed consent.

Measurements

The presence of AMPA and RF in serum, saliva, and faeces in the MUCOSA study was tested by in-house enzyme-linked immunosorbent assay (ELISA) using peptides containing different modifications on a CCP2 (Cyclic Citrullinated Peptide 2) backbone. Measurements in the PFJ trial were performed in accordance with the protocols used for faeces samples in the MUCOSA. In the IntestRA study, modified commercial anti-CCP assays (CCPlus® Immunoscan, Svar Life Science) were used to measure ACPA IgA in ileal wash and saliva samples. Autoantibody analyses in IntestRA focused on ACPA only. Total IgA levels and anti-E. coli antibodies were also measured in mucosal secretions by ELISA. For anti-E. coli, ELISA plates were coated with E. coli lysates. After blocking and adding undiluted faecal extracts, horseradish peroxidase (HRP)-labelled detection antibodies were used stepwise before visualization with ABTS (supplementary data 1). Furthermore, analysis of inflammatory markers in saliva (total protein and matrix metalloproteinase-8 levels (MMP-8)) and faeces (calprotectin) was performed in the MUCOSA study using ELISA kits (Total MMP-8 ELISA kit R&D systems; DMP800B and Calprotectin ELISA kit Orgentec; ORG580) according to manufacturer's instruction.

Saliva samples were homogenized and centrifugated before use to remove any debris. To be able to detect autoantibodies in faecal matter, protein fractions were prepared by diluting the faeces 1:5 in faeces dilution buffer (phosphate-buffered saline + 0.05M EDTA + 1.66mM phenylmethylsulfonyl fluoride (PMSF) + 0.1mg/ml soybean trypsin inhibitor (Sigma)). Samples were mixed vigorously for 10-20 minutes until homogeneous and spun down. Ileal wash samples were centrifuged and frozen within one hour after collection.

When testing for AMPA positivity, all samples were also measured simultaneously on the unmodified control peptide to investigate whether binding was specific for the post-translational modification. In the MUCOSA and PFJ, a sample was considered AMPA-positive when both of the following criteria were met: 1) the value measured on the modified peptide was higher than the cut-off based on the mean + 2 times the standard deviation (SD) of the signal of healthy controls on that peptide, and 2) the optical density (OD) of the signal measured on the modified peptide was >2 times higher than the OD of the same sample measured on the unmodified peptide. In the IntestRA study OD signals on the arginine control were first subtracted from the ACPA OD values. Thereafter, a cut-off was calculated in a similar fashion.

More information about sample processing and autoantibody detection can be found in supplementary data 1 (MUCOSA and PFJ) and 2 (IntestRA). Mann-Whitney U tests, Chi-square tests or Fisher's exact tests, as appropriate depending on the kind of data, were performed to compare antibody positivity and inflammatory markers between groups.

Results

AMPA in saliva

Mucosal autoantibodies were investigated in three independent cohorts, of which the clinical characteristics are listed in Table 1. First, autoantibodies were measured in serum and saliva of participants in the MUCOSA study. 17% of ACPA-seropositive RA patients in the MUCOSA had detectable ACPA IgA in saliva, while ACPA could not be detected in saliva of ACPA-seronegative patients or healthy donors (Figure 1A) (not significant (ns)). In the Swedish IntestRA study, ACPA IgA was found in saliva of 40% of the ACPA-seropositive patients (Figure 1B), while none of the IBD patients or healthy controls were positive (p=0.03 compared to healthy). However, the presence of AMPA was not limited to ACPA IgA. Also, anti-CarP IgA and AAPA IgA could be detected in saliva of seropositive RA patients in the MUCOSA (Figure 1C-D), although the number of patients positive for salivary autoantibodies was low (9%) for both autoantibodies. RF IgA could be found in saliva 46% of ACPA-seropositive of RA patients (Figure 1E) (p=0.001 compared to healthy) and was present more frequently compared to salivary ACPA IgA.

Notably, when examining reactivity to the modified peptides (citrulline, homocitrulline and acetylated lysine) and unmodified peptides (arginine and lysine respectively) in saliva in more detail, a substantial number of seropositive RA patients, but not seronegative patients or healthy controls, had high reactivity to the modified peptide, but also showed a similarly high degree of reactivity to the unmodified peptide (Figure 2A-C). When the reactivity to the modified and unmodified peptide was similar, antibody binding was not specific for the PTM and samples were considered AMPA negative. The high signal measured on the unmodified peptide in saliva samples is markedly different from serum, where background signals are usually very low (Figure 2D-F). In the IntestRA unmodified peptide signals were more equally distributed among RA patients and controls (Figure 2G) (ns).

Table 1: Patient characteristics of all cohorts at inclusion.

	MUCOSA		IntestRA			Plants For Joints	
	RA	Healthy	RA	Healthy	IBD	RA	OA
	n=47	n=21	n=20	n=10	n=9	n=42	n=10
Age, mean ± SD	59 ± 13	48 ± 16	61 ± 9	62 ± 9	30 ± 8	55 ± 12	62 ± 6
Female, n (%)	37 (79)	13 (62)	15 (75)	7 (70)	6 (67)	36 (86)	10 (100)
Disease duration, years, median (IQR)	14 (8-16)	-	0.7 (0-12)	-	-	6.5 (3-15)	-
Smoking ever, n (%)	26 (55)	6 (29)	12 (60)	4 (44) (n=9)	3 (33)	-	-
ESR, mm/h median (IQR)	9 (2-34)	-	13 (8-20)	6 (4-24)	8 (4-15)	15 (8-32)	-
DAS28, median (IQR)	2.6 (1.7-3.6) (n=45)	-	3.0 (2.0-4.0) (n=10)	-	-	3.9 (3.2-4.5)	-
Serum antibody positivity[a]							
ACPA IgG, n (%)	36 (77)	0 (0)	20 (100)	0 (0)	0 (0)	42 (100)	-
Anti-CarP IgG, n (%)	21 (45)	0 (0)	-	-	-	-	-
AAPA IgG, n (%)	21 (45)	0 (0)	-	-	-	-	-
ACPA IgA, n (%)	18 (38)	0 (0)	20 (100)	0 (0)	0 (0)	-	-
Anti-CarP IgA, n (%)	4 (9)	0 (0)	-	-	-	-	-
AAPA IgA, n (%)	5 (11)	0 (0)	-	-	-	-	-
RF IgM n (%)	34 (72)	3 (14)	-	-	-	-	-
RF IgA, n (%)	21 (45)	1 (5)	-	-	-	-	-

AAPA, anti–acetylated protein antibodies; ACPA, anti–citrullinated protein antibodies; CarP, carbamylated protein antibodies; DAS28, Disease Activity Score in 28 joints; ELISA, enzyme-linked immunosorbent assay; ESR, erythrocyte sedimentation rate; IBD, inflammatory bowel disease; IQR, interquartile range; MUCOSA, MUCosal Origin of Serum Autoantibodies in rheumatoid arthritis; OA, osteoarthritis; RA, rheumatoid arthritis; RF, rheumatoid factor. All serum autoantibody measurements in the MUCOSA study are done with in-house ELISAs. [a]All serum autoantibody measurements in MUCOSA are done with in-house ELISAs.

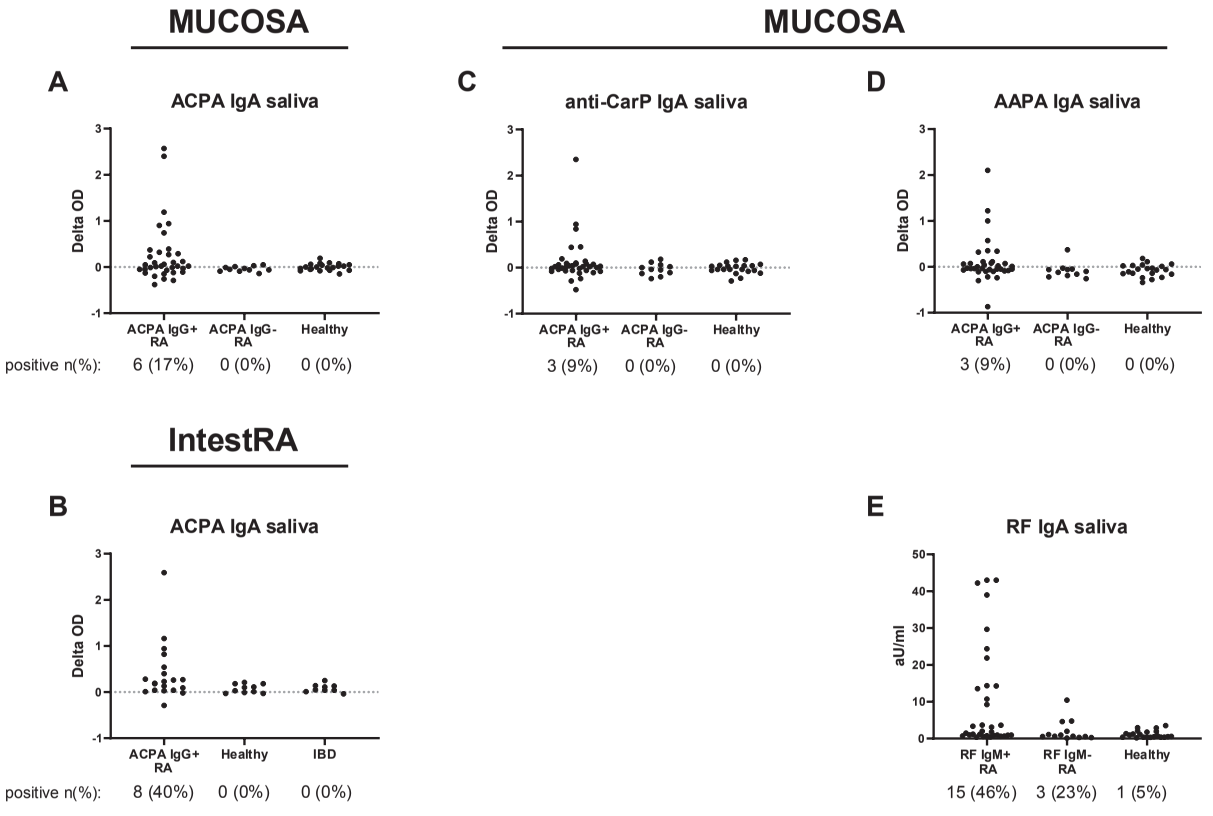

Figure 1: Autoantibody measurements in saliva. A ACPA IgA in saliva in MUCOSA, B ACPA IgA in saliva in IntestRA, C anti-CarP IgA, D AAPA IgA, E RF IgA in saliva in MUCOSA. Delta OD (difference in OD between modified peptide and unmodified peptide) for AMPA and aU/ml (arbitrary units per ml) for RF are depicted. Groups on X-axis are based on diagnosis and seropositivity (graph E uses RF IgM seropositivity to define groups). The number (%) of positive patients for that specific autoantibody is given.

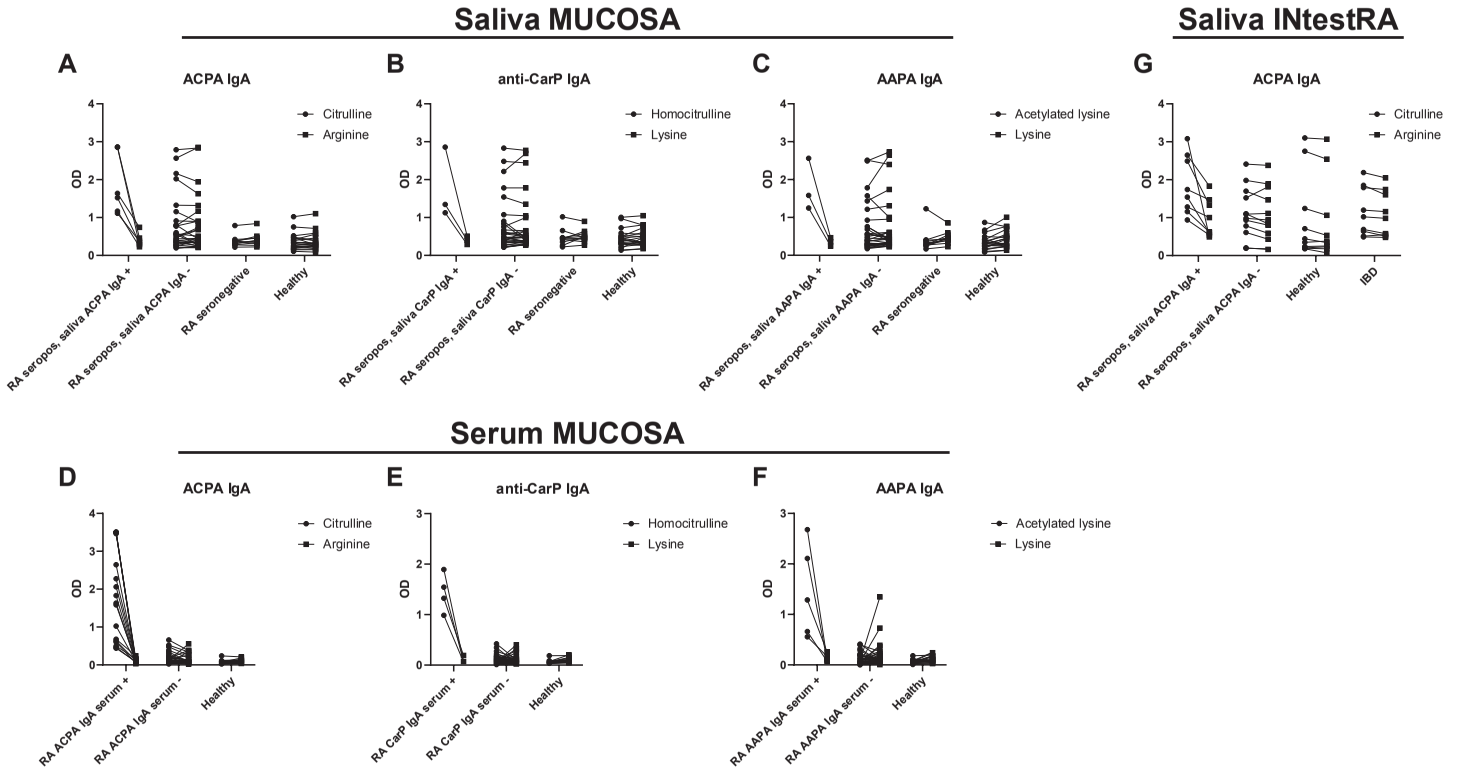

Figure 2: Paired optical density (OD) values on the modified and unmodified peptide for each AMPA. Paired OD values on the modified and unmodified peptide for each AMPA in saliva (A-C) and serum (D-F) of patients and healthy donors in MUCOSA and in saliva (G) of patients and controls in IntestRA. A-C First group of each graph shows all seropositive RA patients who tested positive for that specific AMPA in saliva, while the second group depicts all seropositive RA patients who tested negative for that specific AMPA in saliva. D-F First group shows patients who were positive for that specific AMPA IgA in serum, while the second group shows patients negative for that specific AMPA IgA in serum.

Next, we investigated whether the presence of autoantibodies in saliva was related to the amount of total IgA in these samples, as salivary IgA levels can differ between individuals and over time (27). There was no significant difference in salivary total IgA between seropositive RA patients who were positive for AMPA in their saliva and those who were negative (Supplementary Figure 1). Therefore, it seems that prevalence of IgA AMPA in saliva cannot solely be attributed to differences in salivary total IgA levels, but may rather point to inherent differences between patients.

AMPA profile in saliva and serum

Different types of AMPA in saliva tend to co-occur. Among the 7 saliva AMPA positive patients in the MUCOSA, 2 were triple positive for ACPA, anti-CarP and AAPA and 1 patient was anti-CarP and AAPA double positive (Figure 3). Furthermore, 6 saliva AMPA positive patients were also saliva RF IgA-positive. The presence of AMPA in saliva always coincided with the presence of that specific AMPA in serum, although the isotype could differ (Figure 3). For example, one patient is positive for AAPA IgA in saliva, while AAPA IgG but no AAPA IgA could be detected in serum. Similar findings were made for rheumatoid factor. However, 3 RA patients who tested positive for RF IgA in saliva, tested negative for both RF IgM and IgA in serum. This suggests a local origin and subsequent secretion of autoantibodies rather than leakage from serum antibodies to the saliva. Furthermore, the amount of IgG measured in these saliva samples is on average ± SD, 9 ± 8 μg/ml, a thousand-fold lower than serum IgG (reference levels 7-16 g/L), making contamination of saliva samples with serum autoantibodies in measurable amounts less likely. Thus, the saliva autoantibody profile displays similarity to, but does not necessarily originate from, the serum autoantibody profile.

Saliva AMPA and local inflammation

Next, we investigated whether the presence of salivary autoantibodies was associated with oral inflammation. Total protein content, matrix metalloproteinase-8 levels (MMP-8) and total IgA levels in saliva have been determined as markers of local inflammation (27, 28). In the MUCOSA, RA patients in general had slightly lower total IgA levels in saliva compared to healthy donors, while there was a non-significant trend towards higher MMP-8 and total protein values in RA patients. There were no significant differences in all three salivary inflammatory markers between RA patients who were positive for ACPA in their saliva (median (IQR): total protein 1380 μg/mL (1057-1747), total IgA 343 μg/mL (253-562), MMP-8 123 ng/mL (33-150)) and those who were not (median (IQR): total protein 1411 μg/mL (1040-1621), total IgA 259 μg/mL (169-386), MMP-8 83 ng/mL (26-138)) (Supplementary Figure 2A-C). As the number of ACPA saliva-positive patients is small, salivary inflammatory markers were also compared between ACPA-seropositive RA patients, ACPA-seronegative RA patients and healthy

donors (Supplementary Figure 2D-F), which showed similar results. As smoking directly affects the oral mucosa, the relation between autoantibody positivity in saliva and smoking was examined. No significant relation between smoking and the presence of autoantibodies was seen in saliva (Supplementary Table 1), despite the significant association between RF IgM-seropositivity and smoking in the MUCOSA study (p=0.04) and a similar trend for ACPA-seropositivity (Supplementary Table 1).

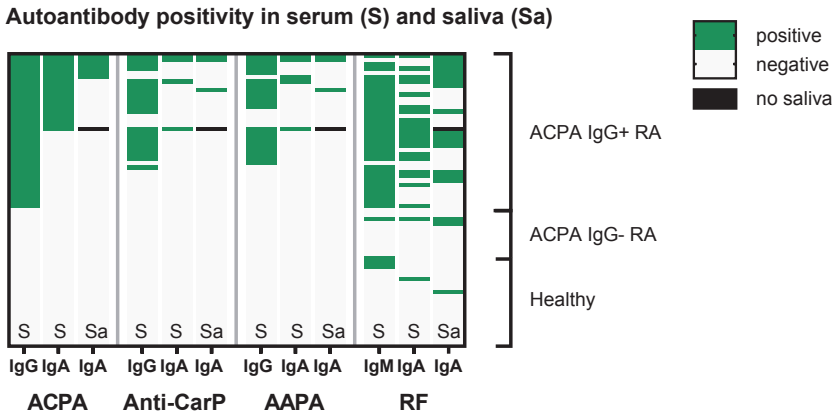

Figure 3: Autoantibody profile in serum (S) and saliva (Sa) in MUCOSA. Positivity for autoantibodies in serum and saliva. Each row depicts a study participant. Black field: no saliva available due to hyposalivation.

AMPA in the intestinal tract

In the MUCOSA, no ACPA, anti-CarP or AAPA were found in faeces samples of RA patients or healthy controls (Figure 4A-C), as there was almost no difference between the signals on the modified and unmodified peptides. Also in the PFJ, no AMPA was found in faeces (Figure 4D-F).

Like in saliva, reactivity to the modified and unmodified peptides was evaluated separately in faeces as well. Multiple ACPA-seropositive RA patients, ACPA-seronegative RA patients and controls showed an OD >1 to the citrullinated peptide in their stool, but also to unmodified arginine-containing peptide, which was thus considered as non-specific binding/AMPA negative (Figure 5A-B). Similar results were found for anti-CarP and AAPA (Figure 5C-F) in both cohorts. The high OD signals on the unmodified peptides in faeces are in contrast to saliva, where only seropositive RA patients showed a high background (Supplementary Figure 3).

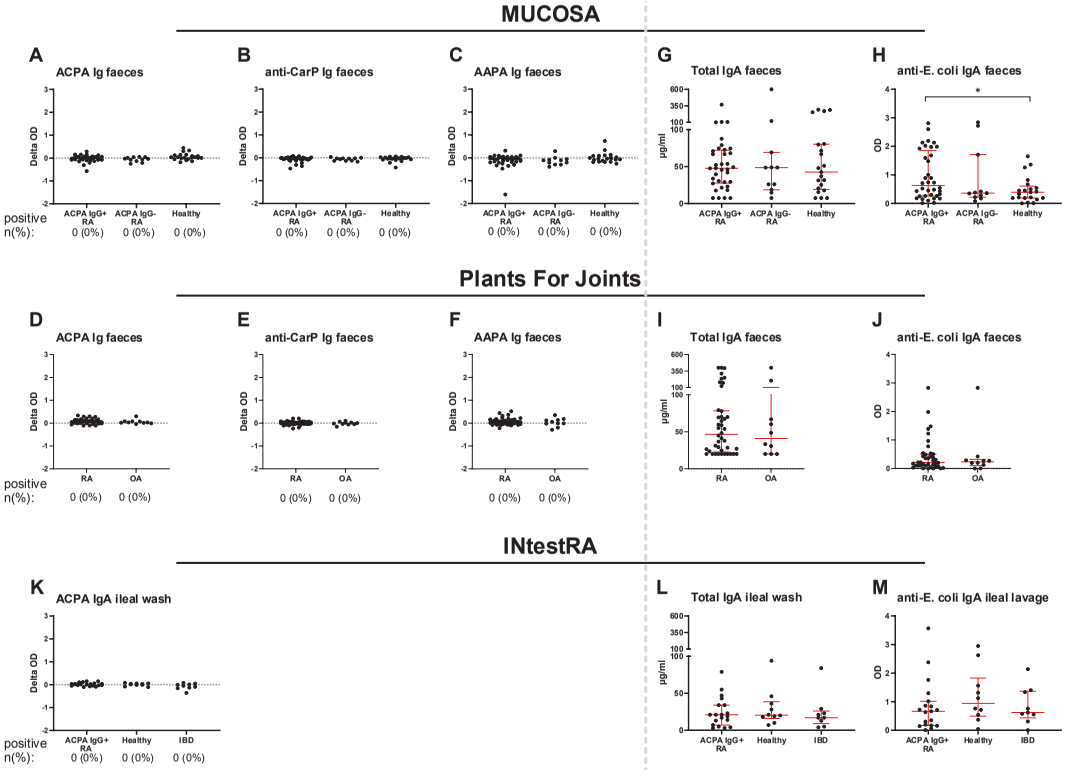

Figure 4: AMPA Ig in faeces and ileal lavage of RA patients. A-C ACPA, anti-CarP and AAPA Ig respectively, in faeces from patients and healthy donors in MUCOSA. Groups on X-axis are based on diagnosis and seropositivity, D-F AMPA Ig in faeces of PFJ, G/I Total IgA levels in µg/ml and H/J OD on the anti-E. coli IgA ELISA in the same faeces samples, K ACPA IgA in ileal lavage samples from IntestRA, L Total IgA levels and M anti-E. coli IgA OD in the same ileal lavage samples. For AMPA, the number (%) of positive patients is given. For AMPA the Y-axis depicts difference in OD (delta OD) between the modified and unmodified peptide. Reds bars show the median and interquartile range. *p<0.05

To determine whether the faecal supernatants contained sufficient amounts of immunoglobulins to fall within the detection range of our ELISAs, total IgA levels were measured. Faecal supernatants in MUCOSA contained a median of 48 μg/ml total IgA, with no significant differences between seropositive RA patients, seronegative RA patients and healthy donors (Figure 4G). Similar results were found in the PFJ (Figure 4I). These total IgA levels are roughly comparable to the amount of total IgA in the diluted saliva samples used for AMPA ELISA. Furthermore, anti-E. coli IgA was used as additional (antigen-specific) control. High anti-E. coli signals could both be detected in RA patients and healthy donors. Interestingly, anti-E. coli reactivity was significantly higher in seropositive RA patients compared to healthy donors in MUCOSA (p=0.04) (Figure 4H), although numbers are small. In the PFJ no significant difference was observed (Figure 4J). These data indicate that the methods used are able to detect the presence of (antigen-specific) antibodies in faeces samples in general, and that the lack of AMPA signal is not due to the absence of total IgA in these samples.

To substantiate our findings regarding the intestines, we also investigated ileal wash samples, collected via colonoscopy in the independent IntestRA cohort. Antibodies in such samples might be less prone to degradation compared to faeces. However, also in the ileal wash samples no ACPA IgA was detected (Figure 4K), while there was total IgA (median 20.5 μg/ml) (Figure 4L) and anti-E. coli IgA (Figure 4M) detectable. ODs for both the modified and unmodified peptides were overall low (Figure 5G). Total IgA levels and anti-E. coli antibody signals were also slightly lower compared to the faeces samples, possibly due to dilution by the lavage fluid instilled in the ileum used to collect these samples.

Calprotectin in faeces of RA patients

Since previous reports have suggested that RA might be characterized by a leaky intestinal barrier, gut dysbiosis and inflammation (3, 11, 29, 30), intestinal inflammation was investigated in the MUCOSA. Calprotectin was measured in faeces of both RA patients and healthy donors by commercial ELISA (Orgentec) (Supplementary Figure 4A). Values above 200 μg/g are considered significantly elevated and reflect active inflammatory intestinal disease. 6/47 RA patients and 1/21 healthy control had calprotectin values above 200 μg/g and an additional 7 patients had slightly elevated calprotectin levels between 100-200 μg/g. However, 5/7 subjects with strongly elevated calprotectin levels and 4/7 subjects with slightly elevated calprotectin levels reported regular NSAID use (Supplementary Figure 4B) (ns, p=0.07). NSAIDS are known to cause gastro-intestinal mucosal damage (31) and it has been reported that 2 weeks of NSAID use in healthy individuals can lead to significantly elevated faecal calprotectin levels (32).

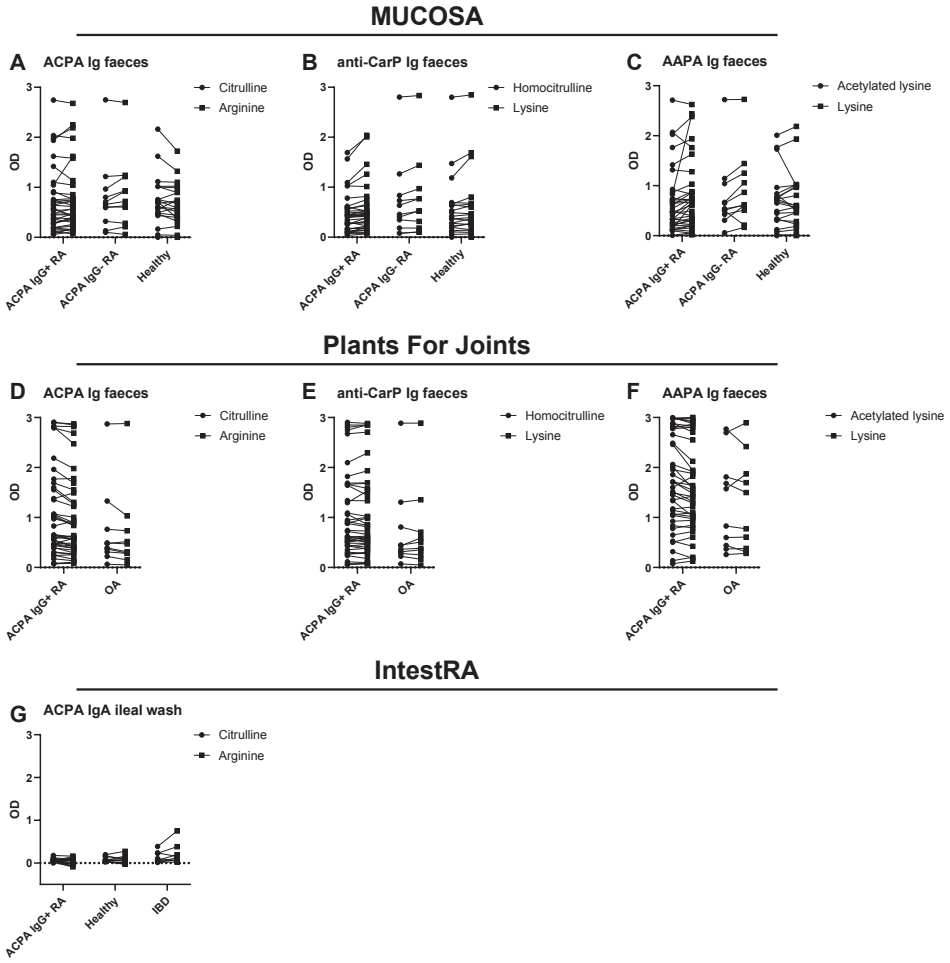

Figure 5: Paired optical density (OD) values on the modified and unmodified peptide for each AMPA Ig in faeces of patients and controls. A, C, E in MUCOSA and B, D, F in PFJ and ACPA IgA in ileal wash in patients and G controls in IntestRA.

Discussion

This study aimed to examine the RA-associated autoantibody profile in various mucosal compartments. In saliva of established seropositive RA patients, we found that ACPA, anti-CarP and AAPA IgA antibodies can all be present, although in modest quantities compared to serum. Differences in the percentage of saliva ACPA positive patients (17-40% in ACPA IgG seropositive RA) between cohorts might reflect the inclusion of only patients with moderate to high ACPA serum levels in

the IntestRA study. When salivary AMPA are present, the breadth of the response is similar to the AMPA serum profile. In contrast, no AMPA were found in faeces of the same RA patients, although the faecal supernatants did contain total IgA and anti-E. coli IgA antibodies. These findings were confirmed in faeces and in ileal lavage samples from two independent cohorts of RA patients. Our findings suggest that secretion of AMPA is limited to certain mucosal sites, with local secretion of all AMPA taking place in the oral cavity, but not to a detectable degree in the lower intestinal tract.

These observations are in line with previous studies showing the presence of salivary ACPA IgA in RA patients (17). ACPA IgA and IgG were also previously found in sputum of seropositive RA patients and first-degree relatives (FDR) of RA patients (16). Part of the FDRs who were positive for ACPA in their sputum, were serum ACPA-negative, indicating that sputum ACPA are produced locally and can precede or occur independently of a serological ACPA response. Our findings provide further evidence that autoantibodies found in mucosal secretions can be secreted locally at mucosal sites, as the AMPA isotype present in saliva was not always measurable in serum. The low amount of IgG present in the saliva samples may further support that both the detected AMPA and RF originated from the mucosal lining of the oral cavity instead of leaking from serum, since leakage would have led to a higher quantity of IgG as the most abundant isotype present in serum. Furthermore, monomeric IgA from serum cannot be actively transported over the mucosal epithelium by the polymeric immunoglobulin receptor, while mucosal derived dimeric IgA can.

These findings raise the question where the initial activation of autoreactive B-cells in RA can take place and which triggers elicit these anti-modified protein responses. Activated B-cells re-enter the tissue where they were activated based on homing marker expression, although there probably is some crossover to other, often anatomically closely related tissues (27). This suggests that the cells secreting AMPA in the oral mucosa, are probably derived from B-cells activated in local lymphoid tissue. Our study shows that the salivary AMPA response not only includes anti-citrullinated protein antibodies, but also antibody responses against carbamylated and acetylated proteins, suggesting the local presence of these antigens. Interestingly, bacteria can acetylate self-proteins (21, 22) and thus might evoke an anti-acetylated bacterial protein response, which could be cross-reactive to acetylated self-proteins. It is hypothesized that via this mechanism the antibody responses against acetylated bacterial content can contribute to diversification and epitope spreading of the AMPA response in RA. Furthermore, in the MUCOSA

seropositive RA patients tended to have a higher reactivity in saliva towards the unmodified peptides, compared to seronegative RA and healthy donors, although this was not as clear in the IntestRA. This higher reactivity towards unmodified peptides in seropositive RA patients could also point to activated humoral immune responses in general in these patients, for example due to decreased barrier function or local inflammation.

Our study did not include a dental examination to determine the presence of periodontitis, inflammation of the gums, which can be caused by bacterial infection. To gather some information on oral inflammation nonetheless, total protein content, MMP-8 levels and total IgA were measured in saliva. No association between these inflammatory markers and ACPA positivity in saliva was seen. This could be explained in several ways: the sensitivity of these markers might be more limited than a dental examination, and gingivitis or periodontitis could have been missed. Alternatively, a true lack of association could suggest that oral production of autoantibodies is independent of simultaneously occurring mucosal inflammation, and would either not require inflammation at all, or could be related to barrier dysfunction and inflammation in the past.

Not only the oral mucosa, but also the gut could represent a large source of citrullinated, carbamylated and acetylated (microbial) proteins. From an immunological point of view, it appears conceivable that a T-cell response against post-translationally modified bacteria (as foreign/non-self) may provide the required T-cell help to activate self-reactive AMPA directed B-cells. Reactivity to intestinal bacteria was found to be a normal property of the human CD4+ T cell repertoire in healthy individuals (33). Moreover, it has been described that monoclonal ACPA derived from individuals at risk for RA can bind bacterial isolates from human faeces (20). Based on these findings, one would have expected to find AMPA in intestinal secretions as well, but we did not find ACPA, anti-CarP or AAPA in faeces or ileal wash samples. This suggests there is no substantial ACPA production in the lower intestinal tract. Anti-E. coli IgA and total IgA were measurable in these intestinal samples, indicating that the methodology is adequate to detect (antigen-specific) antibodies.

Our results suggest that mucosal AMPA production is site specific, with local secretion of AMPA taking place in the oral mucosa, but not substantially in the gut. In addition, earlier studies provide evidence for AMPA responses in the airways (16, 18). This spatial variation might be due to differences in the local micro-environment, such as antigen availability and local inflammatory processes like NETosis (release of Neutrophil Extracellular Traps). However, there are several other reasons why AMPA might not

be detectable in the gut, for example due to strong binding to their antigen, degradation of antibodies by digestive enzymes, or the amount of AMPA being under the detection limit of our assays. The fact that total IgA and anti-E. coli IgA were detectable in faeces and that ACPA were also not present in ileal lavage samples, which might be less prone to degradation, makes it more likely the gut is not a major site of secretion for AMPA. Nevertheless, further research on barrier dysfunction and presence of PTMs in the intestinal tract of seropositive RA patients is warranted, as there are other potential mechanisms via which the intestinal mucosal compartment could contribute to the systemic AMPA response, which are beyond the scope of our study, such as microbiome dysbiosis, decreased intestinal barrier function and trafficking of immune cells primed in the intestine to the systemic circulation. For example, in 1 cohort there was a difference in faecal anti-E. coli reactivity between seropositive RA patients and healthy donors, which could point to increased interactions between gut bacteria and the immune system in RA.

Our study provides new insights in the autoantibody profile at mucosal surfaces, but it comes with some limitations. Most patients had longstanding RA and used various immunosuppressive therapies, which could have influenced the results. Previous studies investigating the effect of anti-rheumatic treatment on serologic AMPA responses have revealed that the presence of AMPA in serum is quite stable under treatment (34), but it is unknown whether mucosal AMPA responses originate from antibody secreting cells with similar (long-lived) characteristics. Positivity for secretory ACPA in serum declined more strongly compared to ACPA IgG after initiation of therapy (35). Furthermore, despite the use of three independent cohorts to verify our findings, the number of patients included is limited. Due the COVID-19 pandemic we were prohibited from collecting paired sputum samples in the MUCOSA, as originally planned. Therefore, whether anti-CarP and AAPA are also present in sputum of RA patients remains unknown.

To the best of our knowledge, our study nonetheless represents the most extensive investigation to date of a large variety of autoantibodies in a most diverse array of bodily fluids. Our results show that ACPA, anti-CarP and AAPA can be secreted locally in the oral mucosa. This suggests local immune responses against post-translational modified proteins, for example in the context of an anti-bacterial response, might contribute to the development and diversification of the AMPA response in RA patients. No support for local AMPA secretion in the lower intestinal tract was found. This study therefore, for the first time, sheds light on one of the possible roles (or potential lack thereof) of the intestinal mucosa in the onset of the AMPA responses in RA.

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Supplementary information

Supplementary data 1: Methodology MUCOSA study and faeces samples Plant For Joints

Patients and sample collection

All patients with a definite diagnosis of rheumatoid arthritis based on the ACR/EULAR 2010 criteria visiting the outpatient clinic of the Leiden University Medical Center (LUMC) were eligible for inclusion, regardless of disease duration or treatment. As a control, healthy donors without any history of inflammatory arthritis were included. All participants were 18 years or older and provided written consent. Individuals with current upper airway infection, presence of oral ulcers, dental treatment within the previous month or known IBD were excluded. Data on smoking and medication use was collected using a digital patient survey. Medication use was verified by status review. Participants were asked to refrain from vigorous exercise for 2 hours prior to saliva collection and refrain from food or beverage (except water), smoking, chewing gum and brushing their teeth for 1 hour prior. Before starting saliva collection, the participants were asked to rinse the mouth with water. Saliva was collected by 'passive drooling': the donor leaning forward allowing saliva to drip into a test tube for 10 to 15 minutes. Saliva samples were incubated at 56°C for 45 minutes as a precautionary measure to prevent spreading of SarS-CoV-2 virus particles. After incubation samples were homogenized and spun down at 7000g for 10 minutes. Supernatants were collected and stored at -80°C until further use. After thawing, saliva samples were spun down for 5 minutes at 5000g directly before use.

Faeces was self-collected at home within 2 weeks of saliva and serum donation. The faeces was immediately stored in a house-hold freezer for a maximum of 2 days, before it was transferred on dry ice to a -80 freezer. Saliva and faeces samples containing macroscopic blood were excluded.

To prepare protein fractions of the faeces sample to use on ELISA, circa 1 gram of faeces was diluted in 5ml/gram faeces dilution buffer (PBS + 0.05M EDTA + 1.66mM PMSF + 0.1mg/ml soybean trypsin inhibitor (sigma)). Glass beads (2mm, Merck) were added, and samples were mixed vigorously for 10-20 minutes until homogeneous and spun down at 2000g for 10 minutes at 4°C. Supernatants were collected and centrifuged for 10 minutes at 10.000g. Supernatants were aliquoted and stored at -20°C until further use. Faeces supernatants were spun down for 5 minutes at 10000g directly before use. The same protocols for preparing the faeces samples as well as for the ELISAs on faecal extracts were used in the Plant For Joints study.

AMPA measurements using ELISA

ELISAs were performed using 384 Well Flat Bottom High Bind Microplates (Corning), unless stated otherwise. For AMPA detection, biotinylated CCP2 (patent EP2071335) or similar peptides where the citrulline was replaced by a homocitrulline (CHcitP2) or acetylated lysine (CAcetylP2) were used. Control peptide containing an arginine (CargP2) instead of citrulline, or a lysine (ClysP2) instead of a homocitrulline or acetylated lysine were coated on the same plate. The peptides including the patent protected CCP2 and CargP2 were provided by Dr. J.W. Drijfhout (Dept. of IHB, LUMC). After each step, ELISA plates were washed with PBS/0.005% Tween 20. All ELISAs are visualized with ABTS/H_2O_2. For ACPA, anti-CarP and AAPA measurements in saliva and faeces a modified version of the serum assay was used. The biotinylated modified or control peptides were coated in a concentration of 1 µg/ml in PBS/0.1%BSA on streptavidin coated plates (microcoat, standard capacity; 604500) for serum or on 384 well microplates pre-incubated overnight at 4°C with 1 µg/ml streptavidin (invitrogen) for saliva/faeces and incubated for 1 hour at room temperature (RT). Serum was diluted 1:50 in PBS/0.05% Tween/ 1% BSA (PBT) (for IgG) or PBS/1%BSA/50 mM TRIS/0.05% Tween, pH 8.0 (PBTT) (for IgA) and incubated for 1 hour at 37°C (ACPA, anti-CarP) or overnight at 4°C (AAPA). Saliva was diluted 1:4 in PBS/0.05% Tween/2%Casein (PTC) and incubated for 2 hours at 37°C. Faeces homogenates were added undiluted and incubated overnight at 4°C on ice. A pooled serum standard was used to calculate arbitrary units, and positive and negative serum controls were included on each plate. After washing, plates were incubated with rabbit anti-human-IgG-HRP (DAKO, P0214) 1:4000 (anti-CarP, AAPA) or 1:8000 (ACPA) in PBT for serum or goat anti-human-IgA-HRP (Invitrogen; A18781) 1:5000 for serum and 1:3000 for saliva in PBTT for 1 hour at 37°C. For faeces, goat anti-human-Ig-HRP (Bethyl) 1:2000 in PBTT was added an incubated for 3.5h at 4°C on ice. Blanks were subtracted and samples were considered AMPA positive when they were above the cut-off and the OD (optical density) of the modified peptide was larger than 2 times the OD on the unmodified peptide. The cut-offs were determined based on the mean plus 2 times the standard deviation of the OD of healthy controls. In case the cut-off was below the linear range of the standard, the lowest point of the linear range was used as cut-off.

Rheumatoid factor ELISA

For RF IgM and IgA ELISA, ELISA plates (Nunc maxisorp plates (VWR) for serum) were incubated overnight at room temperature with 10 µg/ml human IgG for RF IgM (Jackson Immunoresearch; 009-000-003) or 5 µg/ml rabbit IgG for RF IgA (Nordic-MUbio) and blocked for 1 hour at 37°C with PBS/1%BSA (RF IgM) or PBS/2%Casein (RF IgA). Serum samples were diluted 1:100 in PBT for RF IgM and 1:185 in PBTT for RF IgA and incubated for 1 hour at 37°C. Saliva samples were diluted 1:4 in PTC and incubated

for 2 hours at 37°C. A commercial standard (N/T Rheumatology control SL/2, Siemens) was used to calculate arbitrary units. Plates were incubated with goat anti-human-IgM-HRP (Millipore; AP114P) 0,3 µg/ml in PBT or goat anti-human-IgA-HRP 1:5000 in PBTT for 1 hour at 37°C.

Total IgA and total IgG ELISA

For total IgA level measurements in saliva and faeces, ELISA plates were coated with goat anti-human-IgA-Fc (Bethyl; A80-102A) 10 µg/ml in bicarbonate/carbonate coating buffer (pH 9.6). All incubation steps were performed for 1 hour at RT, except blocking with PBT which was done for 30 minutes at RT. Two dilutions were used of both saliva and faeces: saliva was diluted 1:400 and 1:800 and faeces 1:80 or 1:100 and 1:400 in PBTT. A serial dilution of purified secretory IgA (Bio-rad PHP133, 1mg/ml) was used as standard. Goat anti-human-IgA-HRP (Bethyl; A80-102P), diluted to 50 ng/ml in PBTT, was used for IgA detection. Total IgG levels in saliva were measured in a similar fashion, using goat anti-human-IgG-Fc (Bethyl; A80-104) diluted 10 µg/ml in bicarbonate/carbonate coating buffer (pH 9.6) as coating antibody and goat anti-human-IgG-HRP (Bethyl; A80-104P), diluted to 50 ng/ml in PBTT as detection antibody. A serial dilution of human IgG (Jackson Immunoresearch; 009-000-003), starting with 0.25ug/ml was used as standard. Saliva samples were diluted 1:50 and 1:200 in PBTT.

Total protein and MMP-8 measurement in saliva

For total protein measurements in saliva, Pierce BCA protein Assay kit (Thermoscientific) was used in combination with Nunc maxisorp plates. Saliva samples were diluted 1:2 in PBS before use and measurements were performed conform manufacturer's protocol. Total MMP-8 ELISA kit (R&D systems; DMP800B) was used to determine matrix metalloproteinase 8 levels (MMP-8) in saliva. The kit was used according to manufacturer's instructions. Saliva samples were diluted 1:15 and a log-log standard curve was used to calculate MMP-8 levels.

Anti-E. coli and calprotectin ELISA

Escherichia coli (E. coli) of strain BL21 (kind gift of Can Amaran) were cultured locally manufactured M9 medium (containing KH_2PO_4 (3 g/L), Na_2HPO_4 (6 g/L), NaCl (0.5 g/L), $MgSO_4$ (1 mmol/L), $CaCl_2$ (0.1 mmol/L) and lysed using French press at 10,000 psi. The lysis procedure was performed three times. The solution with lysed bacteria was spun down at 21,000g, 4°C for 60 min, and the supernatant was collected. Concentration was measured with spectrophotometry. ELISA plates were coated with 10µg/ml E. coli lysate overnight at 4°C. After blocking with PBT for 1 hour at 37°C, undiluted faecal extracts and a pooled serum standard were added and incubated overnight at 4°C. Serum samples and purified secretory IgA were taken along as controls. Two HRP-

labelled detection antibodies were used. First, goat anti-human-IgA-HRP was added and after washing, polyclonal rabbit anti-goat Ig-HRP (DAKO, P0449) was used. Both antibodies were diluted 1:1000 in PBTT and incubated for 1 hour at 37°C. Afterwards, signals were visualized with ABTS. Calprotectin in faeces was measured using a commercial calprotectin ELISA kit (kindly provided by Orgentec; ORG580) according to manufacturer's instruction.

Supplementary data 2: Methodology IntestRA study

Study subjects and samples

20 patients with RA (10 with disease duration <1 year and 10 with established RA), 10 patients with Crohn's disease and 10 healthy controls from the County of Dalarna, Sweden, were included between 2016 and 2019 in the IntestRA study. Only RA patients with a moderate or high level of IgG anti-CCP in serum were included, as these were expected to more likely have detectable ACPA in the intestine. Patients with known Crohn's disease with engagement of terminal ileum and a scheduled colonoscopy were recruited consecutively from the endoscopy department's waiting list. Indication for colonoscopy was assessment of disease activity. Persons participating in a screening study for colorectal cancer were asked to also contribute with samples from terminal ileum. None of these patients were diagnosed with colorectal cancer during the procedure.

Serum and saliva samples were collected at the rheumatology clinic. Participants were asked to restrain from eating, drinking other liquids than water, brushing teeth or smoking one hour before saliva sampling. Saliva was collected using passive secretion during 10 min, the sample was kept on ice and centrifuged 5 minutes at 5000 g. Serum samples were centrifuged 5 minutes at 5000g.

A colonoscopy was performed in all patients with at least 5 cm intubation of terminal ileum. 50 mL PBS was first instilled into the intestinal lumen using a catheter through the working channel of the endoscope. Intestinal fluid was then aspirated through the catheter. Ileal wash fluid samples were centrifuged 5 minutes at 5000 g, frozen within one hour after collection and stored at -80°C until further analyses.

IgA ACPA in saliva

Commercially available serum IgG-class anti-cyclic citrullinated peptide (anti-CCP) enzyme-linked immunoassays (ELISA) tests (CCPlus® Immunoscan, Svar Life Science) were modified to analyse IgA ACPA in saliva. All samples were analysed in duplicate. Saliva samples were thawed at room temperature and spun down (11000g at 4°C for 10 minutes) directly before use, to remove non-soluble material. The supernatant was

diluted to a final concentration of 1:20. The secondary antibody, polyclonal rabbit anti-human-IgA-HRP (DakoCytomation) was diluted 1:200. To adjust for non-specific IgA adsorption, all samples were tested against a control peptide (cyclic arginine peptide, CAP, EuroDiagnostica AB). Anti-CCP and anti-CAP analyses were performed in parallel and anti-CAP background levels were subtracted from anti-CCP values (delta optical density measurements).

IgA ACPA in ileal wash

Commercially available serum IgG anti-cyclic citrullinated peptide (anti-CCP) enzyme-linked immunoassays (ELISA) tests (CCPlus® Immunoscan, Svar Life Science) were modified to analyse IgA ACPA in ileal wash. All samples were analysed in duplicate.

Ileal wash samples were thawed at room temperature and diluted to a final concentration of 1:5. Samples were incubated 1 hour at room temperature on the pre-coated 96-well plates. The secondary antibody, polyclonal rabbit anti-human-IgA-HRP (DakoCytomation) was diluted 1:200, added to the wells for 1h at RT. The plate was washed and the substrate Tetramethylbenzidine (TMB, Sigma Aldrich) added. The plate was read at 450nm. A positive control and blanks were included on the plate. Blanks were subtracted. To adjust for non-specific IgA adsorption, samples were tested against a control peptide (cyclic arginine peptide, CAP, EuroDiagnostica AB). Anti-CCP and anti-CAP analyses were performed in parallel and anti-CAP background levels were subtracted from anti-CCP values (delta optical density measurements). Samples were considered ACPA positive when they were above the cut-off determined based on the mean plus 2 times the standard deviation of the OD of healthy controls.

IgG and IgA ACPA in serum

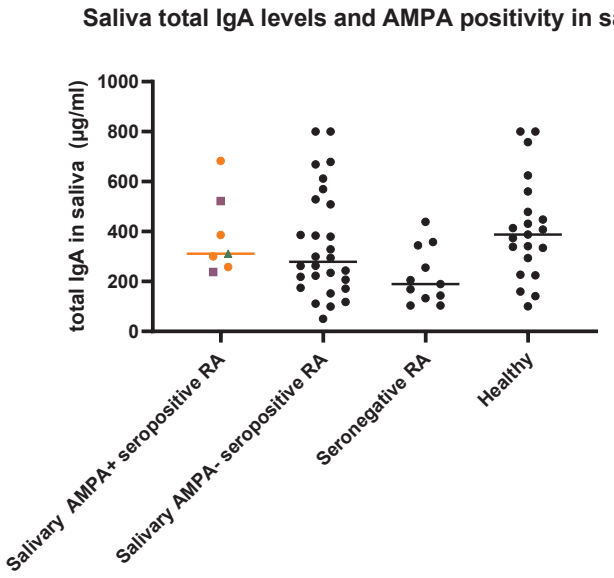
Serum samples were analysed for IgG ACPA according to the manufacturer's instruction (CCPlus® Immunoscan, Svar Life Science), with cut-off set at 25 U/mL. IgA ACPA was analysed using the same pre-coated CCP-plates, adding serum diluted 1:100. The secondary antibody of the kit was replaced with polyclonal rabbit anti-human-IgA-HRP (DakoCytomation) diluted 1:2000. A serum with high level of IgA ACPA was used to create a standard curve and cut-off levels for positive serum tests of IgA ACPA was set to 25 arbitrary units (AU)/mL.

Total IgA in saliva and ileal wash samples

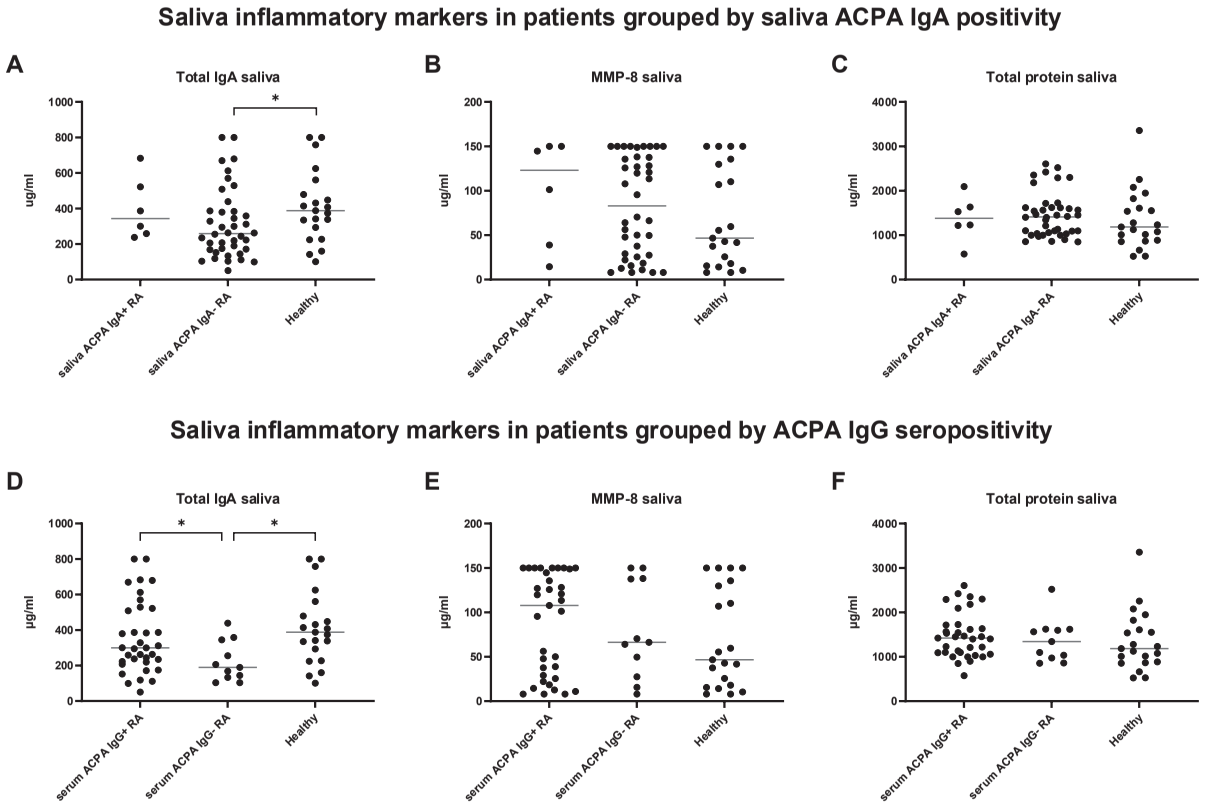
For total IgA level measurements in saliva and ileal wash, the IgA Saliva ELISA (IBL International, DM 59171) was performed according to the manufacturer’s instructions. Saliva was analysed in dilution 1:1000 and Ileal wash in dilutions 1:100 and 1:500.

Anti-E. coli IgA in ileal wash samples

Anti-E. coli IgA in ileal washes samples was measured using the same in-house ELISA as used for the faeces samples with minor modification. ELISA plates were coated with 10µg/ml E. coli lysate overnight at 4°C. After washing and blocking with PBT for 1 hour at RT, ileal washes, diluted 1:5, a positive control and a pooled serum standard were added and incubated overnight at 4°C. The plate was washed and goat anti-human-IgA-HRP (Invitrogen), diluted 1:1000 in PBTT, was added. Following incubation for 1 hour at 37°C the plate was washed and the substrate, TMB, added. The plate was read at 450nm (TECAN).



Supplementary Figure 1: AMPA positivity in saliva related to total IgA levels (µg/ml) in these saliva samples, collected in the MUCOSA study. Patients were divided into groups based on both saliva AMPA positivity and ACPA-seropositivity. The first column shows seropositive RA patients positive for any AMPA in saliva. Colour coding is used to indicate for which AMPA they were positive in saliva; Orange: saliva ACPA single-positive, anti-CarP negative and AAPA negative patients, green: saliva ACPA negative, anti-CarP and AAPA double-positive patient, purple: saliva ACPA, anti-CarP and AAPA triple-positive patients. Error bar shows the median.

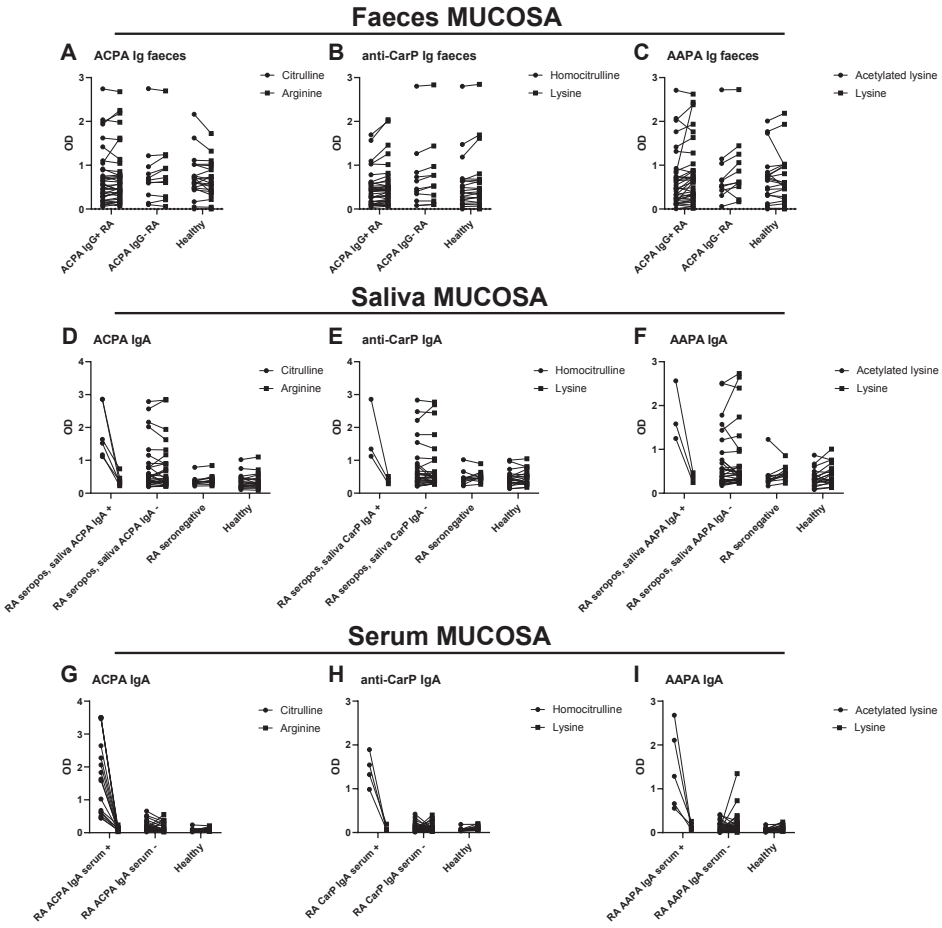


Supplementary figure 2: Inflammatory markers in saliva of patients in the MUCOSA study. A-C Saliva-ACPA IgA positive RA patients, saliva-ACPA IgA negative RA patients and healthy donors are compared. D-F Similar analyses were performed for ACPA seropositive RA patients, seronegative RA patients and healthy donors. The inflammatory markers measured in saliva are total protein levels, metalloproteinase-8 levels (MMP-8) and the amount of total IgA present in saliva. Mann-Whitney U tests were used to compare levels between groups. Bars depict the median, *p<0.05.

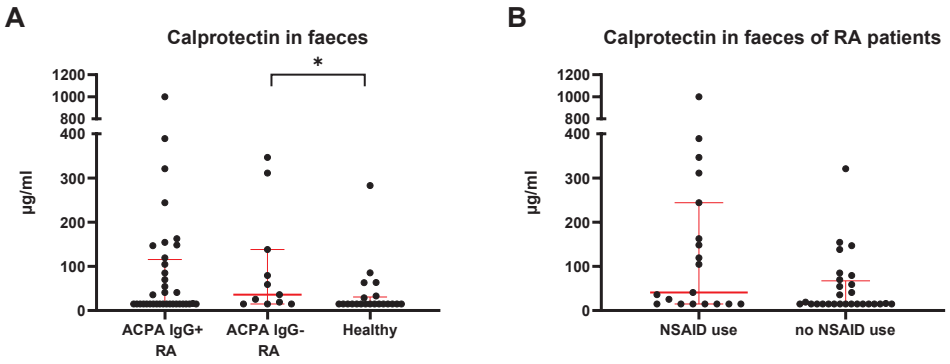
Supplementary table 1: The association between serum and saliva autoantibody positivity and smoking in RA patients in the MUCOSA study.

MUCOSA study	Never Smoked n=21	Ever Smoked n=26	p-value
ACPA IgG serum positive, n (%)	14 (67)	22 (85)	0.15
ACPA IgA serum positive, n (%)	8 (38)	10 (39)	0.98
RF IgM serum positive, n (%)	12 (57)	22 (85)	**0.04**
RF IgA serum positive, n (%)	5 (24)	16 (62)	**0.01**
ACPA IgA saliva positive, n (%)	4 (19)	2 (8) (n=25)	0.39*
RF IgA saliva positive, n (%)	10 (48)	8 (32) (n=25)	0.28

Significance was tested with chi-square test or 2-sided Fisher's exact test (indicated with *). Smoking was defined as ever- vs never-smokers.



Supplementary figure 3: Paired signals on the modified and unmodified peptide for each AMPA in faeces, saliva and serum collected in the MUCOSA study. A-C Faeces: The first group in each figure shows ACPA IgG seropositive RA patients, the middle group shows ACPA IgG seronegative RA patients. D-F Saliva: The first group in each figure shows ACPA IgG seropositive RA patient positive for that AMPA IgA in saliva, the second group shows ACPA IgG seropositive RA patient who are negative for that AMPA IgA in saliva, the third group shows ACPA IgG seronegative RA patients. G-H The first group shows RA patients positive for that specific AMPA IgA in serum, the second shows RA patients negative for that specific AMPA IgA in serum. The last column of all figures includes healthy donors.



Supplementary figure 4: Calprotectin levels in faeces samples collected in the MUCOSA study. A Seropositive RA patients, seronegative RA patients and healthy donors and B RA patients with and without NSAID use are compared. Calprotectin levels are measured by ELISA. Values above 200 µg/g are considered to be significantly elevated. NSAID used was based on the medication list in the electronic medical file and/or self-reported use as indicated in the patient survey. All NSAIDs (no information on dosage or frequency available) except for low dose acetylsalicylic acid as platelet aggregation inhibitor, was included in the NSAID group. Mann-Whitney U tests were used to compare levels between groups. Red bars depict median with interquartile range. *p<0.05