Effects of habitual exercise on saliva immunoglobulin A in older adults

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Abstract

Introduction: Saliva immunoglobulin A (IgA) is considered the first line of defense against microbial antigens. Factors such as age and physical exercise modulate the mucosal immune system; including saliva IgA responses. Decreased levels of saliva IgA in elite athletes as well as high age have been correlated to increased susceptibility for upper respiratory tract infections; thus, there is value in further investigating the relationship with high age, physical exercise and IgA modulation.

Objective: This study investigated differences of the levels of total and secretory IgA (SIgA) in sedentary older adults and habitually active older orienteers.

Method: Whole saliva and physical activity measurement were collected from 25 sedentary older adults and 29 physically active older orienteers, aged 65 and above. Secretory and total IgA was then evaluated using enzyme linked immunosorbent assay (ELISA).

Result: Total IgA levels had a median of 108,8 mg/L for orienteers, and 99,5 mg/L for sedentary older adults. Further, median of total IgA levels in relation to total protein content in saliva was 5,9 mg/L and 8,0 mg/L for orienteers and sedentary older adults respectively. SIgA levels could not be reliably calculated due to large inter-variability.

Conclusion: No significant difference was found between the two groups for total IgA level or total IgA in relation to total protein content in saliva. However, many confounding factors could influence the result, e.g. gender, sampling method and exercise level. With consideration to the wide ranges and high inter-variability found, larger sample size and further method optimization is suggested in the future.

Key words: Saliva IgA, older adults, aging, physical exercise, mucosal immune response
**Abbreviations**

BSA – bovine serum albumin
CV – coefficient of variation
ELISA – enzyme-linked immunosorbent assays
Fab – variable part of immunoglobulin
Fc – constant part of immunoglobulin
H – heavy chain of immunoglobulin
H$_2$SO$_4$ – sulfuric acid
Ig – immunoglobulin
IgA – immunoglobulin A
J-chain – joining chain
L – light chain of immunoglobulin
MIgA – monomeric immunoglobulin A
PBS – phosphate buffered saline
PBT - phosphate buffered saline with tween
pIgR - polymeric Ig receptor
SC – secretory component
SIgA – Secretory immunoglobulin A
TMB - tetramethylbenzidine
URTI – upper respiratory tract infection
VO$_{2peak}$ – maximal oxygen uptake
1RM – one repetition maximum
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1. Introduction

1.1 Immunoglobulins

Immunoglobulins are antibodies secreted by B lymphocytes (i.e. plasma cells) and are an important part of the immune system. The immunoglobulins are proteins composed of two identical heavy polypeptide chains (H) and two identical light polypeptide chains (L). Both the heavy chain and the light chain together create a variable region (Fab) and a constant region (Fc) of the immunoglobulin. The variable region binds specifically to an antigen while the constant region mediates the immunoglobulin’s effector function through binding to different effector cells such as phagocytes, neutrophiles and eosinophiles [1].

Immunoglobulins (Ig) are divided into five isotypes, which can exist in either a membrane bound or a secreted form. These isotypes - IgA, IgD, IgE, IgG and IgM – all have different heavy chains and thus exert different effector functions. The different effector functions includes antibody dependent cell cytotoxicity (ADCC), neutralization of microbes, opsonization of pathogens for enhanced phagocytosis, activation of the complement system and mast cell activation [1]. Furthermore, immune responses that can be measured in saliva, such as fluctuations in IgA levels, is often viewed as being representative for the whole mucosal immune system [2], and IgA in particular has been proposed as a highly suitable marker for overall mucosal B-cell function [3].

1.2 Secretory Immunoglobulin A

Secreted IgA is mainly in dimeric form were the tail pieces are joined by a joining (J) chain. Secretory IgA (SIgA) is secreted in intestinal mucus, gastric fluids, tears and saliva and act as an important part of the mucosal barrier. The SIgA producing plasma cells are located in lamina propria directly beneath the basolateral membrane of the epithelial cells [4]. When secreted, SIgA bind to a polymeric Ig receptor (pIgR) on the basolateral surface of epithelial cells [4,5]. Together with the receptor, SIgA is then transported through the cell to its apical surface and subsequently released by enzymatic cleavage, leaving a part of the receptor on the SIgA that becomes the secretory component (SC) [4]. This specific transport makes SIgA the dominant immunoglobulin in secretions, however monomeric IgA (MIgA), IgG, IgD and IgE are also found in smaller amounts, primarily due to passive transport from plasma [6].

SIgA acts as a first line of defence by several mechanisms, such as: 1) inhibiting adherence and penetration of microbial organisms to mucosal surfaces, 2) facilitating their elimination by binding antigens from the basolateral surface of epithelium and 3) binding and neutralizing
antigens at the intracellular or interstitial level [7]. Both the free and bound SC has been shown to neutralize toxins and inhibit adhesion of gram negative bacteria to the epithelial surface [5]. SIgA have low capacity to act as an opsonin or to activate the complement system and therefore does not induce inflammation that could increase the penetration of microbes [7]. Factors known to influence IgA level in saliva are, for example: age, various stressors, sensory stimulation, food ingestion, drugs, smoking and degree of hydration [5,8].

Due to the various mechanisms in combating several pathogenic microorganisms, saliva IgA is thus consider to be an important part of the mucosal immune system.

1.3 Effect of exercise on salivary immunoglobulin A levels
It is well recognized that regular and single bouts of exercise influence the immune system in different ways, depending on e.g. regularity, intensity and type of the training. Intense training has been connected to suppressed immune system in several research studies. Elite swimmers have been found to have lowered resting levels of saliva IgA after the training season compared to before the season [9,10], suggesting a chronic depletion of the mucosal immune system due to prolonged intense exercise. Gleeson et al (1995) have also show an acute decrease of the saliva IgA post-exercise after each training session during the competitive season in elite swimmers, while saliva IgA increased post-exercise in controls (non-elites) [10]. On the contrary, recreational joggers and competitive runners who had a single bout of moderate to intense exercise (at 55% and 75% VO$_{2peak}$) showed no significant difference between pre-exercise and post-exercise IgA level in relation to total protein in saliva. However, when the competitive runner’s training was continued for three consecutive days (with same intensities as mentioned above), they showed a post-exercise decrease in saliva IgA in relation to total protein on day two and three. [9]

In older adults, regular long-term exercise, at non-elite level, has been connected to an increase of saliva IgA concentration and secretion rate [11,12], where both a non-significant [11] and a significant increase [12] in saliva secretion rate has been seen. Similar results were found in a study measuring the relation between voluntarily performed, what they called “free-living”, daily physical exercise and saliva IgA, namely: that the older adults with the highest daily activity had significantly higher SIgA and SIgA-secretion rate than those with the lowest daily activity [13]. The same relationship has also been seen when measuring acute SIgA responses post-exercise following a low-moderate intensity training in older adults [14] as well as in younger adults [9,15].
In contrast to the elite swimmers [9,10], the increasing IgA levels shown in older and younger adults could be interpreted as a positive modulation of the immune system due to exercise, however, this relation could also be attributed to the differences in exercise intensity. Yet, when testing the acute changes in elderly women with different intensities, an increase in salivary IgA has been seen, both at the lower intensity (50% of 1RM) and at the higher intensity (80% of 1RM). [16]

In general it seems that very intense training during longer episodes, i.e. training with a load close to overtraining, is correlated to a decreased level of SIgA while moderate training, both during shorter and longer time periods, is correlated to an increased level of SIgA.

The effect of exercise on IgA immunity is an important thing to consider in relation to the general health of the population, since a majority of infections is initiated at mucosal surfaces [2]. Especially since researchers have found a correlation between training, saliva IgA levels and susceptibility for contracting upper respiratory tract infections (URTI) - where moderate training has been correlated to a decreased risk [17] and intense training to an increased risk [10,18].

1.4 The older adults’ immune system
The immune system undergoes many changes with age, a phenomenon commonly referred to as immunosenescence. Immunosenescence includes thymic involution resulting in diminished capacity to generate new T-cells [19], and a poorer response to vaccines [20], the latter suggesting a decreased function of the humoral immune response. However, the immunoglobulin concentration in serum seems to increase with increasing age [21], probably due to maturation of the immune system and increasing antigen load [22]. Though, in saliva, IgA concentration seems to increase with age up to 60 years, but at age 61-70 there seems to be a trend towards decreased saliva IgA concentration in unstimulated whole saliva [23]. This trend is also supported by Miletic et al, showing that 60-80 year olds have a significant lower saliva IgA concentration in comparison to younger adults (20-30 years old) [24]. However in stimulated whole saliva the opposite relation has been seen, i.e. persons in ages ≥65 years had a higher saliva IgA concentration than did 18-65 years old. These contradictory results could possibly be attributed to the fact that stimulated saliva have been shown to have a higher resting secretion rate than resting secretion of unstimulated whole saliva [25].

Nevertheless, there seems to be a positive correlation with higher ages and increased incidence of URTI, especially in infections such as community-acquired pneumonia
associated with influenza virus [26], suggesting a decreased function of the mucosal immune system in the older adults.

All this taken in consideration, one could assume that moderate exercise would positively modulate the mucosal immune response, indicated by the level of saliva IgA. A positive effect of exercise on the immune system could therefore possibly aid older adults to be less susceptible to infectious diseases, such as URTI’s, and thus increase the general health in the aging population.

2. Objective

2.1 Aim
The primary objective of this study is to further expand the knowledge on the effect of exercise on saliva IgA in older adults, by comparing levels of total and secretory IgA in sedentary older adults and habitually active senior orienteering athletes (ages 65 years and above). The secondary objective is to establish optimization of analysis of saliva IgA at Örebro University.

2.2 Hypothesis
The habitually active senior orienteers have a significant increased total and secretory IgA compared to the sedentary older adults.

2.3 Null hypothesis
There is no significant difference of total and secretory IgA between habitual active senior orienteers and sedentary older adults.

3. Method And Materials
This study was performed as a pilot study for the initial establishment and optimization of the analysis of total and secretory IgA levels in the original study “Efficacy of dietary supplementation with Lactobacillus reuteri DSM 17938 for digestive health in an elderly population, dnr 2012/309” [27]. The current pilot study was designed as a cross-sectional study, were stratified sampling was used to obtain the targeted groups from the larger population sampled in the original study [27]. The two groups were selected based upon self-estimated physical activity from the Frändin-Grimby questionnaire (see appendix 1) were they
rank their own activity from one to six (one is the lowest activity level, i.e. barely any activity at all, and six is the highest, i.e. heavy training regularly, several times a week) based on standardized guidelines. The limit for sedentary elderly was a mean score (i.e. mean of summer and winter activity score) of three or below from the questionnaire, while the orienteers had a limit of a mean of three or above (were three represents 2-4 hours easier training each week). This post-collection stratification was done in order to limit the chance of the two study populations to have too similar activity levels. Since the study aims to investigate the effect of habitual exercise on saliva IgA levels, and thus lack a systematically regulated exercise intervention, this study can also be considered to be of a natural experimental design.

3.1 Subjects
The subjects were 25 independently living sedentary older adults and 29 physically active senior orienteers (for characteristic information, see table 1 in result) recruited by adds in the local newspaper and information meetings held at local orienteering competitions respectively. All subjects have provided their informed consent before study inclusion.

3.2 Saliva sampling and preparation
Saliva samples were collected according to standardized instructions in the subjects’ home. The subjects spat 1-1.5 ml into a 15 ml falcon tube, sealed the sample and immediately placed the tube into the freezer at -20°C. All samples were then collected in a cooled transport by the researcher team and then stored at -80°C until analyses started. The samples were heat inactivated in 56°C water for 30 minutes and subsequently centrifuged at 5000G in 15 minutes before analysis, in order to clear the samples from waste products, e.g. contaminating food stuff.

3.3 ELISA for detection of salivary IgA immunoglobulins
For analysis of IgA concentrations in the saliva samples, enzyme-linked immunosorbent assays (ELISA) was used with transparent 96-well half area Costar 3690 microplates (Thermo Fisher Scientific Inc., Loughborough, England). The plates for detecting SIgA were coated with 100µL/well with monoclonal anti-human secretory component clone GA-1 (Sigma Chemical Co. St Louis, USA, art nr: I6635) diluted 1/10.000 in PBS (phosphate buffered saline) (AppliChem, Darmstadt, Germany). The plates for detecting total IgA were coated with 100µL/well with polyclonal rabbit anti- human IgA
(DAKO, Glostrup, Denmark, art nr: A-0262) diluted 1/50.000 in PBS. The coated plates were then incubated for two hours in a damp chamber at 37°C and then for another three hours at 4°C. After the incubation, the plates were then washed in PBT (PBS-tween) (Calbiochem, Darmstadt, Tyskland) and for the blocking, 100μL/well of 0,5% BSA (bovine serum albumin) (Sigma Chemical) diluted in PBS was added. The plates where then incubated in a damp chamber at 37°C for 2 hours and then overnight in 4°C.

Saliva samples was diluted 1:500 and 1:1000 for SIgA and 1:2000 and 1:4000 for total IgA in 0,5% BSA in PBT. If any sample could not be measured within appropriated ranges of analysis, further dilutions were made. Each sample was added in duplicates of 50μL/well. Duplicates of control and standard solutions were included on every plate to control inter- and intra-validity of the plates. The standard solution consisted of human IgA (Sigma Chemical Co. St Louis, USA, art nr: I-1010) diluted in PBT in eight dilutions (see table 1 in appendix 2), from concentration 1000ng/mL to 7,8ng/mL. A blank sample (consisting of 50μL PBT) was also included on every plate in order to check for the amount of unspecific binding.

After one hour of incubation in damp chamber at 37°C, the plates were washed with PBT and 50μL of detection antibody (Antihuman IgA peroxidase conjugated, art nr: A0295) diluted 1/25’000 with PBT, were added to each well. Followed by incubation for one hour in damp chamber at 37°C and then washed with PBT, 50μL/well of tetramethylbenzidine (TMB) (Thermo Fisher Scientific Inc., Loughborough, England), which was used as a substrate, was added at set time intervals and then the plate was shaken in darkness for 30 minutes. At same time intervals, the reaction was stopped with addition 75μL/well of 1,8M H₂SO₄ (VWR International, Leuven, Belgium).

To estimate the IgA concentration in each well, the light absorbance were measured at 450nm, using an EnSpire® multimode plate reader (PerkinElmer, Turku, Finland) with the software EnSpire Workstation version 4.10.3005.1440 (PerkinElmer) at 40 minutes after TMB was added to the first well. A sample was judged as trustworthy if the samples had a difference of 0,25 units or lower between the duplicates.

For analyses, a reference standard curve was created on a separate plate, using the same human IgA (Sigma Chemical, art nr: I-1010) that was used as the standard curve on all the plates. The reference standard curve was diluted in 18 steps (table 2, appendix 2) from concentration 1000ng/mL to 1,95ng/mL, in order to create a more exact and standardized curve equation. The concentration of the saliva samples measured in this study was then calculated based on the equation originated from the linear points of the reference standard curve, calculated in Microsoft Excel.
Total IgA and secretory IgA were detected by anti-human antibody directed against the alpha chain and secretory component respectively. That means that total IgA detection includes secretory as well as monomeric and polymeric IgA while secretory only detects secretory IgA.

3.4 Bradford for detection of total protein
Standard proteins levels were prepared according to the Coomassie (Bradford) protein assay kit (Thermo Scientific, art nr: 23200) instructions. The sample size was then adjusted to fit the half-area plates (transparent 96-well half area Costar 3690 microplates). 125µL of Coomassie reagent was first added to each well and then 2,5µL of standards, blank, control and saliva samples were added in duplicates. Saliva samples were diluted 1:0 and 1:1 in milliQ H2O. The samples were then mixed on a plate shaker for 2 minutes followed by 10 minutes incubation in darkness. Absorbance was then read at 595nm, using the same plate reader as for the ELISA protocol described previously. A sample was judged as trustworthy by the same standards as SIgA and total IgA, i.e. if the sample had a difference of 0,25 units or lower between the duplicates. Total proteins were then set in relation to total IgA in the analysis to minimize the sensitivity of measurements of absolute saliva IgA values due to oral dryness.

3.5 Statistics
Since the concentration of antibodies in saliva (total IgA and SIgA) could not be considered as normally distributed (and the fact that the measurements were unpaired), the non-parametric test Mann-Whitney U-test was chosen. All statistics and graphs were calculated using GraphPad Prism 6 and GraphPad InStat 3 (GraphPad Software Inc., La Jolla, USA).

3.6 Ethics
The participants signed an informed consent developed for the original study [27] prior to data collection and saliva sampling. All participants were clearly informed that they had the right to withdraw at any point from the study, without stating their reason. This pilot study has been ethically approved, as being a part of the original study’s protocol, by the Uppsala Regional Ethical Review Board (www.epn.se) (dnr 2012/309).
4. Results

4.1 Sample characteristics

The sample characteristics for the two population groups are presented in table 1. Six samples of total IgA (five for sedentary and one for orienteer group) were excluded due to unreliable measurement such as: saturated absorbance or too large variability between duplicates (i.e. more than 0.25 units in difference). Hence, the final number of studied and analyzed individuals were 20 sedentary older adults and 28 senior orienteers. The difference of physical activity was statistical significant (p>0.0001) between the groups according to a Mann-Whitney U-test.

<table>
<thead>
<tr>
<th>Population group</th>
<th>Senior Orienteers (n=28)</th>
<th>Sedentary older adults (n=20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender, F=females, M=males, (%)</td>
<td>F=13 (46), M=15 (54)</td>
<td>F=15 (75), M=5 (25)</td>
</tr>
<tr>
<td>Age in years, median (range)</td>
<td>68 (65-81)</td>
<td>71 (65-77)</td>
</tr>
<tr>
<td>Physical activity score, median (range)</td>
<td>4* (3-6)</td>
<td>2 (2-3)</td>
</tr>
</tbody>
</table>

*statistical significant difference seen in the physical activity score between the two groups (p<0.0001)

4.2 Total IgA levels

The median level of total IgA concentrations in saliva for the senior orienteers were 108,8 mg/L (21,3- 238,7) and the sedentary older adults 99,5 mg/L (43,3- 288,0), presented in figure 1. No significant difference in IgA levels were found between the two groups (p=0.9345).

Figure 1: Total IgA concentration is presented in mg/L for both senior orienteers and sedentary older adults in a box-and-whiskers plot displaying median, inter-quartile range and total range.
4.3 Ratio total protein to total IgA
Senior orienteers had a median ratio of total protein to total IgA of 5.9 mg/L (3.0 – 28.4) and sedentary older adults had a median 8.0 mg/L (3.0 – 19.8) (see figure 2), yet the difference in median ratios between the two groups was not found to be significant (p=0.4225).

![Ratio Total Protein to Total IgA](image)

Figure 2: Ratio between total protein and total IgA is represented in a box-and-whiskers plot (displaying median, inter-quartile range and total range) for both senior orienteers and sedentary older adults. The total protein (mg/L) is divided by total IgA concentration (mg/L) in order to get the ratio of the amount of IgA per total protein as shown on the y-axis.

4.4 Inter-variability
The variability coefficient (CV) of the control samples were 39.2% for secretory IgA and 9.1% for total IgA.

4.5 Secretory IgA
Due to internal variability, inconsistency during the limited time of lab-procedures and too high CV (CV=39.2%), secretory IgA was excluded from analyses.

5. Discussion
According to the result presented in this study, no significant differences of total saliva IgA levels, neither absolute values nor ratio to total proteins contents, were found between sedentary older adults and senior orienteers. Hence, this study does not support the hypothesis
stating that “habitually active senior orienteers have a significant increased total and secretory IgA compared to the sedentary older adults”, and thus the null hypothesis cannot be falsified.

There are other studies supporting the results from this study, finding no significant increase of saliva IgA, after a 30-minute walk session [28], a three week training intervention [29] or a 60 min cycling exercise [30]. However, in several studies investigating saliva IgA in relation to engagement in physical activity over longer time periods, a significant change has been observed [11,12,17], contradicting the findings in this present study.

The reason for such contradictory results might be explained by the exercise levels being too similar between the two study groups, even though there was a statistical significant difference in activity between the groups. Several researches have shown that even a small increase in activity level could increase saliva IgA [14]. Taking this into consideration, the sedentary older adults might have been active enough to elicit some increase in salivary IgA, especially since the physical activity cut-off was quite narrow in this study. Also, the saliva sampling in this study was conducted in the winter season for both groups and thus, its results might be influenced by the seasonal physical activity in the groups rather than a mean value representing a whole year.

Another possible reason for why no significant change was seen in this study, compared to other research studies measuring the effect of activity level on saliva IgA over longer time periods [11,12,17], could possibly be found in a study performed by Shimizu et.al. (2007). Shimizu and his group found that voluntarily performed physical exercise, so called free-living activity, did elicit a significant increase in saliva IgA levels for the elderly population that was the second most active (Q3) compared to the least active group (Q1). However, when comparing the third (Q2) and first (Q4) most active population, a non significant change was measured in comparison to the least active group (Q1) [13]. Applying this observation on the findings of this study could enunciate that if the sedentary group had a mean activity level representative to the Q2-group in Shimizu’s study, there would be no significant difference between sedentary and orienteers - regardless if the activity level of the orienteers were representative to Q3 or Q4 [13].

A second thing to consider about the exercise level is the accuracy if the measurements of physical activity in this study and the validity of the questionnaire used. The participants were asked to rate their activity level for the winter and summer season respectively, meaning that they would need to recall and assume their activity level representing a long period of time. A
self-reported questionnaire is always susceptible to the subjectivity in the measurements; what one would rate as high activity, another person could possibly rate as low. However, due to standardized instructions, subjectivity is limited, though not eliminated. Instead of questionnaires, step count could possibly be used in future studies to obtain a more accurate measurement of activity level.

An additional matter to consider is the large range and intra-variability in the measures, both within the groups and between samples; this could suggest that a larger sample is needed to further investigate the relationship between habitual exercise and saliva IgA levels. Salivary IgA have a CV of 5-10% when using ELISA method [3], which was also seen in this study when measuring total IgA (CV 9.1%), but not secretory IgA (CV = 39.2%). This would mean that more optimization of the method is needed for secretory IgA analysis. Strategies to further minimize inaccuracies in the method are: 1) using standardized pre-coated plates, 2) quadruplicates of the sample, 3) further optimizing the blocking of unspecific bindings and 4) optimize the binding of SIgA to the coating antibodies. It is also important to consider the sampling method, since many factors that influence saliva IgA [8] was not accounted for in this study. The use of unstimulated whole saliva, on the other hand, is favorable due to that a majority of the studies are using this as a standard [10,16,18,23,24,28-31], making it easier to generalize between studies.

Gender was not equally represented between the two groups and since gender differences, considering saliva flow rate (higher in men) and SIgA concentration (lower in men), have been observed in earlier studies [12,13] this could possibly influence the results of this study. These gender differences would likely be due to the fact that a higher saliva flow rate is directly connected to a lower SIgA concentration [13,25] according to the principal of dilution (i.e. higher saliva flow rate would dilute the concentration of IgA in saliva, hence a lowered saliva IgA concentration).

However, the dilution principal does not apply to the SIgA-secretion rate since SIgA-secretion rate is not directly dependent on saliva flow rate, and indeed no significant gender difference has been seen considering SIgA-secretion rate [12,13]. Therefore, one could assume that the relation between total protein content in saliva and total saliva IgA could still be a valid result in this study, since some researches consider total protein to negate some effect of oral dryness [32,33] and hence saliva flow rate.
Age was almost equally distributed amongst the groups (median 71 and 68 years), suggesting a minimal influence of this confounding factor on the findings in this present study. Also, previous studies have shown that age differences in older adults (60-69 versus 70+ years) make no significant difference for salivary IgA levels [12].

To summarize, following changes could be done in order to further increase the reliability of this study: 1) measure activity level by step count, 2) increase the study population size, 3) further optimize the ELISA method used for IgA analysis, especially concerning SLgA 4) further standardize sampling method and 5) improve sex distribution between groups.

6. Conclusion
No significant difference in total saliva IgA level between sedentary older adults and habitually active senior orienteers could be found in this study. Comparison of SLgA levels between the two groups could not be performed due to problems with the ELISA detection, which implicate that further optimization of the method is required for the analysis of SLgA. In conclusion, additional studies with larger sample size and improved methods should be executed in order to generate reliable results of total and secretory IgA measures in physically active and sedentary older adults, which might be generalized to larger populations.

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