MIKAEL SONESSON
ON MINOR SALIVARY GLAND SECRETION IN CHILDREN, ADOLESCENTS AND ADULTS
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MIKael SONESSON

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To Sara, Adam, Povel
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This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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**ABSTRACT**

The minor salivary glands are of great importance for maintenance of homeostasis in the oral cavity. These glands continuously secrete substances which lubricate and protect the oral tissues, contributing to comfort and health. The minor salivary glands contribute approximately 7-8 per cent of the total volume of saliva. Flow rate and composition seem to vary according to anatomical location. Current knowledge about the minor salivary glands is derived primarily from studies on adults. The overall aim of this thesis was to study age-related changes in minor gland saliva, from childhood to adulthood. By increasing the knowledge of minor gland secretion, we hopefully better understand how different mucosal locations are lubricated and protected in individuals of different ages and various health statuses. The project comprises four papers.

In **Paper I**, the flow rate and numerical density of the labial and buccal minor glands of pre-school children, adolescents and adults were investigated. Saliva was collected on filter paper discs and the flow rate was measured by the Periotron-method®. The numerical density was assessed by PAS-staining.

*Key findings:* The flow rate of the buccal glands was significantly lower in children than in adults and the number of labial glands was significantly higher in children than in the other age-groups.

In **Paper II**, the composition of minor gland saliva of the three age groups (Paper I) was analysed (by ELISA-technique), with reference
to the mucins MUC5B and MUC7, representing some of the major components of innate salivary immunity.

*Key findings:* Children did not differ from adolescents and adults with respect to MUC5B content in labial gland saliva, but had less MUC7 than the adults. In the buccal gland saliva, detectable amounts of the mucins were found in only a few of the participants.

In **Paper III**, the content of the adaptive immune component (salivary IgA) in minor gland saliva of pre-school children, adolescents and adults was measured by the ELISA technique. The salivary IgA-concentration in whole saliva of the three age-groups was also estimated.

*Key findings:* The IgA-concentration was significantly lower in the labial glands and the whole saliva of the children than in the adults.

In **Paper IV**, age-dependent differences of other innate components were studied in pre-school children, adolescents and adults, by analysing the amount of glycoprotein 340 (gp-340) in minor gland and whole saliva, using the ELISA technique. The content of sialic acid, a common terminal structure of glycoproteins, was analysed using the ELLA technique.

*Key findings:* With respect to minor gland saliva, no differences were disclosed among pre-school children, adolescents and adults. However, the gp-340 content of whole saliva was significantly higher in the children than in the adults.

The above investigations of properties of minor salivary glands in children, adolescents and adults seems to be the first to present data on age-dependent variations in gland density and secretions from healthy individuals. The results show high gland density, mature innate immunity and an ongoing maturation of adaptive immunity in the saliva of children. The report provides a reference for further comparative studies on minor gland saliva of younger individuals in health and disease.
POPULÄRVETENSKAPLIG SAMMANFATTNING


Avhandlingen söker svar på följande frågeställningar:
Studie I. Finns det åldersrelaterade skillnader i flöde från små salivkörtlar och finns det åldersrelaterade skillnader i antal småkörtlar?
Studie II. Finns det åldersrelaterade skillnader i mängden av framträdande ospecifika försvarskomponenter (mucinerna MUC5B och MUC7) i saliv från små körtlar?
Studie III. Finns det åldersrelaterade skillnader i koncentrationen av specifika försvarskomponenter (saliv-IgA) i småkörtel- och helsaliv?
Studie IV. Finns det åldersrelaterade skillnader i mängden av ytterligare en viktig ospecifik försvarskomponent (Gp-340) samt
Huvudfynden i studierna är:

- Barnen hade lägre flöde från små körtlar i kindslemhinnan jämfört med de vuxna. Vidare uppvisade barnen fler körtlar i läppen jämfört med de vuxna.
- Barnen och de vuxna hade samma innehåll av MUC5B, men barnen hade mindre innehåll av MUC7 i saliv från små körtlar i läppen. Endast ett fåtal individer uppvisade muciner i saliven från små körtlar i kinden.
- Barnen hade lägre koncentration av saliv-IgA i saliv från små körtlar i läppen och i helsaliv, jämfört med de vuxna.
- Barnen och de vuxna uppvisade liknande mängder av gp-340 och sialinsyra i småkörtelsaliven men barnen hade större mängd gp-340 i helsaliven. De vuxna hade större mängder av gp-340 och sialinsyra i saliven från små körtlar i kinden jämfört med körtlarna i läppen.

Förskolebarnens småkörtelsaliv innehåller samma mängd av några viktiga komponenter tillhörande det ospecifika immunförsvar som de vuxnas, medan det specifika immunförsvaret tycks fortfarande vara under utveckling hos förskolebarnen. Vidare har förskolebarnen lägre salivflöde från de små körtlarna i kinden och tätare mellan körtlarna i läppen än de vuxna.

Genom denna grundläggande kunskap kan genomförandet av nya jämförande studier av hur saliven fungerar hos yngre medicinsk eller odontologiskt belastade individer och av omhändertagande av patienter med störningar i saliven utformas. Skillnader i salivsekretion mellan barn och vuxna är också viktiga att utreda bland annat som eventuell förklaringsmodell för åldersvariationer i hur orala sjukdomstillstånd mellan åldrarna uttrycks.
INTRODUCTION

Saliva
Saliva has multiple roles: it is important not only for protection of the oral cavity, but also for general health, digestion and well-being. Saliva is secreted by exocrine glands, anatomically located in different parts of the maxillofacial region: the two main sources are the major and the minor salivary glands. Whole saliva, or oral fluid, is a mixture of glandular secretions and gingival crevicular fluid.

Salivary glands
The major salivary glands are the parotid, submandibular and sublingual glands, which together produce more than 90 per cent of saliva (1). The parotid glands are bilateral, located anterior to the external auditory meatus; the saliva drains into the oral cavity via ducts located buccal to the maxillary second molars. The submandibular glands are located beneath the tongue, with ducts lateral to the lingual frenulum. The sublingual glands are also located beneath the tongue, anterior to the submandibular glands; saliva drains into the oral cavity via ducts which terminate in rows of minor orifices (Fig. 1) (2).

The minor salivary glands, also called mucosal glands, are located in the labial, buccal, palatal and lingual regions of the oral cavity and account for 7-8 per cent of the total volume of saliva (1, 3). The glands are surrounded by blood vessels, nerve and muscle fibres (1). Each individual gland comprises a cluster of cells connected by a duct to the oral cavity (Fig. 2). Like the major
glands, the minor salivary glands are formed during the first trimester of pregnancy: oral epithelium proliferates into the underlying ectomesenchyme and forms ductal and terminal secretory end pieces (1).

Similar minor exocrine glands are found in most mucosal surfaces of the human body i.e. the eyes and the respiratory, uterine, urinary and gastrointestinal tracts.

Fig. 1. Schematic picture of major salivary glands. Illustration: Bo Veisland.

Fig. 2. Schematic picture of a minor salivary gland. Illustration: Bo Veisland.
Salivary gland regulation
Salivary secretion is influenced by several physiological conditions and is regulated by nervous systems (4), hormones (5) and neuropeptides (6). The salivary glands are innervated by the autonomic nervous system and both the major and minor glands are stimulated by parasympathetic nerve fibres of the seventh (facial) and the ninth (glossopharyngeal) cranial nerves. Unlike the minor glands, the major glands are also directly innervated by sympathetic nerve fibres from the second thoracic segment of the spinal cord (6-8).

When a neurotransmitter adheres to a receptor on an acinar cell, e.g. when acetylcholine adheres to a muscarinic receptor, the gland starts to synthesize isotonic primary saliva, by transforming capillary blood into interstitial fluid. As it flows through the glandular duct, the tonicity of the primary saliva is modified, from isotonic to hypotonic, before draining into the oral cavity (6, 7, 9-11).

Salivary secretion
The daily volume of secreted saliva is approximately 0.6 to 1.0 L in adults and 0.5 L in children (12-14). Saliva constantly bathes the oral cavity, lubricating and moistening the tissues and delivering components of the innate and adaptive immune system, such as mucins and immunoglobulins (15-17) (Table 1). Several of these components are incorporated into the pellicle on the hard and soft tissues (18, 19).

The saliva from major glands is rich in phosphate-binding proteins, buffering bicarbonates and calcium and has several functions considered to be important for the maintenance of homogeneity of dental tissues. Saliva is also important for the digestive process: mastication of food stimulates secretion of saliva from the major glands, causing an increase in salivary flow and in amylase concentration. The increased flow and the elevated amylase concentration facilitate clearance of food remnants and digestion, respectively (9).

The minor glands continuously secrete saliva (3) rich in organic substances such as proteins and glycoproteins (20-23) which have gel-forming properties and create a protective lubricating layer of
mucus (24-26), which is important for oral comfort. The composition of saliva from different mucosal areas seems to vary (21, 27), and the glandular cell types differs in different locations (28, 29).

Protective components in minor gland saliva

The adaptive component of the immune system in saliva consists of different classes of immunoglobulins (IgA, IgE, IgG, IgM), mainly immunoglobulin A (IgA) (20, 30, 31). The buccal glands are reported to have particularly high concentrations of IgA (21, 27). Innate immune protection is often mediated by various mucins or other classes of glycoproteins (24, 32), which bind and agglutinate bacteria, mostly via their carbohydrate moieties. Glycoproteins contains a large number of oligosaccharides covalently bound to the protein core, often exposing sialic acid as a terminal structure (33). Notably, these sialylated structures act as free radical scavengers and are also responsible for interactions with microorganisms and host ligands (34-36).

Analogous microbial interactions have been attributed to the salivary glycoproteins MUC5B and MUC7, due partly to the presence of sialic acids and fucose on the terminal part of their carbohydrate chains, making them receptors for micro-organisms (37-39). Glycoprotein 340 (gp-340), also known as salivary agglutinin, is one of the bacterial-binding glycoproteins (40, 41) and has been shown to bind to oral streptococci (42).

Mucins

All mucosal surfaces are coated with a film comprising different types of mucins. The main mucins in the oral cavity are MUC5B (formerly referred to as MG1) and MUC7 (formerly referred to as MG2) (26, 39, 43-45).

MUC5B is a high-molecular-mass oligomeric glycoprotein, with a molecular mass of up to 44 MDa (26), characterized by serine-threonine-proline (STP)-rich domains, highly substituted with O-linked oligosaccharides. The carbohydrates comprise almost 80 per cent of the weight and contribute to the structure of the molecule and protect the protein core against proteolysis (33). The carbohydrate structure and terminal epitopes of MUC5B have a
high degree of heterogeneity, which might have important implications for the biological properties of the molecule (37, 46). Between the carbohydrate fractions, the polypeptide backbone consists of unglycosylated hydrophobic cysteine-rich domains (47).

MUC5B is found on both soft (24) and hard surfaces (18, 48) and has lubricating properties, attributed to its water-binding capacity. It has been suggested that MUC5B acts as a matrix-retainer on the oral surfaces for other protective proteins such as IgA, lactoferrin and lysozyme, (49, 50), which facilitates the elimination of foreign substances and organisms.

MUC7 is a smaller unit, with a molecular mass of 150 to 250 KDa, also containing STP-rich domains substituted with approximately 70 per cent carbohydrates (39, 45). It is suggested that MUC7 interacts with oral micro-organisms (36, 51), through its ability to self-associate (52, 53), forming larger complexes which agglutinate and eliminate the micro-organisms.

Gp-340
Gp-340 is present in tears, respiratory tract fluids and in the gastrointestinal tract (54, 55). It is a glycoprotein with a molecular mass of 300-400 kDa and a carbohydrate content of approximately 45 per cent (40). Gp-340 seems to be identical to salivary agglutinin and shows great similarities with Deleted in Malignant Brain Tumours 1 (DMBT1), and is encoded by the same gene, the dmbt1 gene (55, 56). It has been shown that gp-340 may modulate the bacterial composition due to its ability to inhibit colonization of Streptococcus mutans (42). Gp-340 also interacts with other salivary components, e.g. salivary IgA, creating complexes which agglutinate different types of bacteria (57, 58).

Glycosylation
Glycoproteins in humans consist of a protein core decorated with oligosaccharides (glycans). The oligosaccharides are covalently attached, most often via N- or O-linkages, to the protein core. The N-glycans are covalently linked to an asparagine residue, commonly involving an N-Acetylglucosamine (GlcNAc) residue and the O-glycans are frequently linked via N-Acetylgalactosamine (GalNAc) to a hydroxyl group of a serine or threonine residue, to
the protein core. The O-linked oligosaccharides have three sections: core, backbone and peripheral regions. The core section consists of permutations of Gal/GlcNAc/GalNAc and the backbone of repeating sequences of Gal-GlcNAc (33). The peripheral region consists of different types of carbohydrates such as sialic acid, fucose, constituting sometimes blood group determinants, or sulfate (37, 38, 59, 60).

The proteins undergo glycosylation primarily in the rough endoplasmic reticulum (N-linked) and Golgi apparatus of the cells (O-linked). The oligosaccharide pattern differs, probably due to the diversity in available glycosyltransferases (33).

**Salivary IgA**

IgA is involved in mucosal immunity on all the mucosal surfaces of the body (17), mostly in the form of secretory IgA, a dimer connected by a polypeptide (J chain) and surrounded by a proteolysis-resistant secretory component (SC). Monomeric IgA may also be present (mostly originating from the gingival exudate) (16, 61). Approximately 30–35 per cent of all salivary IgA in adult whole saliva is secreted by the minor salivary glands (20).

Salivary IgA binds to various micro-organisms, disrupting adhesion (61-63). The concentration seems to vary in different mucosal areas. Some studies have reported high concentrations in particularly buccal and palatal gland saliva in adults (21, 27).

The formation of IgA consists of a series of events. Briefly, the micro-organism is engulfed and digested into small peptides which are absorbed by specialized membranous cells (M cells) on the mucosal-associated lymphoid tissues (MALT) in tonsils and/or adenoids (Waldeyer’s ring) and in the small intestine (Peyer’s patches). After absorption, the substance is transported by antigen-presenting cells (APC) which process and present the antigen to regulatory T cells. These cells stimulate B cells to differentiate into precursors of IgA-secreting plasma cells which then differentiate into plasma cells, which produce IgA antibodies against the original antigen (17). Secretory IgA is then formed when the IgA molecule adheres to the secretory component in the membrane of the glandular cell, thus the molecule becomes more resistant to proteolytic degradation (17).
Age-dependent differences in salivary secretion

Saliva flow rate seems to increase in the growing individual (64), but for the ageing individual, there are divergent data: some studies report a decrease in flow rate in middle-age (65-67), while other investigators have failed to find any differences in rates between young and older adults (68).

Some of the whole saliva components are reported to change in concentration early in life. For example an increase of MUC5B and a decrease of MUC7 have been observed during infancy (69). In ageing individuals, however, the total quantity of mucins seems to decline (70, 71).

The salivary IgA-concentration in whole saliva and serum is reported to increase during infancy and childhood (30, 72, 73), reaching adult levels at four to twelve years of age (16, 74). In later life (60–80 yrs), a gradual decrease in concentration is reported (75-78).

Age, gender and site-dependent differences in minor glands

In adults, the data on minor salivary gland flow rates in relation to age are contradictory: some studies report comparable flow rates from minor glands in young and older individuals (21, 79-82), while others report a decrease with age (83, 84).

There are also conflicting reports about age-dependent differences in salivary IgA, the major adaptive immune component in saliva. Eliasson et al. (21) observed that the concentration in minor gland saliva was higher in older than younger subjects (> and <65 yrs respectively), while the opposite was reported by Smith et al. (83): lower values in older than in younger subjects (>55 yrs and 17-24 years respectively).

Minor salivary gland flow rates and IgA-concentration are reported to be lower in women than in men (21, 79, 84). However, some studies report comparable IgA-concentrations in men and women (83).

Furthermore site-dependent differences, with lower flow rates from labial than buccal glands have also been observed (21, 27, 79).

There are no corresponding studies of minor gland saliva in children. Thus little is known about the salivary secretion rates or
the innate and adaptive immune components of minor gland saliva in children, or in comparison with corresponding values in adults.

**Measurement of minor gland secretion**

Minor gland saliva is viscous and secreted only in small volumes. Sampling is thus a challenge. Estimations of salivary volume have been made by different absorption techniques, using filter paper discs (85, 86), synthetic discs (87) or surgical sponges (83) in combination with gravimetric, electromagnetic methods (Periotron-method®) or semiquantitative methods. Syringes and capillary tubes have also been used (88, 89). Volumetric measurements have also been made from photographs of droplets of saliva formed on mucosa, or saliva-coloured dots, depicting droplets of saliva, on chromatology papers (80, 90). For subsequent qualitative analysis of samples, various biochemical techniques are applied, such as radial immunodiffusion (RIA) (20, 85), enzyme-linked immunosorbent assay (ELISA) (27, 31) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (25).

The Periotron-method® was originally developed for measuring crevicular fluid (91). The method has high precision and reproducibility in estimating small volumes and has been modified for research on saliva (79, 81, 86, 92). Briefly, this electromagnetic instrument creates a voltage between two plates on the measuring instrument and measures the resistance of the salivary molecules in the filter paper, corresponding to a loss in voltage, and transforms the loss in voltage to relative values shown on the instrument display. These figures constitute the basis for calculation of the volumes secreted. The method provides both an estimation of flow rate by measuring the salivary volume in the filter paper and biochemical analysis of the salivary components collected on the paper. An important advantage of the technique is that the equipment is easy to handle and thus appropriate for studies of mucosal gland secretions in children.

**Final remarks**

Biological and pathological responses in children have frequently been extrapolated from the findings of studies on adults. As reported above, no published studies on minor salivary glands in
children, seem to be available. Documentation of minor salivary gland secretion in young individuals is fundamental to our understanding of the maturation of the salivary protective systems. Based on the hypothesis that the secretions change between childhood and adulthood, the present clinical investigations have been designed to study flow rate, gland density, and some of the major innate and adaptive immune components in the saliva of children and adults. The studies have been conducted under similar, controlled conditions, using the same methods. Furthering our knowledge of minor gland secretion should lead to improved understanding of the mechanisms by which the oral tissues are lubricated and protected in individuals of different ages and states of health.
AIMS

As data on minor salivary glands in children seem to be unavailable, the overall objective of this thesis was to investigate various salivary factors in 3-yr-old children, 14-yr-old adolescents and 20 to 25 yr-old adults. The specific aims were:

- To compare the secretion rate of labial and buccal glands in children and adults (Paper I).
- To compare the labial and buccal gland density in children and adults (Paper I).

- To compare the content of MUC5B and MUC7, commonly occurring components of the innate immune system, in labial and buccal gland saliva in children and adults (Paper II).

- To compare the concentration of salivary IgA, the major component of the adaptive immune system, in labial and buccal gland saliva in children and adults (Paper III).
- To compare salivary IgA-concentrations in whole saliva of children and adults (Paper III).

- To compare the content of gp-340, a bacterial-binding component of the innate immune system, in labial, buccal and whole saliva of children and adults (Paper IV).
- To compare the content of sialic acid, a terminal carbohydrate of glycoproteins, in labial, buccal and whole saliva in children and adults (Paper IV).
SUBJECTS AND METHODS

Subjects
Papers I-II
In all, 90 participants were recruited to the study. There were three age-groups: 3-yr-olds, 14-yr-olds and 20 to 25-yr-olds. Each age-group comprised 30 subjects, equal numbers of males and females. The subjects were healthy and not taking any medication at the time of examination, except for five adult females using contraceptives. Three subjects occasionally used antihistamines, but not during the week before they participated in the study. All subjects, with the exception of two Asian children, were of Caucasian origin. The 3-yr-olds and the 14-yr-olds were randomly selected from the patient stock at the School of Dentistry, University of Malmö, Sweden and the adult group comprised students at the University of Malmö.

Saliva sampling and measurements were carried out between February and July 2001. The participants were instructed, either directly or through their parents, not to eat, drink or brush their teeth for at least 1 h prior to sampling. In order to reduce the influence of circadian variations (75), all measurements were taken between 9 and 12 a.m. The adolescent and adult subjects were instructed not to use tobacco on the day of the appointment. Before study start, all participants of legal age, and the parents of the children and adolescents, gave written informed consent to participation.
Papers III-IV
Another saliva sample was collected, under the same conditions as for Papers I and II.

The subject selection procedure, instructions to the participants, saliva collection time and oral hygiene were the same as for the previous collections. Before study start, all participants of legal age, and the parents of the children and adolescents, gave written informed consent to participation. Eighty-seven of the 90 subjects were Caucasians and three, one in each age group, were of Asian origin. Saliva sampling and measurements were undertaken between March and July 2005.

Studies I-IV were approved by the Ethics Committee of the University of Lund, Sweden (Dnr. LU 437-00 and LU 766-02).

Methods
Papers I-II
Measurement of salivary secretion from minor glands
Unstimulated (resting) saliva was collected from the left labial and buccal mucosal areas of the oral cavity. In the labial mucosal area, measurements were made near the mid-line of the lower lip, halfway between the vermillion border and the vestibule. In the buccal mucosal area, measurements were made at the level of the parotid gland duct and approximately midway between the duct and the angle of the lips.

The mucosa in the area of measurement was gently dried with a small cotton pad and then immediately covered with a filter paper (SialoPaper Ø 8 mm, Oraflow Inc., Smithtown, NY, USA), the buccal area for 5 s and the labial area for 15 s. To ensure mucosal contact, the paper was held under light finger pressure; the surgical glove on the examiner’s hand protected the paper. To measure the volume of collected saliva, a Periotron 8000® instrument (Oraflow Inc.) was used. Before measurement of the volume of saliva, the instrument was adjusted by placing the filter paper between the sensors. After harvesting, the paper was immediately placed in the device and the reading (0-199 units) was recorded. Secretion, expressed as $\mu l \, cm^{-2} \, min^{-1}$, was then calculated from the regression formula $y = 0.011x - 0.036$ where $y$ is the volume in $\mu l$ and $x$ the displayed Periotron 8000® digits. The regression formula was
estimated by creating a standard curve made up of different volumes of distilled water, aspirated by a Hamilton Syringe™ (model 7001KH), onto filter papers. The formula describes a linear relationship between aspirated volumes and the readable digits. The reproducibility of the instrument was tested regularly during the saliva collection period.

**Measurement of numerical density of minor glands**

The number of active glands was assessed according to Gaubenstock et al. (90). After measurement of salivary secretion, the labial and buccal mucosa on the right side was dried with a cotton pad and covered with a pre-cut circular disc (Ø 10 mm) of chromatography filter (Millipore Corporation, Bedford, MA, USA). The disc was immediately removed and the mucosa was left exposed for 20 s, allowing droplets of saliva to form. A new disc was applied under light pressure and then immediately removed and transported to the laboratory for analysis.

The discs were submerged in 0.104 M periodic acid, followed by Schiff’s reagent and washed in 0.04 M sodium metabisulphite solution. The glycoproteins of the salivary droplets took on a dark red stain (Fig. 3). The number of secretory glands was assessed under light microscopy (16x) and expressed as the number of secretory minor salivary glands per cm².

Despite a number of pre-study tests of varying intervals for salivary droplet formation and different staining techniques, reliable numerical assessments were not achieved for the buccal mucosal area.

*Fig. 3. Disc with salivary droplets treated with periodic acid and Schiff’s reagent, representing gland density. Photo: Hans Herrlander.*
Assessment of minor gland secretion of MUC5B and MUC7

After measurement of minor gland flow rate (Paper I) the filter papers were placed in Eppendorf tubes™ with 150 µl 4 M guanidinium chloride (GuHCl) and stored at below -80°C until biochemical analysis. Four papers from the labial and buccal mucosal areas, respectively, were used to obtain an adequate volume of saliva for analysis.

The amount of MUC5B was assessed by ELISA, using the polyclonal antiserum LUM5B-2. The antiserum was raised against a sequence within the cysteine-rich domain, in the central exon of MUC5B and outside the glycosylated domain, to avoid epitope shielding (93). The amount of MUC7 was estimated by the use of the polyclonal antiserum LUM7-1, raised against specific amino acid motif (94). The MUC5B used as a standard, was purified using density-gradient centrifugation of whole saliva and the MUC7 standard was also pooled out from a density gradient, although these fractions contained a mixture of proteins. The standard curves for MUC5B and MUC7 were made by serial dilutions and showed a linear relationship between the serially diluted standard and the absorbance.

Papers III-IV

Minor gland and whole saliva collection

In papers III and IV both minor gland saliva and whole saliva were collected.

Unstimulated (resting) minor gland saliva was collected from lower labial and buccal mucosal areas, as described above. The collection times were 120 s (labial) and 60 s (buccal). After volume measurements as described above, the filter papers were placed in Eppendorf tubes™ containing 150 µl phosphate buffered saline (PBS), pH 7.2 and stored at below -80°C until biochemical analysis. One paper per area was used. All samples were collected by the same investigator as in previous saliva sampling, assisted by a biochemical technician.

At the same appointment, minor gland saliva sampling was followed by collection of whole saliva. The participants were seated, leaning forward slightly, so that the saliva could drain passively for 1-5 min. into a polypropylene tube (Sarstedt,
Nümbrecht, Germany). Children sat in their parents’ laps. After collection, the samples were stored at below -80°C.

**Assessment of salivary IgA, glycoprotein 340 (gp-340) and total protein in saliva**

To assess the concentrations of salivary IgA in small sample volumes, a modified sandwich ELISA using polyclonal antibodies was carried out (95). The gp-340 content was determined through ELISA, using polyclonal antiserum (96). Total protein concentration of whole saliva was assessed by a Protein Assay (Bio-Rad). The protein assay is a colorimetric assay, based on a shift in color reflecting protein concentration. An absorbance microplate reader (ELx800; BioTek Instruments, Winooski, VT, USA) was used to measure the reactivity of the salivary IgA, total protein and gp-340. The results for salivary IgA and total protein were plotted against specific standard curves of colostrum or bovine serum albumin, respectively. Gp-340 was pooled out of a density gradient, containing a mixture of proteins, serially diluted and used as standard curve. Measurements were performed within the linear range of the standard curves.

**Assessment of carbohydrates**

Salivary samples were analyzed by ELLA, using a mixture of the lectins *Sambucus nigra*, recognizing sialic acid (NANA-2-6) and *Maakia amurensis*, recognizing sialic acid (NANA-2-3) (97). The results of carbohydrate reactivity were plotted against a standard curve made by serial dilution of purified MUC5B, analyzed for carbohydrate content, to check that the absorbance values were within the range of the standard.

In a supplementary analysis of whole saliva, fucose content was analysed using the *Ulex europaeus* agglutinin I lectin, recognizing fucose (α-fuc).

**Recovery from the filter papers**

To maximize the recovery of mucins, disulphide bonds were abolished by adding 10 mM dithiothreitol (DTT), in 6 M GuHCl, 0.1M Tris-HCl buffer (pH 8.0), for 1 h at 37°C, followed by
alkylation by addition of iodacetamide (IAA). The reduced samples were then centrifuged (18,000 x g, 10 min) and analyzed.

To estimate the recovery of mucins, equal volumes of whole saliva from one individual were placed on filter papers and in tubes containing 150 µl 4 M GuHCl. The filter papers were then placed in the Periotron 8000® for volume assessment. The filter papers, together with the saliva in the tubes, were then treated according to the above protocol. Recovery from the filter papers was 73 and 72 per cent for MUC5B and MUC7, respectively.

Pilot studies were also undertaken on salivary IgA, to reduce the absorption of the protein in the filter papers. The highest recovery was achieved by preparing the papers with 0.05% polysorbate 20 (Tween20) in PBS before use. 4M GuHCl was tested but was shown to interfere with the biochemical analysis. The recovery differed by only 1-2 per cent between saliva containing low and high concentrations of salivary IgA, respectively. The mean recovery was 35 per cent.

**Statistical methods**

*Sample size calculation*

As no data on salivary secretion and mucins in minor gland saliva in children were available, sample size calculation in papers I and II were based on an assumption that a mean difference between age-groups of approximately 50 per cent might be of clinical relevance. Assuming a standard deviation within the group of approximately 50 per cent and a power of 90 per cent at a significance level of 5 per cent, the sample size was estimated to n = 30. In Paper III a mean difference in salivary IgA concentration of 3.5 mg 100 ml⁻¹ was assumed to be clinically relevant. Assuming a SD of 4.0 mg 100 ml⁻¹ within the age-groups, the resultant sample size, at 5% significance and a power of 90% was calculated as n = 28. With reference to analysis of gp-340 and sialic acid in paper IV, there were no existing data on age-dependent differences. After pre-study tests, an assumption on sample size was made. To achieve a power of 90 per cent, the required sample size was calculated to be 25 individuals. A mean difference of 100 per cent between the age-groups and a SD of 100 per cent were chosen.
Descriptive data
The data were analyzed by the GraphPad Prism (GraphPad Software Inc. San Diego, CA, USA) version 4.0 software program and SPSS (SPSS, SPSS Inc., Chicago, IL, USA). For numerical data the arithmetic mean and standard deviations (SD) were calculated.

In papers I and II, a two-way analysis of variance, with comparisons by Tukey’s post-hoc test, was used to test the influence of the factors age and gender on secretion, the number of secretory glands and MUC5B and MUC7 content. Differences at the 0.05 level of significance were considered to be statistically significant. In Papers III and IV, a one-way analysis of variance was applied, with comparisons by Tukey’s test, to calculate the effects of age on the concentration of salivary IgA and total protein.

To investigate the effect of gender on salivary IgA-concentrations or the gp-340 and the sialic acid contents within each age-group (papers III, IV), Mann-Whitney U-tests were used because of the lower number of individuals in the subgroups.

Paired t-tests were carried out to analyze the intra-individual differences in concentrations between the mucosal sites (papers I, III, IV). Intra-individual correlations were tested by Pearson’s correlation test. P-values below 0.05 were considered to be statistically significant (papers III, IV).
RESULTS

Paper I
Minor gland salivary secretion rates
The flow rate from buccal glands was statistically significantly lower in the 3-yr-olds than in the 20 to 25-yr-olds. There were no age-dependent differences in the salivary secretion of the labial glands, nor were there any statistically significant intra-group gender differences with respect to either buccal or labial gland secretions. In all three age groups, the labial secretion rate was significantly lower than the buccal rate (Table 1).

Numerical density
The mean density of the labial secretory glands was significantly higher in the 3-yr-olds than in the 14 and the 20 to 25-yr-olds. Moreover, the density in the 14-yr-olds was significantly higher than in the 20 to 25-yr-olds (Fig. 4).

![Fig. 4](image-url)  
Fig. 4. Plots of numerical density of glands in the three age-groups (dark line = mean, glands/cm²). The 3-yr-olds had significantly higher numbers than the 14-yr-olds and the 20-25 yr-olds (P < 0.01).
Supplementary calculation of labial gland secretion rates in relation to gland density showed a significantly lower secretion rate per gland in the 3-yr-olds than in the adults. These calculations are based on the assumption of a bilateral symmetry of secretion from labial glands (82). No intra-group gender differences were disclosed.

Paper II
Mucins in minor gland saliva
No significant age-related differences in MUC5B content of the labial gland saliva were noted (Table 1). The subgroup 3-yr-old males had the lowest MUC5B content, significantly lower than that of the 3-yr-old females. However, compared to the adults, the labial gland saliva of the 3-yr-old group as a whole contained significantly less MUC7.

In contrast, analysis of buccal gland saliva disclosed measurable amounts of MUC5B in only nine 3-yr-olds, two 14-yr-olds, and none of the adults. Measurable amounts of MUC7 were found in only nine 3-yr-olds, three 14-yr-olds, and two of the adults.

Assuming bilateral symmetry in secretion from labial glands, as mentioned above (paper I), further calculation of MUC5B and MUC7 in relation to numerical gland density showed a significant increase with age in both MUC5B and MUC7 secretion from individual glands (Figs. 5, 6).

![Fig. 5. Content of MUC5B per gland (mean, SEM, abs/µl/gland). The 3-yr-olds had significantly lower values than the 20-25-yr-olds (P < 0.05).](image)

![Fig. 6. Content of MUC7 per gland (mean, SEM, abs/µl/gland). The 3-yr-olds had significantly lower values than the 20-25-yr-olds (P < 0.01).](image)
Paper III
Salivary IgA in minor gland saliva
The concentration of salivary IgA in labial gland saliva was significantly lower in the 3-yr-olds (Table 1) than in the other age-groups. In the adult group, females had significantly lower concentrations than males.

In buccal gland saliva, no statistically significant inter-group differences in mean salivary IgA concentration were observed. In the adult group, the buccal gland concentration was significantly lower in females than in males.

Comparisons of salivary IgA concentrations in the buccal and labial glands revealed no statistically significant differences, except in the group of 3-yr-olds as a whole, where the concentration in the labial glands was significantly lower.

There were no statistically significant intra-individual correlations in salivary IgA between the different types of saliva in any of the age groups.

Salivary IgA in whole saliva
In whole saliva, the lowest concentration of salivary IgA was detected in the 3-yr-olds (Table 2); the difference between this group and both the older age-groups was statistically significant. Salivary IgA-concentration expressed as a ratio of total protein was also significantly lower in the 3-yr-olds than in the 14-yr-olds.

Paper IV
Gp-340 in minor gland saliva
The gp-340 content of labial and buccal gland saliva respectively, did not differ significantly among the age-groups (Table 1). This applied not only to the total group of subjects, but also to the subgroups of males and females.

Comparison of the gp-340 content of buccal and labial glands revealed a statistically significant difference in the adults, with statistically significantly higher values for the buccal glands. There was no intra-individual correlation in the gp-340 content of the different types of minor gland saliva.
Table 1. Summary of age-dependent findings on minor salivary gland components (Papers I-IV).

<table>
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<tr>
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<tbody>
<tr>
<td><strong>Labial glands</strong></td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>All (mean)</td>
<td>2.9</td>
<td>2.6</td>
<td>2.5</td>
<td>0.21</td>
<td>0.19</td>
<td>0.17</td>
<td>0.09*</td>
<td>0.11</td>
<td>0.21</td>
<td>0.037*</td>
<td>0.126</td>
<td>0.128</td>
<td>539</td>
<td>549</td>
<td>501*</td>
</tr>
<tr>
<td>SD</td>
<td>1.4</td>
<td>1.8</td>
<td>1.4</td>
<td>0.20</td>
<td>0.16</td>
<td>0.11</td>
<td>0.09</td>
<td>0.13</td>
<td>0.20</td>
<td>0.035</td>
<td>0.128</td>
<td>0.134</td>
<td>446</td>
<td>574</td>
<td>431</td>
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<tr>
<td><strong>Buccal glands</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>All (mean)</td>
<td>7.7*</td>
<td>10.1</td>
<td>11.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.112</td>
<td>0.123</td>
<td>0.176</td>
<td>539</td>
<td>633</td>
<td>820</td>
<td>438</td>
<td>644</td>
<td>782</td>
</tr>
<tr>
<td>SD</td>
<td>4.4</td>
<td>4.9</td>
<td>4.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.140</td>
<td>0.139</td>
<td>0.224</td>
<td>539</td>
<td>513</td>
<td>591</td>
<td>398</td>
<td>524</td>
<td>555</td>
</tr>
</tbody>
</table>

1. The 3-yr-olds had significantly lower secretion rates than the adults ($P < 0.01$).
2. The 3-yr-olds had significantly lower MUC7 content than the 20 to 25-yr-olds ($P < 0.04$).
3. The 3-yr-olds had significantly lower salivary IgA concentration than the 14-yr-olds or the 20 to 25-yr-olds ($P < 0.01$).
4. The 20 to 25-yr-olds had significantly lower gp-340 content in the labial than in the buccal glands ($P < 0.05$).
5. The 20 to 25-yr-olds had significantly lower sialic acid content in the labial than in the buccal glands ($P < 0.01$).
Table 2. Components of whole saliva (Papers III-IV)

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Paper III</th>
<th></th>
<th>Paper IV</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Salivary IgA (mg/ml)</td>
<td>3</td>
<td>0.090</td>
<td>0.091</td>
<td>0.179</td>
<td>0.149</td>
<td>0.170</td>
<td>0.099</td>
</tr>
<tr>
<td></td>
<td>Gp-340 (abs/µl)</td>
<td>3</td>
<td>33.99</td>
<td>12.50</td>
<td>29.35</td>
<td>18.88</td>
<td>20.17</td>
<td>12.70</td>
</tr>
<tr>
<td>14</td>
<td>Sialic acid (abs/µl)</td>
<td>14</td>
<td>0.44</td>
<td>0.60</td>
<td>0.65</td>
<td>0.28</td>
<td>0.65</td>
<td>0.28</td>
</tr>
<tr>
<td>20-25</td>
<td>Total protein (µg/µl)</td>
<td>20-25</td>
<td>3.36</td>
<td>3.28</td>
<td>2.15</td>
<td>3.28</td>
<td>2.15</td>
<td>2.31</td>
</tr>
<tr>
<td></td>
<td>Fucose (abs/µl)</td>
<td>20-25</td>
<td>3.28</td>
<td>3.28</td>
<td>2.28</td>
<td>2.28</td>
<td>2.28</td>
<td>2.61</td>
</tr>
</tbody>
</table>

A Supplementary analysis

* The 3-yr-olds had significantly lower concentrations of salivary IgA than the 20-25 yr-olds (P<0.05).
* The 3 yr-olds had significantly higher amounts of gp-340 than the 20-25 yr-olds (P<0.01).
* The 3-yr-olds had significantly lower concentrations of total protein than the 20-25 yr-olds (P<0.05).
Gp-340 and total protein in whole saliva
Compared with the older age-groups, the gp-340 content of whole saliva was statistically significantly higher in 3 yr-olds (Table 2). Moreover, the ratio of gp-340 to total protein in whole saliva was significantly higher in the 3-yr-olds than in the adults. The total protein concentration was significantly lower in the 3-yr olds than in the adults (Table 2).

Sialic acid in minor gland saliva
The sialic acid content of labial and buccal gland saliva respectively did not differ significantly among the age-groups (Table 1). This applied to analysis of the total age-group as well as to the subgroups of males and females.

Comparison of the sialic acid content of buccal and labial gland saliva revealed a statistically significant difference within the adult group, with statistically significantly higher values for buccal gland saliva (Table 1). No intra-individual correlations were observed in the sialic acid content of the different types of minor gland saliva.

Sialic acid in whole saliva
The sialic acid content of whole saliva did not differ significantly among the age-groups (Table 2). However, expressed as a ratio of total protein, a decrease with age was noted and the difference between the 3-yr-olds and the adults was statistically significant. When gp-340 was expressed as a ratio to sialic acid in minor gland and whole saliva, the proportion of gp-340 did not change.

Whole saliva was further analyzed with respect to the content of fucose, a common terminal carbohydrate of gp-340, revealing a trend towards higher values in the 3-yr-olds than in the other age-groups. Expressed as a ratio of total protein, statistically significantly higher values were disclosed in the 3-yr-olds than in the older age-groups (Table 2).
DISCUSSION

This series of studies was undertaken in order to produce scientific evidence to support clinical observations that the saliva coating the oral mucosa in young (pre-school) children is more viscous than in adults. A search of the literature for published studies on the secretion of the minor salivary glands disclosed that most of the available data originate from studies on adults, primarily on flow rates in the elderly and patients undergoing medical treatment. No clinical study on the secretion of minor salivary glands in healthy, growing individuals was found in the literature.

As described in the introduction, a variety of methods has been applied to determine the flow rates of minor salivary glands in adults. Most studies have been conducted on the readily accessible labial glands. However, because of the variety of methods used, it is difficult to compare the results of the studies. The Periotron® method is now applied in studies of salivary flow rates (21, 27, 79, 86, 92, 98). It is uncomplicated to use and involves no discomfort for the subjects.

As the youngest age-group in the studies, 3-yr-olds were selected on the basis of children’s ability to comply during the measurement procedures: 3-yr-olds, but not younger children, are generally able to co-operate during an oral examination. The selection of 14-yr-old adolescents met the requirement of an age-group between childhood and adulthood, with an established permanent dentition. The 20 to 25 yr-olds represented young adults.
Methodological aspects

Collection of whole saliva from pre-school children is truly a challenge. It is difficult for young children to comply with instructions to refrain from swallowing the saliva and not to move the tongue or other muscles in the lower facial region. Thus the risk that the samples in fact comprised slightly stimulated, rather than resting saliva, could not be excluded, and this might have influenced the present results. This risk has also been discussed in studies on adults (99).

During measurement of minor salivary glands, the filter paper was held against a well-defined mucosal area, under fingertip pressure. To avoid contamination, the investigator wore a surgical glove, in accordance with Eliasson et al. (79). This method of assessing mucosal flow rates might involve some risk of glandular stimulation: it has previously been shown that mechanical stimulation increases flow rates from the glands (100). The magnitude of stimulation of minor glands is unknown, but as one investigator undertook all measurements, the degree of stimulation should have been constant throughout.

Whether the estimated flow rates using the Periotron® reflect the “true” unstimulated secretion rate has been questioned. Compared to other studies, the values are relatively high, especially for buccal areas (101). It has been suggested that when different areas of the mucosa are being investigated, variations in density of minor glands throughout the mucosa might influence the flow rates (102). These authors also suggested that while the flow rates estimated by the Periotron®-method should definitely not be extrapolated to give the total salivary flow rate, the method was appropriate for comparative studies of flow rates at specific mucosal sites (102).

Associated with the filter paper method of sampling saliva is the risk of complex bindings between salivary components, or retention of salivary molecules within the filter papers. Different extraction buffers were used to improve recovery. To extract the glycoproteins, the disulphide bonds were abolished by DTT and to prevent the mucins from reclaiming their molecular structure, IAA was added (Paper II). To enhance recovery of IgA (Paper III), the papers were soaked in Tween20, thus saturating the binding areas and reducing the surface tension of the papers.
In the mucin study (Paper II), recovery was approximately 72 per cent, which is marginally lower than data on protein recovery (27, 98). In the IgA study (Paper III) the yield was lower (~35 per cent), but recovery from papers with low or high salivary IgA-concentrations showed negligible differences (1-2 per cent), thus making the comparisons between age-groups feasible: the primary aim of all the investigations was to make group comparisons.

Another potential risk associated with the use of filter papers is leakage of interstitial fluid through the epithelia onto the paper (103). However, as the epithelia of the lip and cheek consist of several dense cellular layers and basement membranes, it seems reasonable to assume that the volume of fluid passing out of the tissues is minimal.

In the studies of mucins and gp-340 (Paper II and Paper IV) the polyclonal antisera LUM5B-2, LUM7-1 and anti-gp-340 were used to detect MUC5B, MUC7 and gp-340, respectively (93, 94, 96). All antibodies were raised against specific peptide sequences in unglycosylated stretches of the glycoproteins. The specificity of the polyclonal antibodies and the treatment of the samples with different extraction buffers before analysis have reduced the risks of carbohydrate masking the mucins and gp-340. Polyclonal antibodies were also used in the salivary IgA study (Paper III). The antibodies were raised against peptide epitopes on the α1- and α2-chains on the heavy chains of the IgA molecules. The use of polyclonal antibodies, interacting with several specific sites on the molecules, increases the chances of detecting the target molecules.

To our knowledge there are no previous studies on sialic acid in minor gland saliva. In some studies on agglutinins in minor gland saliva, antibodies against e.g. specific carbohydrate epitopes, such as sialic acid on the glycoprotein, were used (104, 105). This might indirectly describe the presence of sialic acid. Moreover, other components, such as secretory-IgA, contain sialic acid, but only in minor amounts; sialic acid has been shown to constitute the smallest part of the carbohydrates present in secretory IgA (106). To maximize the detection of sialic acid (Paper IV), a mixture of the lectins *Sambucus nigra* and *Maakia amurensis* which recognize only sialic acids, were used (97).
Minor glands
Secretion rates (Paper I)

The secretion rate of buccal glands was significantly lower in 3-yr-olds than in adults. In all age-groups, the calculated values for flow rates in buccal glands were about three times as high as in labial glands.

The cause of age-dependent differences only in buccal secretion rates is not clear. One explanation for the lower rates in children might be an age-dependent difference in sensitivity to glandular stimulation: compared to adults, the flow rates of labial glands in 3-yr-olds might be stimulated more readily in response to application of the filter paper to the mucosa. This issue has been addressed in general discussions of advantages and disadvantages of filter paper measurements (80, 82).

Another explanation for the lower flow rate in children might be the lower numerical density of buccal glands. However, as the density of minor glands in the lower labial mucosa was higher in the 3-yr-olds than in the adults (Paper I), it is more likely that these glands are not fully developed in young children, i.e. the glandular acini and ducts are smaller and have yet to reach maximum secretory capacity (Paper I). This has been discussed for major glands (14).

The site-dependent differences in secretion rates is in line with a previous report (79) might reflect differences in gland density, secretion pattern or responsiveness to the measurement procedures in the different mucosal areas. These variations in minor gland secretion may reflect variations of protection in different regions of the oral cavity.

No differences in flow rates were observed with respect to gender. This finding is in accordance with a study by Won and co-workers (98). Other studies have reported lower secretion rates among women (79, 84). However, the group characteristics, in these two studies are different and the lower flow rates applied to women in the older age-groups.

In the present studies, the flow rates for adults are within the range of earlier studies using the Periotron®. However, the reported data on labial flow rate vary five-fold (21, 79, 81, 82, 86, 98). There may be several explanations. The different measurement
techniques cause varying degrees of glandular stimulation. Moreover, even when the anatomical area to be measured is well-defined, the investigators may have applied the paper at different sites of the mucosal area; as discussed above, variations in the distribution of glands over the area may result in differences in flow rates. Seasonal variations may also have influenced the results: Kavanagh et al. (107) have reported seasonal fluctuations in the flow rates of whole saliva. However, as the measurements of flow rate in Paper I were carried out in accordance with some of the previous Periotron®-based studies (21, 27, 79), comparison of the results of Paper I with these studies should disclose the process of maturation of minor gland secretion from childhood to old age (Table 1).

Numerical density (Paper I)
The numerical density of the labial glands decreased with age. In adults, the average density was approximately 5.4 secretory glands per cm². The density of secretory glands in the adolescents (8.2) was also significantly lower than in the children (14.4), but significantly higher than in the adults. The values for adults are in accordance with previously presented data (80). Hypothetically, the higher degree of active secretory glands in children might be attributable to differences in sensitivity of the glands to stimulation, with higher sensitivity in children than in adolescents and adults, as discussed above. However, it seems more reasonable that the actual number of glands is constant and the decrease in numerical density is related to the general growth of the oral cavity, increasing the distance between the glands.

Using periodic acid-Shiff (PAS) staining it was not possible to calculate the numerical density of glands in the buccal area. This might be attributable to the low concentration of mucins in buccal gland saliva (Paper II): periodic acid oxidizes the carbohydrates on the glycoproteins collected on the papers, thus almost no mucins are available for staining. An immunohistochemical study by Riva et al. (29) showed that the buccal glands contain more seromucous cells than the labial glands; under such conditions, the saliva would be expected to have lower concentrations of glycoproteins as mucins. Also different concentrations of
Coomassie Brilliant Blue staining (108) were tested but no distinct dots were shown. Another explanation for the absence of distinct dots in the papers used in the buccal area could be the texture of the mucosa. The epithelium of the buccal mucosa is reported to be thicker than the labial mucosa, with more pronounced fissures (109), thus allowing the saliva to spread more extensively over the buccal area, before being soaked into the filter paper.

**Innate components in minor gland saliva (Papers II, IV)**

In labial gland saliva, the 3-yr-olds had approximately the same amount of MUC5B as the adolescents and adults, but significantly lower amounts of MUC7 than the adults (Paper II). With respect to the buccal glands, detectable amounts of MUC5B and MUC7 were observed only in a minority of the participants.

The results with respect to MUC5B from labial glands (Paper II) might imply that the secretion has reached adult levels as early as the age of three years. However, calculations of secreted amounts of MUC5B and MUC7 in relation to labial gland density revealed a significantly lower mucin secretory capacity of individual labial glands in the 3-yr-olds than in the adults. An age-dependent increase in mucin secretion by the individual gland seems reasonable, as gland density decreases and concurrently the total area to be “lubricated and protected” increases: from 118 cm$^2$ in 5-yr-olds to 215 cm$^2$ in adults (14, 110). The finding that MUC7 content of labial gland saliva is lower in children than in adults suggests that MUC7 is produced primarily by the palatal, the submandibular and sublingual glands.

Detectable amounts of MUC5B in buccal gland saliva were found in only nine of the children, two of the adolescents and none of the adults; detectable amounts of MUC7 were found in nine of the children, three of the adolescents and two of the adults. These findings suggest that MUC7 secretion by buccal glands decrease with age in contrast to labial glands were the content of MUC7 increase with age. These results might indicate that the secretion of MUC5B and MUC7 differ between the different glands, during growth.

Only limited data are available about the MUC5B and MUC7 content of saliva from different types of minor glands. MUC5B has
been detected in palatal saliva and MUC7 in both labial and palatal gland saliva (22, 23, 25, 26, 46, 111, 112).

As mentioned above, mucins were detected in the buccal gland saliva of only a few subjects (Paper II): this indicates site-dependent differences in production of these glycoproteins. As the mucins are reported to have anti-microbial properties (36, 51, 113-115), it is reasonable to assume that intra-oral variation in bacterial colonization might occur (116). Interestingly, in vitro studies have shown that adsorption of MUC5B to different surfaces increases with increased concentration (18). This tends to support the above-mentioned concept of intra-oral variation in biological activities.

Gp-340 was detected in labial and buccal gland saliva (Paper IV), a finding consistent with earlier reports on minor gland saliva (105). Although no age-dependent differences were observed with respect to the gp-340 content of labial or buccal gland saliva (Paper IV), in the adult group the gp-340 content was greater in buccal than in labial saliva. This is not readily explained, but as mucins in buccal saliva were detected in only a few adults, the observation indicates a regional variation in glycoprotein secretion by the minor glands; the differences in composition provide protective properties specific to the area in which the minor gland is located. Moreover, agglutinins are more frequent in premolar than incisor pellicle (104); this supports the findings in Paper IV of higher gp-340 content of buccal than labial gland saliva. While the lower gp-340 content of adult labial saliva might imply a weaker defence against microbes in this area, the lower gp-340 content might be compensated for by higher content of other components, such as mucins (Paper II).

The sialic acid content of labial and buccal gland saliva did not differ significantly among the age-groups or between the subgroups of males and females. In adults, the buccal gland values were higher than for the labial glands. The MUC5B, gp-340 and sialic acid content (Paper II, IV) of labial gland saliva did not differ significantly between children and adults, which might indicate that the content of sialic acid reflects the total glycoprotein content of minor gland saliva. In buccal gland saliva, mucins were detected in only a few subjects, suggesting that the glycoproteins which accommodate the sialic acids, and also glycosylation, might vary.
In females, the glycosylation of glycoproteins might fluctuate, as the amount of carbohydrates has been shown to increase in whole saliva and in other mucosal secretions during ovulation (117, 118). In the present studies, the menstrual cycle in the female subgroups of adolescents and adults was not recorded and this may have influenced the results.

An increase of sialic acid in overweight and obese children and a decrease in individuals with gingivitis and periodontitis have been reported (119, 120). The physiques of the participants, determined from their weights, seem to be normal. However, the samples were not analyzed with respect to bacterial and bacterial enzyme content. As bacteria and bacterial neuraminidases hydrolyze sialic acid (120), a high content of bacteria and bacterial enzymes might have reduced the levels of bound sialic acid. On the other hand, the use of filter papers should have reduced microbial contamination of the minor gland saliva samples.

**Adaptive component in minor gland saliva (Paper III)**

The major finding was a lower concentration of salivary IgA in labial saliva of children than in adolescents and young adults (Paper III). No differences were observed between adolescents and adults. In the children, the concentration in the labial glands was lower than in the buccal glands. No such difference was observed in the other age-groups.

The results indicate that the major components of the adaptive salivary immune system in minor gland saliva are still under development in children but had reached maturity in adolescents. Similar findings are reported for tears (121) and whole saliva (16, 74). As mentioned above, there are conflicting data on IgA-concentration in the ageing individual (21, 83), but other immunoglobulins might develop differently; the IgG-concentration has been reported to be relatively unchanged during ageing (83). The IgG-concentration in minor gland saliva in children has yet to be determined.

No differences were observed in the IgA-concentration of buccal and labial gland saliva in adolescents and adults. There are previous reports of higher concentrations in buccal than in labial saliva of adults (27). As the concentration was also reported to
increase with age (21), one explanation for the differences between
the present and previous reports on adults might be that the
concentration in buccal gland saliva continues to increase with age,
more so than in the labial saliva; compared with the subjects in the
present studies, the participants in the previous studies were older
and thus the site differences might be more pronounced. The
higher IgA-concentrations in buccal than in labial gland saliva in
the 3-yr-olds might be attributable to higher antigenic loading and
antigenic stimulation (122) in the buccal than in the labial area,
leading to an increase in concentration in buccal saliva.

In the present study, the intra-individual correlation of salivary
IgA-concentration between different minor gland saliva was tested,
but no correlation was found. Similar observations have been
reported in studies on palatal and labial gland saliva, together with
parotid saliva. One explanation proposed by the authors was
differences in lymphocyte homing between the glands (31) which
also might explain the results of the present study.

In the adult group, gender differences were observed for salivary
IgA-concentration (Paper III): women had significantly lower
concentrations than men in both labial and buccal gland saliva.
This is in agreement with previous studies on minor gland saliva
(21, 27), but in other types of secretions such as tears, no such
gender differences in IgA concentration are reported (121). While
all gender comparisons should be interpreted with caution because
of the low number of subjects in the subgroups, the differences in
the present study might reflect a hormonal influence on IgA
secretion in minor glands.

**Whole saliva**

**Adaptive and innate immune components (Papers III-IV)**
The present study disclosed significantly lower IgA-concentration
in whole saliva of children than in adolescent and adults, in
accordance with the results for minor glands (Paper III). The
concentration of IgA in saliva and serum increases rapidly during
the first year of life (72, 123) and the increase in salivary IgA-
concentration seems to continue until at least six to twelve years of
age (16, 74, 78). Later, during ageing, the concentration in whole
saliva seems to be unaffected or decreased (75).
The salivary IgA-concentrations in minor gland saliva and whole saliva were similar with those reported in a study on stimulated saliva by Eliasson et al. (27). Stimulation might have decreased the salivary IgA-concentration (124). However, whole saliva is a mixture of fluids, originating mainly from major and minor glands, but also harbouring not only a wide range of micro-organisms but also components of crevicular fluid and damaged mucosa, which together might have influenced the concentration. It is therefore difficult to compare concentrations found in whole and minor gland saliva.

The importance of salivary IgA in caries protection is controversial: there are as many studies reporting an anti-caries effect as there are studies reporting the opposite (125, 126). In young children, a maturation of the secretory immune system with impact on cariogenic microorganism, was seen during one year follow-up (127). However, IgA might have besides functions than caries protection, such as the defence of the upper respiratory tract (128). It has been shown that unstimulated (resting) saliva of children suffering from protein energy malnutrition has impaired immunological and agglutinating defence components, which might weaken defence against infections (72, 129). A decrease in IgA has also been reported among passive-smoking young children (130).

In the present study no statistically significant difference in salivary IgA-concentrations between genders was established, but there was a trend towards lower concentrations in women, which is in agreement with previous reports on IgA concentrations in saliva and serum, respectively (131, 132). There may be fluctuations in concentration in women; a correlation between estradiol and IgA concentrations in saliva has been reported (133). No data on the menstrual cycle were recorded in the present study, as mentioned above, but such a correlation might have influenced the results. The total protein content of whole saliva was significantly lower in the 3-yr-olds than in the adults, which is in agreement with the results presented by other observers (73).

The lower content of gp-340 in the whole saliva of adults but not in minor gland saliva might be a result of bacterial-specific binding or more thorough digestion than in children, as it is
assumed that the microflora of adults is more diverse and mature (134, 135). Moreover, in patients with oral diseases such as gingivitis and periodontitis, Shetty et al. (120) demonstrated a reduction of sialic acid in whole saliva, attributable to high levels of bacteria and bacterial neuraminidases.

Sialic acid exists in both free and bound forms. The bound form seems to react more specifically with free radicals than the free form (34, 136) and present results (paper IV) might indicate that the content of bound sialic acid follow the glycoprotein concentration in the different age-groups.

The analysis of sialic acid was supplemented by an analysis of another terminal epitope on mucins and gp-340, namely fucose (37), and showed a trend towards higher amounts in the saliva of children than in to adults (Table 2), which corroborates the findings for sialic acids; together the results might reflect a high glycoprotein content in the saliva of children. Thus although not relevant in saliva, the importance of fucose is highlighted by a proposal that the complement system, an enzyme cascade of serum proteins which participate in e.g. chemotaxis of immune cells, can be activated by binding to gp-340 in the presence of fucose, but not in its absence (Ligtenberg AJ, presentation at 9th ESS, Holland 2011).

It might be assumed that IgA represents the major adaptive immune component in minor gland secretion and the glycoproteins represent the major part of the innate immune system. As minor glands are spread out all over the mucosa and secrete saliva continuously and spontaneously they are important contributors to these lubricant and protective components which bathe the mucosa and the teeth. In the present studies it was found that in children, the most of the components of the innate immune system investigated, were secreted in amounts similar to those in adults. It could be hypothesized that the salivary innate immune system components compensates the ongoing development of the adaptive immune system. A similar “dynamic balance” has been observed among patients suffering from immunodeficiency but also in passive smoking children (130, 137).

Improved knowledge of the physiology and immunochemistry of the minor salivary glands is important for understanding the
complexity of mucosal immunity, not only with respect to developmental aspects, but also in terms of understanding the potential impact and consequences of different medical treatments, pharmacological or surgical, which affect the glands. Today the oral mucosa also constitutes an area frequently used to harvest grafting material for other mucosal areas of the body, such as the genito-urinary tract. Further research is warranted to establish the characteristic features of this donor area.
CONCLUSIONS

The present thesis warrants the following major conclusions:

· The flow rate of buccal salivary glands is lower in 3-yr-olds than in 20 to 25-yr-olds. In all age-groups, the labial flow rate is lower than the buccal. The numerical density of labial glands is higher in 3-yr-olds than in older age-groups.

· There are no age-related differences in the MUC5B content of labial gland saliva. In 3-yr-olds the MUC7 content of labial gland saliva is lower than in adults. Measurable amounts of MUC5B and MUC7 are found in buccal saliva and seems to decrease with age.

· The concentration of salivary IgA in labial gland saliva is lower in 3-yr-olds than in older age-groups. There are no age-related differences in salivary IgA concentration in buccal gland saliva. The concentration of salivary IgA is lower in whole saliva of 3-yr-olds than in adolescents and adults.

· The gp-340 and sialic acid content of labial and buccal gland saliva does not differ among the age-groups. In adults, the gp-340 and sialic acid content are higher in buccal than in labial gland saliva. Three-yr-olds have a higher gp-340 content in whole saliva but the sialic acid content does not differ among the age-groups. The ratio of sialic acid and gp-340 to total protein is greater in 3-yr-olds.
These initial studies on minor salivary glands in children indicate that some components of the salivary immune system in young children are already present in level of adults while others are present in lower concentrations. The labial and buccal minor salivary glands contribute to the lubrication and protection of the oral cavity, but to varying degrees, as the flow rate and also the secretion of several components differ between different mucosal areas. This might lead to differences in lubrication and protection of closely related soft and hard tissues and to site-dependent differences in pathological expressions.
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Minor salivary gland secretion in children and adults

Mikael Sonesson a,*, Lars Eliasson b, Lars Matsson a

a Department of Paediatric Dentistry, Faculty of Odontology, Malmö University, Carl Gustafs väg 34, SE-205 06 Malmö, Sweden
b Department of Oral Pathology, Faculty of Odontology, Göteborg University, Göteborg, Sweden

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Introduction

The minor salivary glands are important in the physiology and pathology of the oral cavity. By a continuous flow of fluid and secretion of various organic substances, the minor salivary glands are involved in the protection of the oral mucosa and contribute to oral comfort and oral health.1 The organic components include several protective proteins and glyco-proteins, such as IgA, lactoferrin, mucins, and lysozyme.2,3

Of the total amount of unstimulated or stimulated saliva, the contribution from the minor salivary glands is reported to be approximately 6—10%.4 A substantial variation in the flow rate of labial glands has been reported in different studies, although most authors estimate it to be below 1 μl cm⁻² min⁻¹.1,2 Regional differences in flow rate have been reported, and Eliasson et al.5 found significant differences between palatal, labial, and buccal sites. At all these sites, the flow rate was 10—20% lower in women.

Summary

The minor salivary glands are of great importance in the physiology and pathology of the oral cavity. So far, studies of the minor glands have concentrated on adults. In the present study, minor salivary gland secretion was studied in the buccal and labial mucosa of 3-year-old children, adolescents and young adults. In addition, the number of glands per surface area was assessed in the labial mucosa. A total of 90 individuals were included, 30 in each age-group. Saliva was collected on filter paper discs and the salivary secretion rate was measured using a Periotron 8000. The number of secreting labial glands was assessed on PAS-stained filter paper discs under a microscope. Salivary secretion in the buccal mucosa was found to be age-related, with a statistically significant lower rate of secretion (P = 0.003) in the 3-year-olds (mean 7.7 μl cm⁻² min⁻¹) compared with the young adults (11.9 μl cm⁻² min⁻¹). No significant differences between the sexes were noted. For the labial glands, no age- or sex-related differences were found. In all age-groups, salivary secretion was significantly higher in the buccal than in the labial mucosal area. A statistically significant difference in number of secreting glands was found between all age-groups, with a decreasing number of glands per surface unit with age. The number of glands was significantly lower in males compared with females in the group of adults. The lower rate of buccal salivary secretion in the young children may imply that the oral mucosa is more vulnerable to external injury and that caries protection on the buccal molar surfaces is lower. Previous studies indicate that adults with a reduced rate of minor salivary gland secretion are more susceptible to caries.
Notably, all studies seem to have been performed on adults. The inorganic and organic composition of the minor salivary glands differs in several respects from that of the major glands. Concerning the organic components, the concentration of secretory IgA seems to be substantially higher in the saliva of labial glands. In addition, mucins are considered a major secretory component of the minor salivary glands.

A correlation between dental caries and minor labial gland secretion has been reported. Thus, Speirs found a significantly lower flow rate in individuals with restored anterior teeth compared with individuals with caries-free anterior teeth, and Gaußenstock observed that the number of salivary glands and their secretion was lower in individuals with caries compared with caries-free individuals.

Concerning the major glands, longitudinal studies have revealed age-related differences in salivary flow rate. Thus, in a study of young individuals, an increase in stimulated flow rate with age was noted. On the other hand, in ageing individuals decreased as well as unchanged flow rates have been reported. No information about salivary secretion of the minor glands in children, including comparisons with that of adults, seems to be available. Therefore, the aim of this study was to compare the secretion rate of the minor glands in children with that in adults in defined mucosal areas. In addition, the number of glands in the labial mucosa was assessed.

Subjects and methods

A total of 90 subjects were enrolled and divided into three age-groups: 3-year-old children, 14-year-old children, and 20–25-year-old young adults. Each group consisted of 15 females and 15 males. All were healthy and did not use any medication at the time of the examination. Three individuals occasionally used antihistamines, but not during the week before examination. Six females took contraceptives. The 3- and 14-year-olds belonged to the patient stock of the School of Dentistry, Malmö University. The adults were dental students or friends of dental students. All measurements were carried out between February and July 2001. The study protocol was approved by the local ethics committee for human studies, and before the start of the study all participants and the parents of minors were informed and signed an informed consent form.

Measurements were performed in two mucosal areas, one labial and one buccal. The salivary secretion rate was measured on the left side of the oral cavity, and the numerical density was assessed on the right side. Previous studies indicate a bilateral symmetry for flow rates. The labial area measured was near the mid-line of the lower lip, halfway between the vermillion border and the vestibulum. The buccal area measured was at the level of parotid excretory duct and midway between the labial angle and the parotid excretory duct.

The participants were instructed, through their parents or directly, not to eat or drink for at least 1 h prior to the measurements. All measurements were made between 9 and 12 a.m. The teenagers and the adults were instructed not to use tobacco on the day of the measurements.

Salivary secretion

Saliva was collected and analysed according to Eliasson et al. Cotton rolls were placed in the upper and lower oral vestibules. The mucosa in the area of measurement was carefully dried with a small cotton pad and then immediately covered with a disc (8 mm) of Sialopaper (Proflow™, Inc., Amityville, NY, USA) (Fig. 1). The disc was gently pressed to the mucosa to avoid unnecessary stimulation of the glands and kept in place for 5 s (buccal area) and 15 s (labial area). Periotron 8000™ measurements were made immediately after the Sialopaper was removed. The times chosen were based on the results of a series of pre-tests made to keep the amounts of saliva collected within the measuring range of the instrument (0–199 units according to the manufacturer). In addition, the ability of the 3-year-old children to co-operate was considered.

The Periotron 8000™ (Proflow™, Inc., Amityville, NY, USA) was used to assess the amount of saliva.
collected. The Periotron 8000\textsuperscript{TM} was calibrated with different volumes of distilled water, aspirated with a Hamilton Syringe\textsuperscript{TM} (model 7001KH). The readings were highly reproducible. The secretion was calculated from the regression formula $y = 0.011x - 0.036$, where $y$ is the volume in μL, and $x$ the displayed Periotron 8000\textsuperscript{TM} digits and is expressed as μL cm$^{-2}$ min$^{-1}$.

**Numerical density of the minor salivary glands**

The number of glands was assessed according to Gaubenstock\textsuperscript{10} after salivary secretion had been measured. Cotton rolls were applied to isolate the test area. The mucosa was dried with a small cotton pad and covered with a pre-cut round disc ($\varnothing$ 10 mm) of chromatography filter (Millipore Corporation, Bedford, MA, USA). The disc was removed immediately, and the mucosa was left untouched for 20 s. The period of 20 s was chosen to allow salivary droplet formation. A new filter disc was held against the mucosa with light finger pressure, then immediately removed and transported to the laboratory for analysis.

The discs were submerged in 0.104 M periodic acid for 3 min, followed by 1.0% Schiff’s reagent for 3 min. Glyco-proteins of salivary droplets stained dark red (Fig. 2). To obtain an optimal background, the paper was washed in 0.04 M sodium metabisulphite solution and then dried. Assessment of the number of secreting glands was carried out under a microscope (16×) and expressed as the number of secreting minor salivary glands cm$^{-2}$.

In spite of a number of pre-tests using different times for salivary droplet formation and different staining techniques, reliable numeric assessments were not achieved for the buccal area. Thus, only data on the number of glands in the labial area is presented.

**Statistical analyses**

A two-way analysis of variance, with comparisons using Tukey’s method, was used to test the influence of factors sex and age on secretion and number of glands. The difference between the rates of buccal and labial secretion were analysed using a paired t-test. A confidence level of 5% was used in all tests.

**Results**

**Salivary secretion**

The mean rate of salivary secretion for the buccal and labial glands is presented in Table 1. For the buccal glands, no statistically significant relationship was found between sex and secretion or

| Table 1 | Salivary secretion in different age-groups in the buccal and labial mucosal area$^a$. |
| --- | --- | --- |
| | 3-year-olds | 14-year-olds | Adults |
| **Buccal area** | | | |
| Female ($n = 15$) | 6.9 (4.4) | 10.3 (5.8) | 11.1 (4.8) |
| Male ($n = 15$) | 8.5 (4.5) | 9.9 (3.9) | 12.7 (4.4) |
| Total ($n = 30$) | 7.7 (4.4)$^b$ | 10.1 (4.9) | 11.9 (4.6) |
| **Labial area** | | | |
| Female ($n = 15$) | 2.8 (1.4) | 2.4 (1.2) | 2.5 (1.5) |
| Male ($n = 15$) | 3.0 (1.5) | 2.8 (2.3) | 2.3 (1.4) |
| Total ($n = 30$) | 2.9 (1.4) | 2.6 (1.8) | 2.5 (1.4) |

$^a$ Salivary secretion, μL cm$^{-2}$ min$^{-1}$, mean (S.D.).

$^b$ Buccal secretion was lower in 3-year-olds than in adults ($P = 0.003$).
between age–sex interaction and secretion. A statistically significant relationship ($P = 0.004$), however, was seen regarding age. Tukey’s test revealed a statistically significant lower rate of secretion in the 3-year-olds compared with the 20–25-year-olds ($P = 0.003$). The difference noted between the 3- and 14-year-olds, however, was not statistically significant ($P = 0.121$). For the labial glands, no significant relationships were noted.

In all age-groups the total mean salivary secretion was significantly higher in the Buccal than the labial area ($P < 0.001$).

**Numerical density of glands**

The mean number of secreting glands per surface unit for the labial mucosa is presented in Table 2. No statistically significant relationship was found between age–sex interaction and number of glands. A statistically significant relationship with number of glands, however, was noted for the variables sex ($P = 0.013$) and age ($P < 0.001$). Tukey’s test found a statistically significant difference between all age-groups, with a decreasing number of glands per surface unit with age.

**Discussion**

The primary aim of the present study was to investigate age-related differences, if any, in salivary gland secretion in the buccal and lower labial areas of the oral mucosa. A significantly lower rate of secretion, assessed per surface unit, was found in the buccal mucosa of 3-year-old children compared with young adults. No age-related differences in secretion, however, were noted for the labial mucosal area. The lower rate of secretion in the buccal area of the 3-year-old children would indicate that the wetting capacity in the young children was lower and thus mucosal protection poorer. Eliasson et al. reported a 10–20% lower fluid output from buccal and labial glands in females than males. In the present study, no difference between males and females was noted, a finding in line with those by Won et al. in young adults. Six females took contraceptives. However, as the salivary secretion in these individuals did not differ from the rest of the individuals in the group of young adults, it was decided not to exclude them from the study.

The minor salivary glands contribute only a relatively small part to the total amount of saliva. Still, the minor glands are considered to be of significant importance for oral comfort and health among other things because of their content of mucins and comparatively high concentrations of antibacterial substances, such as IgA. The lower salivary secretion in the buccal area in the young children could be an indication that the oral mucosa is more vulnerable to external injury and that the caries protection of buccal molar surfaces is lower. Previous studies indicate that individuals with a reduction in the secretion rate of the minor salivary glands are more susceptible to caries.

The method used to assess the numeric density of active glands in the present study was inappropriate for the buccal mucosa as, in contrast to the labial mucosa, no distinct salivary droplets were formed and thus no stained dots could be clearly distinguished on the chromatography paper discs. In the labial mucosa, the number of active glands in the young adults was found to be about 5 cm$^{-2}$, a density similar to that reported by Ferguson. The number of labial glands was significantly higher in the young children compared with the older individuals. The gradual decrease in the number of glands per surface unit with age follows the general growth of the individual, and it is likely that the actual number of glands is constant and the decrease in numeric density is related to growth of the oral cavity. Another explanation for this age-related difference might be that the method used to prepare the test area more easily stimulated salivary secretion in the glands of the younger children than in the adolescents or adults, resulting in a larger number of active glands. No studies on the effect of stimulation of minor glands in children are available.

One explanation for the lower rate of salivary secretion in the buccal mucosa of the young children could be the lower number of glands per surface unit in these ages. However, as the numerical density of the glands in the labial mucosa was found to be higher in the group of 3-year-olds compared with the older age-groups, a lower number of glands in the buccal mucosa seems unlikely. Instead it is perhaps more reasonable to assume that the minor glands are not fully developed in the young child and have not reached their maximal secretory capacity. This is known to be the case for the major glands.

| Table 2 | Number of secreting minor salivary glands in different age-groups in the labial mucosa$^a$.
<table>
<thead>
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<td>3-year olds</td>
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<tr>
<td>Female ($n = 15$)</td>
<td>$16.6 (5.1)$</td>
</tr>
<tr>
<td>Male ($n = 15$)</td>
<td>$12.3 (5.0)$</td>
</tr>
<tr>
<td>Total ($n = 30$)</td>
<td>$14.4 (5.4)^b$</td>
</tr>
</tbody>
</table>

$^a$ Number of glands cm$^{-2}$, mean (S.D.).

$^b$ Number of glands higher in 3-year-olds than in 14-year-olds and adults ($P < 0.001$).

$^c$ Number of glands higher in 14-year-olds than in adults ($P = 0.016$).
A substantial difference between buccal and labial rates of salivary secretion was observed, the buccal secretion rate being 3–4-fold higher than the labial. The difference was similar in all age-groups. These results are in accordance with those of previous studies in adults\textsuperscript{13,20,21} and almost identical with the findings reported by Eliasson et al.\textsuperscript{5} The difference in secretion may reflect differences in gland density or secretion capacity in the different oral mucosal areas. The variation in minor gland secretion may mirror different protective demands in different regions of the oral cavity.

Concerning labial gland saliva, a secretion rate varying between less than 1 and 5 \( \mu \text{l cm}^{-2} \text{min}^{-1} \) has been reported.\textsuperscript{2,20,21} The present findings of 2.5–2.9 \( \mu \text{l cm}^{-2} \text{min}^{-1} \) for the labial glands are in line with those by Won et al.,\textsuperscript{16} but higher than the values reported by Shern et al.\textsuperscript{13,20} and lower than those of Eliasson et al.\textsuperscript{5} The present values for the buccal glands are also somewhat lower than those reported for these glands by Eliasson et al.\textsuperscript{5} The variation between the different studies may be related to study group characteristics. All studies above used filter paper to collect the fluid, and measurements were made with a Periotron\textsuperscript{16}. Still, the degree of gland stimulation during fluid collection may have varied between the different investigations. Previous studies have reported an effect of mechanical gland stimulation on flow rate,\textsuperscript{9,22} and it should be stressed that the present method of assessing the secretion rate implies a certain degree of gland activity. The difference in secretion may reflect differences in gland density or secretion capacity in the different oral mucosal areas. The variation in minor gland secretion may mirror different protective demands in different regions of the oral cavity.

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References

Mucins MUC5B and MUC7 in minor salivary gland secretion of children and adults

M. Sonesson*, C. Wickström, B. Kinnby, D. Ericson, L. Matsson
Faculty of Odontology, Malmö University, SE-205 06 Malmö, Sweden

1. Introduction

Mucins are vital components of the mucous layers covering the epithelial surfaces of the human body. These multifunctional glycoproteins constitute a protective barrier between underlying tissues and potentially noxious external environments. In adults, the mucins MUC5B (originally named MG1) and MUC7 (MG2) have been detected in saliva from the minor salivary glands and the submandibular and sublingual glands,1,2 and the contribution of mucin from the minor glands is likely to be of particular importance for protection and lubrication of the mucosa.

It has been suggested that the gel-forming mucin MUC5B forms a matrix retainer for other protective proteins such as IgA, lactoferrin, and lysozyme on teeth and mucosal surfaces.3-5 Early studies indicate that MUC7 takes part in the human salivary non-immune defence system and interacts with oral microorganisms,6,7 and it appears that MUC7 plays a role in mediating interactions between neutrophils and bacteria.8 The ability of MUC7 to self-associate, creating larger assemblies through non-covalent bonds, has also been proposed to contribute to the agglutinating and eliminating properties of the mucin.9,10

Studies of whole saliva have shown that the amount of mucins seems to decline as people age.11,12 Further, an increase in MUC5B and a decrease in MUC7 in whole saliva during the first year of infancy has been shown.13 Concerning the minor glands, no information about age-related differences in mucin secretion seems to be available. To test the hypothesis that secretion of salivary mucins changes between childhood and adulthood, the present investigation was designed to study the relative amounts of MUC5B and MUC7 in minor salivary gland saliva of children and adults.
in minor gland saliva of children and adults in defined mucosal areas.

2. Materials and methods

2.1. Individuals

A total of 90 individuals were recruited in three categories: 3-year-old children, 14-year-old children, and young adults between the ages of 20 and 25. Each category consisted of 15 females and 15 males. The number of individuals in each group was determined based on the assumption that a mean difference of 0.10 absorbance/µL is of a clinical importance. Assuming a within group standard deviation of 0.12 absorbance/µL, a two-tailed test at a significance level of 5% with a power of 90% will give a sample size of \( n = 30 \) observation in each group (t-test). All individuals, except two Asian children in the youngest age group, were Caucasians. All were healthy and used no medication. Five females took contraceptives. The study protocol was approved by the local ethics committee for human studies and all participants and the parents of the children signed an informed consent form.

2.2. Collection of minor gland saliva

Saliva was collected in the labial and buccal mucosal areas, on the left side of the oral cavity as previously described. Measurements of the labial area were made near the mid-line of the lower lip, halfway between the vermillion border and the vestibulum. Buccal measurements were made at the level of the parotid excretory duct and approximately midway between the labial angle and the parotid excretory duct. The participants were instructed not to eat or drink for at least 1 h prior to the measurements, which were made between 9 and 12 am, and instructed not to use nicotinic products on the day of appointment. After the left buccal and labial mucosae were carefully dried with a cotton pad, Sialopaper (8 mm, Proflow\textsuperscript{TM}, Inc., Amityville, NY, USA) was applied for 15 s (buccal) or 5 s (labial). A pre-test showed that a shorter time was needed for buccal glands in order not to overfill the papers. To get sufficient volume for the mucin assessment, this procedure was repeated four times on each location. After measurements of the amount of collected saliva, using a Periotron 8000 (Proflow\textsuperscript{TM}), the four Sialopapers were placed in a tube with 150 µL 4 M guanidinium chloride (GuHCl) and stored at –85 °C, representing an individual and site specific sample.

2.3. Extraction and assessment of MUC5B and MUC7

The assessment of MUC5B and MUC7 was carried out in an ELISA using the LUM5B-2 antisem\textsuperscript{2} and the LUM7-1 antisem,\textsuperscript{3} respectively. Both antisera were raised against sequences outside the glycosylated domains to avoid epitope shielding. To maximize recovery of mucins, disulphide bonds were abolished by adding 10 mM DTT in 6 M guanidinium hydrochloride, 0.1 M Tris-HCl buffer (pH 8.0), for 1 h at 37 °C followed by alkylation by the addition of iodoacetamide.\textsuperscript{4} The reduced samples were then centrifuged and the supernatants were collected. Samples were coated on multi-well assay plates (3912, Falcon) overnight at room temperature. A negative control, consisting of 4 M GuHCl, was added to each assay plate. The plates were then blocked for 1 h with PBS containing 0.05% (v/v) Tween 20 and 0.5% (w/v) BSA (blocking solution), followed by incubation for 1 h with the LUM5B-2 antisem (1:1000) or the LUM7-1 antisem (1:500) diluted in blocking solution. Reactivity was detected with an alkaline phosphatase-conjugated swine anti-rabbit antisem, diluted 1:2000 in blocking solution, using nitrophenyl phosphate as a substrate. Reactivity was expressed as absorbance at 405 nm after 1 h. All samples to undergo an ELISA for a specific antigen or mucosal area were assayed on the same test plate. In all, four plates were used (labial MUC5B, labial MUC7, buccal MUC5B and buccal MUC7).

To test the efficiency of elution of MUC5B and MUC7 from the Sialopaper\textsuperscript{TM} strips, 2 µL of whole saliva from one individual was applied to each of five strips. The strips were placed in the Periotron for volume measurement and treated and analysed according to the protocol above. In addition, five samples of 2 µL were pipetted from the same pool of whole saliva, placed into tubes, and analysed accordingly. The efficiency of the elution from the strips, determined as the mean ratio of eluted and pipetted mucin, was 73% for MUC5B and 72% for MUC7. The MUCB used as a standard in this study, was purified using density-gradient centrifugation of whole saliva according to Wickström et al.\textsuperscript{5} and the concentration was determined by lyophilisation and weighing. The MUC7 standard was also pooled out from a density gradient, although these fractions contained a mixture of proteins. Standard curves for MUC5B and MUC7 were made by serial dilutions, and showed a linear relationship between the serially diluted standard and the absorbance (Figs. 1 and 2). As no pure MUC7 was available, it was not possible to calculate the absolute concentration of this mucin. For this reason, the amounts of both MUC5B and MUC7 are expressed as absorbance/µL, which allows group comparisons according to the present aim of the study. The value of absorbance/µL was calculated taken into account the dilution factor of the original Periotron assessed samples.

2.4. Statistical analyses

For each test plate, values below the negative control were set to \( 0 (n = 2) \), and values above the standard curve were set to the

![Figure 1](image-url)
highest absorbance value of the curve \( (n = 3) \). To test the influence of the factors gender and age on the amount of MUC5B and MUC7, a two-way analysis of variance, with comparisons by Tukey’s method, was used. Differences at the 0.05 level of significance were considered statistically significant.

3. Results

3.1. Labial mucosa

No significant age-related differences in the amount of MUC5B, expressed as absorbance/\( \mu L \), were noted (Table 1). The lowest amount was found in the group of 3-year-old males, whose values were significantly lower than those of 3-year-old females \( (p < 0.02) \). The median absorbance values for labial gland MUC5B in the various groups represent absolute values of 0.19–0.50 \( \mu g/\mu L \). With a 73% level of elution of MUC5B from the Sialopaper strips, these values correspond to a concentration range of 0.26–0.68 \( \mu g/\mu L \).

Concerning MUC7 an increase with age was noted for the total group of individuals (Table 2). The difference between the 3-year-olds and the adults was statistically significant \( (p < 0.04) \).

3.2. Buccal mucosa

In most individuals no detectable amounts of MUC5B were found. Measurable amounts were observed in nine 3-year-olds, two 14-year-olds, and none of the adults. In those individuals where MUC5B was detected, the amount (absorbance/\( \mu L \)) varied between 0.02 and 0.39.

Neither were any detectable amounts of MUC7 found in most individuals. Measurable amounts were found in nine 3-year-olds, three 14-year-olds, and two of the adults. The amount (absorbance/\( \mu L \)) of MUC7 in these individuals varied between 0.02 and 0.49.

4. Discussion

Salivary mucins are major constituents of the biofilm that covers and protects the oral mucosa and the tooth surfaces, and the protective roles of salivary MUC5B and MUC7 have been stressed in numerous studies. The few previous studies on the presence of mucins in the saliva of minor salivary glands have focused on adults, and so far no studies have been published on the presence of mucins in the minor gland secretions of children.

No significant differences in labial gland MUC5B were seen between the three age groups. The mean amount of MUC7, however, increased significantly with age. The mean amount of MUC7 was twice as high in adults compared with 3-year-olds and for the subgroup of males the mean difference was even more prominent. Thus, the total amount of MUC7 available in this area of the mouth seems to be far lower in the young child than in the adult. As MUC7 is reported to be part of the salivary non-immune defence system and potent against a broad range of micro-organisms, including fungi,10,15 the comparably low amount of MUC7 in the labial area of the young child may be of importance for the defence against oral diseases in this part of the mouth.

Recently, an increase in the quantity of MUC5B and a decrease in the quantity of MUC7 in the whole saliva of infants during the first year of life was reported.18 These findings, and the results from the present study, suggest that the mucin composition of the saliva varies in the developing child. These observations may be important for understanding the biological role of the mucins in the oral cavity during the growth of the child, with significant changes in the oral environment, shifts in nutrition from fluids to solids, and variations in exposure to oral diseases. The possible clinical significance of the mucins may be illustrated by previously described results, where individuals with a high caries experience exhibited a significantly lower level or even absence of high molecular weight mucin and low molecular weight mucin compared with individuals with a low caries experience.17

Previous studies have reported MUC7 to be present in labial and palatal minor gland saliva,1 and MUC5B in palatal minor

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Table 1 – MUC5B in different age groups in the labial mucosal area

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Total (( n = 30) )</th>
<th>Female (( n = 15) )</th>
<th>Male (( n = 15) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Year-olds</td>
<td>0.21 (0.20)</td>
<td>0.28 (0.22)</td>
<td>0.14 (0.17)*</td>
</tr>
<tr>
<td>14-Year-olds</td>
<td>0.19 (0.16)</td>
<td>0.15 (0.15)</td>
<td>0.23 (0.16)</td>
</tr>
<tr>
<td>Adults</td>
<td>0.17 (0.11)</td>
<td>0.17 (0.13)</td>
<td>0.18 (0.11)</td>
</tr>
</tbody>
</table>

Absorbance/\( \mu L \), mean (\( \pm S.D. \)).

* Secretion of MUC5B was lower in 3-year-old males than in 3-year-old females \( (p < 0.02) \).

Table 2 – MUC7 in different age groups in the labial mucosal area

<table>
<thead>
<tr>
<th>Age Group</th>
<th>Total (( n = 30) )</th>
<th>Female (( n = 15) )</th>
<th>Male (( n = 15) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Year-olds</td>
<td>0.09 (0.09)*</td>
<td>0.08 (0.10)</td>
<td>0.03 (0.09)</td>
</tr>
<tr>
<td>14-Year-olds</td>
<td>0.11 (0.13)</td>
<td>0.06 (0.05)</td>
<td>0.16 (0.16)</td>
</tr>
<tr>
<td>Adults</td>
<td>0.21 (0.20)</td>
<td>0.17 (0.21)</td>
<td>0.26 (0.36)</td>
</tr>
</tbody>
</table>

Absorbance/\( \mu L \), mean (\( \pm S.D. \)).

* Secretion of MUC7 was lower in 3-year-olds than in adults \( (p < 0.04) \).
gland saliva. We found MUC7 and MUC5B in saliva from labial and buccal glands, although from buccal glands only in a few individuals. However, MUC7 has also been reported to be undetectable in saliva from minor glands but present in submandibular and sublingual gland saliva. A possible explanation for the difference between the present findings and the negative findings concerning minor gland MUC7 could be, that the antibody used in their study was raised to a carbohydrate epitope, whereas the antibody used in the present study was a polyclonal antibody, raised against a specific amino acid motif in MUC7. Carbohydrate-specific antibodies may not always identify the mucin due to local variations in glycosylation.

MUC5B and MUC7 were found to be present in the labial salivary samples of nearly all individuals in all three age groups. In the buccal area, however, a majority of the samples had undetectable levels of these mucins. Here, MUC5B and MUC7 were found in less than one-third of the young children and in only two to three individuals in the older age groups, and MUC5B did not reach detectable levels in any of the adults. As mentioned above, the present use of peptide specific antisera against the mucins should compensate for possible local differences in glycosylation, which otherwise might mask the mucin content of the minor gland saliva. Structural differences, including minor gland anatomy, and number of sero-mucous cells between the buccal and labial mucous membranes have been reported. Local differences in these respects might explain variations in mucin production, even if methodological reasons for the differences cannot be fully excluded.

The absolute values of labial gland MUC5B are in agreement of previous findings. In future studies, the use of purified MUC7 will allow an estimation of the actual concentration of MUC7 in minor gland saliva.

Our findings indicate a different pattern of mucin secretion from minor glands in different age groups and oral localizations. This in turn may be reflected in biological differences in the biofilm protecting the oral mucosa and the tooth surfaces. Differences in the protein content of the biofilm in various parts of the mouth have been reported previously and may, for example, explain the intra-oral variation in bacterial colonization.

In summary, the present study has shown that the mucins MUC5B and MUC7 are excreted by the labial minor glands in children as well as in teenagers and adults. The amount of MUC7, however, seems to be notably lower in young children compared to the other groups, possibly indicating a difference in the protective biofilm in the labial area. In addition the results suggest a site-dependent variation in minor gland mucin secretion, the majority of the individuals showing no detectable amounts of mucins in the buccal area.

Acknowledgements

This project was supported by the Swedish Patent Revenue Fund for Research in preventive odontology, the Swedish Dental Society, the Faculty of Odontology, the Region Skåne and the Knowledge Foundation (KK-stiftelsen), Sweden.

References


Erratum


In Tables 1 and 2 (p. 16), and in the abstract (p. 15) of the above article, the salivary IgA concentration was incorrectly expressed as mg/100 ml. The correct unit is mg/ml.

The corrections do not change the conclusions of the paper. The authors apologize for the error.
Salivary IgA in minor-gland saliva of children, adolescents, and young adults

Mikael Sonesson1, Kristina Hamberg2, Marie-Louise Lundin Wallengren2, Lars Matsson3, Dan Ericson1

1Department of Orthodontics, Faculty of Odontology, Malmö University, Malmö; 2Department of Cariology, Faculty of Odontology, Malmö University, Malmö and 3Department of Paediatric Dentistry, Faculty of Odontology, Malmö University, Malmö, Sweden

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Immunoglobulin A (IgA) is present in human saliva, tears, intestinal secretions, urine, and other mucosal fluids and helps to defend the mucosa and other tissues against foreign organisms and substances (1–3). Salivary IgA is predominantly in the form of secretory IgA that binds to different molecular components and interferes with the adhesion of microorganisms (4–7). Secretory IgA is a dimer formed by two monomers connected by a polypeptide (j chain) that is surrounded by a proteolysis-resistant secretory component (8). Strategically positioned throughout the oral mucosa, minor salivary glands secrete about 30–35% of all salivary IgA that occurs in adult whole saliva (9–11).

The concentrations of IgA in serum, and of salivary IgA in whole saliva, have been shown to increase during childhood; a large increase occurs during the first year of life, which indicates that the humoral and salivary immune systems are immature at birth (12, 13). BORGIO et al. (14) reported adult levels of salivary IgA in whole saliva of 4–6-yr-old children, whereas BRANDTZÆG (15) reported such levels in 10–12-yr-old children. The concentrations of IgA in the elderly (60–80 yr of age), however, appear to be lower than in young adults (20–30 yr of age) (3, 16, 17).

Conflicting data concerning age-dependent differences in salivary IgA in adult minor gland saliva has been reported. ELIASSEN et al. (18) found higher concentrations of salivary IgA in the buccal and labial glands of older persons (> 65 yr of age) compared with younger persons (≤ 65 yr of age), and SMRTH et al. (17) demonstrated lower concentrations of salivary IgA in the labial glands of older individuals (> 55 yr of age) compared with younger individuals (17–24 yr of age). To our knowledge, no information has been reported on the concentration of salivary IgA in minor-gland saliva of children.

Therefore, the aim of the present investigation was to compare salivary IgA concentrations in the minor-gland saliva of young children (3 yr of age) with those of adolescents (14 yr of age) and young adults (20–25 yr of age). The salivary IgA concentration in unstimulated whole saliva collected from each of the three age-groups was used as a reference.

Material and methods

The initial group comprised 90 individuals in three age groups: 3 yr (n = 30), 14 yr (n = 30), and 20–25 yr (n = 30). The 3- and 14-yr-old participants were selected from the patient lists at the Faculty of Odontology, Malmö University (Malmö, Sweden). Dental students formed the adult group. Eighty-seven individuals were Caucasian, and three were of Asian origin (one in each group). Group size was determined based on the assumption that a mean difference in salivary IgA concentration of 0.035 mg 100 ml−1 (approximately 50% of adult values) might be of clinical
interest. Assuming a within-group SD of 0.04 mg 100 ml⁻¹, a sample size of approximately 28 in each group was required for a two-tailed t-test to give a significance level of 5% and a power of 90%. Inclusion criteria were good health and no use of medication at the time of saliva collection.

During the biochemical analysis of labial saliva, samples from two of the 3-yr-old children, from three of the 14-yr-old adolescents, and from four of the young adults were discarded because of inadequate sample volumes. For the same reason, buccal saliva samples from three of the 3-yr-old children, from four of the 14-yr-old children, and from five of the adults were discarded. Inadequate volumes of whole-saliva from five of the 3-yr-old children were obtained because of poor cooperation and were discarded. For final sample numbers, see Tables 1 and 2.

The local ethics committee for human studies at Lund University approved the study protocol. Before the start of the study, all participants of legal age, and the parents of the minors, signed an informed-consent form.

Collection of minor-gland saliva

Unstimulated saliva was collected from the lower labial and the buccal mucosal areas, as described previously (19, 20). Briefly, the participants were instructed not to eat, drink or brush their teeth for at least 1 h before sampling, according to the guidelines of Hoek et al. (21). To reduce the influence of circadian variations in salivary IgA concentration, all samples were collected between 09.00 and 12.00 h (3). The two older age-groups were instructed not to use nicotine-containing products on the day of the examination.

After careful drying of the left labial mucosa and the buccal mucosa with gauze, filter paper (SialoPaper Ø 8 mm; Oraflow, Smithtown, NY, USA) was placed on the mucosa and left for 120 s (labial) or 60 s (buccal). A Periotron 8000 (Oralfax) assessed the volume of the saliva on the paper. The papers were then placed in Eppendorf tubes (Oldenburg, Germany) containing 150 μl of PBS, pH 7.2, and stored at −80°C. One investigator collected all samples.

Pretreatment of SialoPapers

To reduce absorption of salivary IgA, the filter papers were soaked in 0.05% polysorbate 20 (Tween 20) and air-dried on absorbing paper (Munktell, Stora Kopparberg, Sweden) before sample collection. A pilot study was performed to determine the optimal concentration of Tween 20 (data not shown). A concentration of 0.05% showed the highest recovery of salivary IgA (35%; SD = 6.1%) and was therefore used subsequently. The original salivary IgA

### Table 1

<table>
<thead>
<tr>
<th>Gland type</th>
<th>3-yr-old children</th>
<th>14-yr-old adolescents</th>
<th>20- to 25-yr-old adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labial</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire group</td>
<td>0.037 (0.035)*</td>
<td>0.126 (0.128)</td>
<td>0.128 (0.134)</td>
</tr>
<tr>
<td>Female participants</td>
<td>0.027 (0.026)</td>
<td>0.106 (0.093)</td>
<td>0.097 (0.112)</td>
</tr>
<tr>
<td>Male participants</td>
<td>0.045 (0.039)</td>
<td>0.155 (0.168)</td>
<td>0.160 (0.151)</td>
</tr>
<tr>
<td>Buccal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire group</td>
<td>0.112 (0.140)</td>
<td>0.1232 (0.139)</td>
<td>0.1755 (0.224)</td>
</tr>
<tr>
<td>Female participants</td>
<td>0.121 (0.185)</td>
<td>0.1373 (0.160)</td>
<td>0.0829 (0.090)</td>
</tr>
<tr>
<td>Male participants</td>
<td>0.105 (0.098)</td>
<td>0.1041 (0.109)</td>
<td>0.2610 (0.276)</td>
</tr>
</tbody>
</table>

*The salivary IgA concentration in the labial glands was lower in 3-yr-old children compared with the 14-yr-old adolescents and the 20–25-yr-old adults (P < 0.01).

### Table 2

<table>
<thead>
<tr>
<th>Gland type</th>
<th>3-yr-old children</th>
<th>14-yr-old adolescents</th>
<th>20- to 25-yr-old adults</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole saliva (mg 100 ml⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire group</td>
<td>0.090 (0.091)*</td>
<td>0.179 (0.149)</td>
<td>0.170 (0.099)</td>
</tr>
<tr>
<td>Female participants</td>
<td>0.079 (0.037)</td>
<td>0.190 (0.108)</td>
<td>0.138 (0.070)</td>
</tr>
<tr>
<td>Male participants</td>
<td>0.095 (0.108)</td>
<td>0.169 (0.180)</td>
<td>0.192 (0.110)</td>
</tr>
<tr>
<td>Salivary IgA total protein (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Entire group</td>
<td>19.10 (12.79)†</td>
<td>32.95 (19.67)</td>
<td>25.49 (10.59)</td>
</tr>
<tr>
<td>Female participants</td>
<td>16.50 (10.41)</td>
<td>34.71 (19.36)</td>
<td>23.00 (9.25)</td>
</tr>
<tr>
<td>Male participants</td>
<td>20.44 (19.67)</td>
<td>31.48 (20.35)</td>
<td>26.80 (11.24)</td>
</tr>
</tbody>
</table>

*The salivary IgA concentration was lower in 3-yr-old children compared with the 14-yr-old adolescents and the 20- to 25-yr-old adults (P < 0.01).
concentrations were then estimated by multiplying by a factor of 2.86. The pilot study also included a recovery analysis of minor gland saliva containing either a low or a high concentration of salivary IgA. The mean recovery in the saliva differed by only 1–2% between saliva with a low concentration of IgA and that with a high concentration of IgA. Thus, the same factor (2.86) was considered to be relevant for all samples when calculating the original concentration of salivary IgA.

Collection of unstimulated whole saliva

At the same appointment, after the collection of minor gland saliva, the unstimulated whole saliva was collected. Participants sat, leaning slightly forward, and let saliva drain for 1–5 min into a polypropylene tube (Sarstedt, Nümbrecht, Germany). All samples were stored at −80°C.

ELISA and total protein assay

An in-house, modified, sandwich ELISA was used to assess the concentrations of salivary IgA, according to Krzywkowski et al. (22). The procedure was designed for small sample volumes.

The wells of Immuno 96 MicroWell plates (MaxiSorp surface, Nunc-Immuno Plate; Thermo Fisher Scientific, Roskilde, Denmark) were coated overnight using 100 μl of z1- and z2-chain-specific goat anti-human IgA (5 μg ml⁻¹; Sigma A0642, Sigma Chemical Co., St Louis, MO, USA) in coating buffer (0.05 M carbonate buffer, pH 9.6). The next morning, the wells were washed three times with 200 μl PBS containing 0.05% Tween-20 (PBST) (Bio-Rad, Hercules, CA, USA). Nonspecific binding was blocked using 100 μl of PBST containing 1% BSA (Sigma) for 1 h at 37°C.

After washing, as described above, 100 μl of standards (31.25–250 ng ml⁻¹ of colostrum standard, Sigma) and saliva samples (diluted 1:3,000–1:8,000 in PBST) were added and incubated for 1 h at 37°C. After washing, as described above, 200 μl of 1 mg 100 ml⁻¹ phosphatase substrate (Sigma P8869) was added in diethanolamine buffer and incubated for 30 min at 37°C. The colour reaction was stopped by the addition of 50 μl of 3 M NaOH.

The absorbance of saliva samples was measured, at 405 nm, using an absorbance microplate reader (ELx800; BioTek Instruments, Winooski, VT, USA), and the results were plotted against a colostrum standard curve and multiplied with the dilution factor. The results are presented as mg 100 ml⁻¹ IgA.

Total protein in unstimulated whole saliva was assayed using the DC (detergent-compatible) Protein Assay; Bio-Rad). The samples were diluted between 1:5–1:25. Total protein reactivity was expressed as absorbance at 595 nm after 15 min, and the results are presented as mg 100 ml⁻¹.

Statistical analysis

A one-way ANOVA, with comparisons performed using Tukey’s method, tested the effects of age on the concentrations of salivary IgA and total protein. Paired t-tests were used to analyze the intra-individual differences in salivary IgA concentrations between the different sites. In addition, because of the smaller number of individuals in the subgroups, the Mann–Whitney U-test was performed to analyze the effects of gender within each age-group on the salivary IgA concentrations. Intra-individual correlations were tested using Pearson’s correlation test. P-values of < 0.05 were considered statistically significant.

Results

Labial glands

The mean salivary IgA concentration in labial-gland saliva was lowest among the 3-yr-old children (Table 1); the difference in salivary IgA concentrations between the group of 3-yr-old children and the adolescents and between the 3-yr-old children and the adults was statistically significant (P < 0.01). Among the adults, women had significantly lower concentrations of salivary IgA (P < 0.05) (Table 1).

Buccal glands

No statistically significant differences in the mean concentration of salivary IgA in the buccal glands were found between the age-groups (Table 1). The buccal gland concentration of salivary IgA was significantly lower in women than in men in the group of adults (P < 0.05).

Comparisons of salivary IgA concentrations between buccal and labial glands revealed no statistically significant differences except in the group of 3-yr-old children, where a significantly lower concentration was seen in the labial glands (P < 0.05).

Unstimulated whole saliva

The lowest mean salivary IgA concentrations in unstimulated whole saliva were found among the group of 3-yr-old children (Table 2), and the difference in salivary IgA concentration between the group of 3-yr-old children and the adolescent and adult groups was statistically significant (P < 0.05). In addition, the salivary IgA concentration expressed as a ratio of total protein was significantly lower in the group of 3-yr-old children compared with the 14-yr-old adolescents (P < 0.01) (Table 2). No statistically significant differences between the genders were noted.

No statistically significant intra-individual correlations in salivary IgA were found between the different types of saliva in any of the age groups.

Discussion

To our knowledge, this is the first report to compare the concentration of salivary IgA in minor-gland saliva of children with the concentration of salivary IgA in minor-gland saliva of adolescents and adults. The major finding of the present study was a lower concentration of salivary IgA in labial saliva among 3-yr-old children compared with adolescents and young adults. No
age-dependent differences between the adolescents and adults were found, which is in line with data reported on tears (23). However, a site-dependent difference was observed in the 3-yr-old children: salivary IgA concentrations were lower in the labial glands than in the buccal glands. The 3-yr-old children had about 30% of the adult level of salivary IgA in both labial-gland saliva and whole saliva. The lower concentration of salivary IgA among the 3-yr-old children might indicate that their immune system is still not fully developed.

In the youngest age group, sometimes hesitant cooperation and low quantities of minor gland saliva made user-friendly techniques necessary. Because the high viscosity of saliva might make aspiration in a capillary tube (10, 24) or a polypropylene syringe (25) difficult, the filter paper method was chosen. Filter papers are easy to handle and well documented for small-volume collection (19, 20, 26), although drawbacks include the risk of retention of salivary IgA, mucins, and other proteins. We recovered 35% of salivary IgA and total proteins, which is lower than reported by Eliasson et al. (27), who used a different filter paper. This level of recovery, however, does not affect the comparisons made here. Also, the salivary IgA concentrations obtained in the present study are in line with those reported by Eliasson et al. (27).

Instead of stimulated whole saliva, we chose to collect unstimulated whole saliva because it provides information about the level of salivary IgA that continuously bathes the oral surfaces and because, as a result of uncertain compliance in the youngest age group, unstimulated saliva is easier to collect. Whole-saliva concentrations of salivary IgA in the three age groups presented in this study were within previously reported ranges (28, 29).

The present intra-individual test of correlation of salivary IgA collected from different sites did not reveal any significant correlations. Smith et al. (11) made a similar observation of a poor correlation between parotid-gland (major gland) concentrations and palatine and upper labial-gland (minor gland) concentrations of salivary IgA. The authors suggested that the lymphocytes in the different glands had different origins, in that the lymphocytes of the parotid gland probably derive largely from gut-associated lymphoid tissue and the lymphocytes of the palatine glands derive largely from bronchus-associated lymphoid tissue. The poor intra-individual correlation observed in the present study might reflect similar differences in lymphocyte homing, derivation, and population between the minor glands. Moreover, varying exposure to local antigenic stimuli has been proposed as an explanation of the site-dependent variations in salivary IgA concentrations (30).

The lower concentration of salivary IgA in the labial gland saliva compared with the unstimulated whole saliva could be interpreted as in conflict with some earlier data, which showed higher concentrations of salivary IgA in the labial glands compared with stimulated parotid saliva (10). This could be explained by the fact that the salivary IgA concentration in parotid saliva (and also in submandibular/sublingual saliva) decreases dramatically with increased flow rate (31). Relevant to this study is that the contribution of parotid saliva to unstimulated whole saliva is only about 30% (32). In addition, because, in the present study, saliva from all glands was unstimulated, it contained higher concentrations of IgA (compared with stimulated saliva), and it is possible that IgA from the gingival exudate was also present. Therefore, it is not surprising to find a higher concentration of salivary IgA in unstimulated whole saliva, which has also been reported by Eliasson et al. (27).

The lower concentration of salivary IgA among 3-yr-old children might well reflect a developing immune response in the minor labial glands, similar to the development of humoral immunity (33). The relevance of the total salivary IgA concentration in whole saliva and disease prevalence has been discussed, and studies have reported conflicting data. Kirtaniya et al. (34) reported lower total salivary IgA concentrations among children with high caries activity compared to those with low caries activity. However, Thawonoos et al. (35) found no differences in whole-saliva concentrations of total salivary IgA between caries-free children and children with rampant caries.

In Candida albicans carriers, Epstein et al. (4) suggested that high concentrations of salivary IgA inhibit adherence of the microorganisms. On the other hand, Hiroso et al. (36) found no correlation between the concentration of total salivary IgA and the presence of Candida albicans in young adults. Thus, the importance of total levels of salivary IgA in disease prevention is still controversial. In contrast, specific antibodies for mutants streptococci have been shown to be caries protective in children (37–39).

Statistically significant differences in salivary IgA concentrations between men and women were noted in the labial saliva and in the buccal-gland saliva of 20- to 25-yr-old adults, where the concentrations were significantly lower in women. A previous study on whole saliva in adult sportsmen and sportswomen found lower salivary IgA concentrations in the women (40). The same relationship between the genders has also been reported for IgA in serum, but not in tears (23, 41). All gender comparisons in the present study should be interpreted with care owing to a low number of participants in the subgroups. However, the gender differences in minor gland saliva among the adults might reflect a hormonal influence.

In conclusion, this study found age-dependent differences in the concentrations of salivary IgA in labial-gland saliva and in unstimulated whole saliva, and has provided new information on the changes in minor salivary gland IgA concentrations from childhood to adulthood. The lower values among the 3-yr-old children, compared with the adolescents and the adults, possibly reflect an ongoing maturation of the specific immune response. A site-dependent variation was also noted in the 3-yr-old children, who demonstrated lower values of salivary IgA in labial-gland saliva. Our research group previously reported lower quantities of MUC7 in the labial-gland saliva of 3-yr-old children compared with adults (42), and Bandera-Tarabay et al. (43)
reported lower whole saliva concentrations of mucin in individuals with high caries experience. The clinical relevance of lower salivary IgA and mucin concentrations in the young child remain to be elucidated.

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GLYCOPEPTIDE 340 AND SIALIC ACID IN MINOR-GLAND AND WHOLE SALIVA OF CHILDREN, ADOLESCENTS AND ADULTS

Mikael Sonesson¹, Dan Ericson², Bertil Kinnby³, Claes Wickström³

¹Department of Orthodontics, Faculty of Odontology, Malmö University, Malmö, Sweden
²Department of Cariology, Faculty of Odontology, Malmö University, Malmö, Sweden
³Department of Oral Biology, Faculty of Odontology, Malmö University, Malmö, Sweden

Running title: Gp-340 and sialic acid in human saliva

Correspondence to:
Mikael Sonesson, Department of Orthodontics, Faculty of Odontology, Malmö University, SE-205 06 Malmö, Sweden
Phone: +46-40-6658447
Telefax: +46-40-6658584
E-mail: Mikael.Sonesson@mah.se
Abstract
Glycoprotein 340 (gp-340) is a bacterial-binding glycoprotein observed in major and minor gland saliva. Sialic acid, a common terminal structure of salivary glycoproteins, interacts with microorganisms and host ligands, as well as free radicals. This study investigated the content of gp-340 and sialic acid in minor gland and whole saliva of children (3-yr-olds), adolescents (14-yr-olds) and adults (20 to 25-yr-olds). Labial and buccal gland saliva was collected on filter paper and un-stimulated whole saliva by draining into a tube. The relative amount of gp-340 and sialic acid was determined by ELISA and ELLA, respectively.

In minor gland saliva, no statistically significant differences in gp-340 and sialic acid were seen between the age-groups. Significantly lower amounts of gp-340 and sialic acid were seen in labial saliva compared to buccal among adults \((P < 0.05, P < 0.01)\). In whole saliva, the amount of gp-340 was significantly lower among adults compared to children \((P < 0.01)\). No differences between genders were seen.

Stable gp-340 and sialic acid contents in minor-gland saliva across the age-groups and higher gp-340 content in the whole saliva of the youngest age-group (3-yr-olds) compared with the adult-group may reflect a vital innate factor of immunity in children’s saliva.

Key words: age, gp-340, minor gland, saliva, sialic acid
GLYCOPEPTIDE 340 AND SIALIC ACID IN MINOR-GLAND AND WHOLE SALIVA OF CHILDREN, ADOLESCENTS AND ADULTS

Sonesson M, Ericson D, Kinnby B, Wickström C

Introduction
Saliva constantly bathes the oral cavity and has several protective functions such as lubrication, moistening, and adaptive and innate immune antimicrobial functions. Secretory IgA (sIgA) constitutes the major adaptive immune protection mechanism in saliva (1). Innate immune protection is often mediated by various mucins or other glycoprotein species that, mostly via their carbohydrate moieties, bind and agglutinate bacteria (2, 3). Salivary glycoproteins also possess gel-forming properties that retain fluid and lubricate the mucosa. Glycoprotein contains a large number of oligosaccharides covalently bound to the protein core, often exposing sialic acid as a terminal monosaccharide. Notably, those sialylated structures act as free radical scavengers and are also responsible for interaction with microorganisms and host ligands (4, 5). It is possible that the concentration of sialic acid also reflects the total amount of glycoprotein in saliva as it is abundant in the major salivary glycoproteins.

Glycoprotein 340 (gp-340), also known as salivary agglutinin, is one of the bacterial-binding glycoproteins (6). It is found on several mucosal surfaces in the body, and the same gene, the dmblt1 gene, encodes these scavenger receptor, cysteine-rich (SRCR) glycoproteins (7, 8). Gp-340 binds to oral streptococci (6, 9) and CARLÉN et al. (10) suggested that gp-340 has caries protecting activity due to its ability to inhibit colonization of Streptococcus mutans. Other researchers have suggested that gp-340 interacts with other salivary components, i.e. sIgA (11), creating complexes that agglutinate different types of bacteria (12). Moreover, the salivary glycoproteins MUC5B and MUC7 have been attributed analogous microbial interactions, partly due to the occurrence of blood group substances on the terminal part of their carbohydrate chains (13-15).

In whole saliva, IgA-concentration increases from childhood to adulthood (1, 16, 17). RUHL et al. (18) observed that MUC5B increases and MUC7 decreases during the first year of life. Other
researchers have observed decreases in total mucin concentration among aging people (>65 yr) (19) and also a decrease in sialic acid concentrations during early life and ageing (20, 21).

In minor-gland secretions, MUC5B content in labial-gland saliva is reported to be relatively stable during growth (3–25 yrs of age) (22). IgA concentration in labial-gland secretions, however, is significantly lower in children than in adults-similar to IgA content in whole saliva (17). Given the immune nature-adaptive or innate-of the various salivary molecules, one could hypothesize that innate immune molecules such as MUC5B, which has further functions like lubrication, are more or less constantly secreted with relatively unchanging, or even decreasing, content in the saliva while salivary content of adaptive immune molecules increases during general maturation of the immune system. In addition, site-dependent differences in minor-gland secretion of adaptive and innate immune components seem to occur (17, 22).

CARLÈN et al. (23) and BIKKER et al. (24) studied gp-340 in major- and minor-gland saliva of adults, but the presence of gp-340 in minor-gland saliva of children and adolescents has not been investigated. Knowledge concerning age-related variations in gp-340 as well as sialic acid in minor gland saliva, is lacking. Thus, the aim of this study was to analyze the content of gp-340 and sialic acid in labial and buccal minor-gland saliva of 3-yr-olds, 14-yr-olds, and 20–25-yr-olds. Gp-340, sialic acid, and total protein content in whole saliva was also determined.

Materials and methods
Ninety individuals were recruited in these age-groups: 3 yrs (n = 30), 14 yrs (n = 30), and 20–25 yrs (n = 30). Sample size was based on the assumption that a mean between-group difference of 100% of absorbance ul⁻¹ was clinically important. So, assuming a within-group SD of 100% absorbance ul⁻¹, sample size of about 25 per group would be needed to attain a statistical power of 90%. The 3- and 14-yr-old study participants were randomly selected from patient lists. The adult group comprised dental students at the Faculty of Odontology, Malmö University, Malmö, Sweden. All subjects were healthy and used no medication at the time of the examination, except three females in the adult group who took contraceptives. No
adolescent or adult was wearing an orthodontic appliance at the time of saliva collection.

Before study start, all participants of legal age and the parents of children and adolescents signed an informed-consent form. The local ethics committee for human studies at Lund University approved the protocol (ref. no. LU 766-02).

**Collection of minor-gland saliva**

Briefly, the participants were instructed not to eat, drink, or brush their teeth for at least 1 h before sampling (25). To reduce the influence of circadian variations in salivary components, all samples were collected between 9 am and noon (26).

The saliva was collected from lower labial and buccal mucosal areas on the left side of the mouth with pre-treated filter paper (SialoPaper® 8 mm; Oraflow Inc., Smithtown, NY, USA) as previously described (17). The paper was placed on dried mucosa and left for 120 s (labial) or 60 s (buccal). A Periotron 8000 (Oraflow) assessed the volume of saliva on the paper. The papers were then placed in Eppendorf® tubes with 150 ml phosphate buffer saline (PBS), pH 7.2. All samples were stored at −80°C or colder, until analysis of gp-340 and sialic acid. Due to the small volumes of minor-gland saliva, the number of analyzed samples was reduced (Table 1).

**Collection of whole saliva**

As previously described (17), resting whole saliva was collected after minor-gland saliva sampling, on the same visit. Participants leaned slightly forward and let saliva drain into a polypropylene tube (Sarstedt AG & Co., Nümbrecht, Germany) for 1–5 min. All samples were weighed and stored at −80°C until analysis of gp-340, sialic acid, and total protein.

**Biochemical analysis**

To extract glycoproteins from the filter papers, 6M guanidinium hydrochloride, 0.1 M Tris-HCL buffer (pH 8.0) containing 10 mM dithiothreitol (DTT), and CHAPS were added for 5 h at 37°C, followed by the addition of iodoacetamide (27). The samples were then centrifuged (18,000 x g, 10 min) and analyzed with ELLA (28) and ELISA (22). All samples to undergo analysis for a specific compo-
nent or saliva type were assayed on the same test plate (labial gp-340, labial sialic acid, buccal gp-340, buccal sialic acid, whole saliva gp-340, whole saliva sialic acid) as single sample, and whole saliva total protein, in duplicate.

**Enzyme-linked lectin assay (ELLA)**

Salivary samples diluted between 1:500 and 1:10,000 in PBS (pH 7.2) were coated onto 96-well assay plates (3912, Falcon, Franklin Lakes, NJ, USA) overnight at room temperature. A negative control was added to each plate. Plates were blocked for 1 h in a blocking solution of PBS containing 0.05% (v/v) Tween-20 (PBST) (Bio-Rad, Hercules, CA, USA) and 1% (w/v) BSA (Sigma Chemical Co., St Louis, MO, USA). They were then incubated for 1 h with a mixture of two different horseradish peroxidase (HRP)-conjugated lectins, (EY Laboratories Inc., San Mateo, CA, USA) (2 mg/ml) diluted in blocking solution with 0.1 mM CaCl₂. The HRP-conjugated lectins used were the *Sambucus nigra* (SNA) lectin, which recognizes alpha-2,6-linked sialic acid (NANA-2-6), and the *Maakia amurensis* (MAA) lectin, which recognizes alpha-2,3-linked sialic acid (NANA-2-3). SNA and MAA were mixed in equal concentration (50/50) to ensure detection of different linked sialic acids. The SigmaFast OPD (O-phenylenediamine dihydrochloride) tablet set (Sigma-Aldrich, St. Louis, MO, USA) was used as a substrate. Carbohydrate reactivity was expressed as absorbance at 450 nm after 30 min.

**Enzyme-linked immunosorbent assay (ELISA)**

ELISA was performed as previously described (22). Samples and a negative control, diluted as in the ELLA analysis, were coated onto multiwell assay plates (3912, Falcon), overnight at room temperature. Plates were blocked for 1 h in a blocking solution of PBS containing 0.05% (v/v) PBST and 1% (w/v) BSA and incubated for 1 h with the polyclonal anti-gp-340 (gift of Professor David Thornton, Manchester, UK) diluted 1:1000 (29). Detection was carried out using an HRP-conjugated swine anti-rabbit antiserum (Dako Denmark A/S, Glostrup, Denmark) diluted 1:2000 in blocking solution with SigmaFast OPD tablet set (Sigma-Aldrich) as a substrate. Reactivity was expressed as absorbance at 450 nm after 30 min.
Gp-340 was pooled out from a density gradient, containing a mixture of proteins, serially diluted and used to construct a standard curve (22). By serial dilution of purified MUC5B, analyzed for carbohydrate content (30), a sialic acid standard curve was made. The curves were used to confirm that the absorbance of the samples were within the linear range of the curve. The contents of gp-340 and sialic acid in the samples were determined by multiplying the absorbance with the dilution factors and presented as absorbance per µl. In addition, contents of gp-340 and sialic acid in whole saliva are presented as ratios to total protein.

**Total protein assay**

Total protein in whole saliva was assayed (Protein Assay, Bio-Rad Laboratories, Inc.). The samples were diluted between 1:5 and 1:25 and total protein reactivity is expressed as absorbance at 595 nm after 15 min. Serially diluted bovine serum albumin was used as to construct a standard curve. The results are presented as µg total protein µl⁻¹.

**Statistical analysis**

To test the effects of age on gp-340, sialic acid, and total protein concentration, a one-way analysis of variance, with comparisons by Tukey’s method, was done. Intra-individual differences in saliva concentrations between the different sites were analyzed by means of a paired t-test. Intra-individual correlations were tested by Pearson’s correlation test. P-values below 0.05 were considered statistically significant. Mann-Whitney U test was made to test the effects of gender on the amounts of gp-340 and sialic acid, in the sub-groups.

**Results**

**Minor-gland saliva**

Gp-340 and sialic acid content in buccal and labial-gland saliva differed non-significantly between age-groups, between subgroups of females and males in each age-group (Table 1).

In the adult group, amounts of gp-340 and sialic acid were significantly higher in the buccal than in the labial glands (P < 0.05 and P < 0.01, respectively; Table 1). This variation did not occur in the
other groups. No intra-individual correlations in amount of gp-340 or sialic acid were seen among the different types of minor-gland saliva. In addition, no differences in flow rates between the age-groups were seen, using a collection time of one to two minutes (buccal and labial, respectively) (data not shown).

Whole saliva
The highest amounts of gp-340 occurred in the 3-yr-old group. The difference between 3-year-olds and adults was statistically significant ($P < 0.01$), as was the difference between 3-yr-old females and adult females when the gender subgroups in each age-group were compared ($P < 0.05$, Table 2). The ratio of gp-340 to total protein was also significantly higher in 3-yr-olds compared to adults ($P < 0.05$; Table 3).

Sialic acid content differed non-significantly between age-groups and between the female and male subgroups in each age-group (Table 2). However, the ratio of sialic acid to total protein decreased with age, and the difference between the 3-yr-olds and the adults was significant ($P < 0.01$; Table 3). Of the three male subgroups, adult males had significantly lower ratios of gp-340 to total protein ($P < 0.02$) and sialic acid to total protein ($P < 0.05$) compared with children and adolescents (Table 3).

Total protein concentration was significantly lower in the 3-yr-olds compared to the adults ($P < 0.05$); it was also lower in 3-yr-old males compared to adult males ($P < 0.02$; Table 2).

Expressing gp-340 as a ratio of sialic acid in minor-gland and whole saliva revealed that the portion of gp-340 did not change (data not shown).

No intra-individual correlations in gp-340 or sialic acid were seen between whole saliva and the different types of minor-gland saliva in any age-group.

Discussion
The main finding of this study is that the content of gp-340 and sialic acid, important salivary components of a person’s innate immunity, seems to be stable in minor-gland saliva from age 3 yr to age 20–25 yr.
We previously reported that the concentration of IgA in minor-gland saliva increases with age from 3 yr to 20–25 yr, specifically in labial-gland saliva (17), which is similar to what has been reported for IgA in whole saliva (1, 16). In contrast, this study’s findings on gp-340 and sialic acid in minor-gland saliva and our previous findings on MUC5B in labial-gland saliva (22) indicate that these innate immune components are stable from early childhood to early adulthood.

Hypothetically, the mucosal defense mechanism in the young child might depend more upon non-immune microbial binding glycoproteins, such as gp-340 and MUC5B, thus showing higher amounts of these innate immune molecules, to overcome the period of maturation of the adaptive immune system. High levels of glycoproteins early in life might also point to a biological significance for sialic acid’s free radical scavenger properties in the glycoproteins (31, 5). The OGASAWARA et al. study (5) showed that the bound form of sialic acid reacts more specifically than the free form with free radicals. For this reason, the present study measured glycoprotein-bound sialic acid. This molecule’s scavenging function might be a unique factor in oral innate immunity, which protects the surface epithelium of the oral cavity, whatever age, against changes in cellular components (32, 33).

The bacterial-binding and free-radical scavenger properties of innate immunity might be particularly important for patients suffering from various diseases affecting the adaptive immune system, because the innate system does not seem to be affected by such disorders. Healthy controls and patients (> 7 yrs) with an immunodeficiency were shown to secrete similar amounts of salivary agglutinating factors, which might be a sign of the potential of the innate defense (34).

In contrast to minor-gland saliva, we found gp-340 content in adult whole saliva to be significantly lower compared with in children. Expressed as a protein ratio, this difference was even more pronounced. This lower gp-340 content, which occurs in adult whole saliva but not in minor-gland saliva, might be a result of bacterial-specific binding or more thorough digestion than in children, since adult microflora is assumed to be more diverse and mature (35, 36). In addition, a bacterial-induced reduction of sialic acid, due to high
levels of bacteria and bacterial neuraminidase has been demonstrated in patients with oral diseases such as gingivitis and periodontitis (37). The increased concentration of total protein in adults is in line with data reported by other investigators (38) and might reflect an increase in albumin leakage from gingival fluids due to gingivitis or periodontitis, as a positive correlation between total protein and albumin concentration in these infections has been noted (39). As no data on oral health among the subjects was collected, the influence of these possible explanations could not be excluded. In minor-gland saliva sampling, these methodological problems have a lesser impact because bacterial exposure is minimized.

In the present study, gp-340 was detected in both buccal- and labial-gland saliva and in significantly higher amounts in adult buccal than labial saliva. In a previous study, however, we detected MUC5B and MUC7 in all labial saliva samples but only a few buccal samples (22). These observations indicate that glycoprotein secretion differs between the minor glands. It might be that differences in the composition of minor-gland saliva provide protective properties that are specific for the area in which the minor gland is located. VEERMAN et al. (40), who reported variations in amounts of mucins in the mucous layers of different oral areas, has also discussed this idea. Differences in glandular cell populations and differences in number of sero-mucous cells in minor glands might explain these local variations in saliva quality and glandular secretions (41, 42).

The salivary flow from the minor glands was not actively stimulated at any site, but preparation of the mucosa and use of filter paper during sampling might have functioned as light stimulation and mechanical stress on the mucosa. Such factors have been shown to influence flow rate (43). One risk with using filter paper to collect minor-gland saliva is retention of salivary molecules (17). Regardless, sampling and laboratory procedures were identical throughout the study, making group comparisons possible.

In whole saliva, it is well established that total protein content is inversely related to secretion rate (44). A strong correlation between whole saliva mucins and protein concentration has also been shown (45), indicating the possibility of extrapolating glycoprotein content from measurements of protein concentration. To compensate for any stimulation of secretion rate that might have occurred
during sampling, in particular among the children, we also calculated gp-340 and sialic acid content in ratios to protein. We found that protein concentration increased significantly with age, which is in line with the findings of BEN-ARYEH et al. (38). One drawback in their findings, however, is that the secretion rates were not controlled. Consequently, we present our data as both absolute amounts and as ratios to protein for whole saliva. In addition, assuming the ratio of gp-340 to sialic acid reflects the relation of gp-340 to total amount of glycoproteins, our results support the suggestion that gp-340 content in saliva during the first two and a half decades of life is fairly stable.

In conclusion, this study demonstrates that gp-340 and sialic acid content in minor-gland saliva is stable from 3 to 20–25-years of age. A site-dependent difference in the secretion of gp-340 and sialic acid in adults was also observed, with higher amounts in buccal than in labial saliva. Whole saliva in the 3-yr-old group contained more gp-340 compared to adult whole saliva. These observations of innate immunity in the children’s saliva may reflect an important function, primarily that of protecting the oral tissues when the adaptive immune system is still under development.

Further studies on the glycosylation of major- and minor-gland saliva of children, compared to adults, are needed to (i) determine how the innate salivary immune system develops during growth and (ii) develop strategies for young patients with general diseases or who are undergoing treatment that affects the specific immune response.

**Acknowledgements**

This study was funded by The Crafoord Foundation (20090733), the Swedish Patent Revenue Foundation, TePe Oral Health Care (Malmö, Sweden), the Knowledge foundation, and the Faculty of Odontology at Malmö University (OD27-2009/275).
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Table 1
*Gp-340 and sialic acid (abs µl⁻¹) in minor-gland saliva*

<table>
<thead>
<tr>
<th></th>
<th>3-yr-old children</th>
<th>14-yr-old adolescents</th>
<th>20–25-yr-old adults</th>
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<tbody>
<tr>
<td></td>
<td>mean</td>
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<td>n</td>
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<tr>
<td><strong>Labial gland saliva</strong></td>
<td></td>
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<tr>
<td><em>Gp-340</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>539</td>
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<tr>
<td>Female</td>
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<td>524</td>
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</tr>
<tr>
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<td>308</td>
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<tr>
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<td></td>
</tr>
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<td>671</td>
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</tr>
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<td></td>
</tr>
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<tr>
<td>Male</td>
<td>365</td>
<td>311</td>
<td>10</td>
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</table>

¹The 20–25-yr-olds had significantly lower amounts of gp-340 in the labial glands compared to the buccal glands (*P < 0.05*).
²The 20–25-yr-olds had significantly lower amounts of sialic acid in the labial glands compared to the buccal glands (*P < 0.01*).
Table 2

Gp-340 (abs µl⁻¹), sialic acid (abs µl⁻¹), and total protein (µg µl⁻¹) in whole saliva

<table>
<thead>
<tr>
<th></th>
<th>3-yr-old children</th>
<th>14-yr-old adolescents</th>
<th>20–25-yr-old adults</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>SD</td>
<td>n</td>
</tr>
<tr>
<td><strong>Gp-340</strong></td>
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<tr>
<td>All</td>
<td>33.99</td>
<td>12.50</td>
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<tr>
<td><strong>Sialic acid</strong></td>
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<td>11</td>
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</table>

*The 20–25 yr-olds had significantly lower amounts compared to the 3-yr-olds (P < 0.01).*

*The 20–25 yr-old females had significantly lower amounts compared to the 3-yr-olds (P < 0.01).*

*The 3-yr-olds had significantly lower concentrations compared to the 20–25-yr-olds (P < 0.05).*

*The 3-yr-old males had significantly lower concentrations compared to the 20–25-yr-old males (P < 0.02).*
**Table 3**  
*Gp-340 and sialic acid as a ratio (x10^3) of total protein in whole saliva*

<table>
<thead>
<tr>
<th></th>
<th>3-yr-old children</th>
<th>14-yr-old adolescents</th>
<th>20–25-yr-old adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean</td>
<td>SD</td>
<td>n</td>
</tr>
<tr>
<td><strong>Gp-340/total protein</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>0.10</td>
<td>0.05</td>
<td>18</td>
</tr>
<tr>
<td>Female</td>
<td>0.09</td>
<td>0.04</td>
<td>7</td>
</tr>
<tr>
<td>Male</td>
<td>0.10</td>
<td>0.06</td>
<td>11</td>
</tr>
<tr>
<td><strong>Sialic acid/total protein</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All</td>
<td>0.55</td>
<td>0.39</td>
<td>18</td>
</tr>
<tr>
<td>Female</td>
<td>0.58</td>
<td>0.53</td>
<td>7</td>
</tr>
<tr>
<td>Male</td>
<td>0.54</td>
<td>0.31</td>
<td>11</td>
</tr>
</tbody>
</table>

^eThe 20–25-yr-olds had significantly lower ratios compared to the 3-yr-olds (*P* < 0.05).
^fThe 20–25-yr-old males had significantly lower ratios compared to 3-yr-old males (*P* < 0.02).
^gThe 20–25-yr-olds had significantly lower ratios compared to the 3-yr-olds (*P* < 0.01).
^hThe 20–25-yr-old males had significantly lower ratios compared to the 3-yr-old males (*P* < 0.05).
MIKAEL SONESSON
ON MINOR SALIVARY GLAND SECRETION IN CHILDREN, ADOLESCENTS AND ADULTS

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MALMÖ UNIVERSITY
205 06 MALMÖ, SWEDEN
WWW.MAH.SE