Correlations between total protein, lysozyme, immunoglobulins, amylase, and albumin in stimulated whole saliva during daytime

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The correlations between salivary proteins and the daytime variations are not known. The present study investigated the within-subject variation of correlations and concentrations between lysozyme, IgA, IgG, IgM, albumin, amylase, and total protein in stimulated whole saliva of healthy adults in the course of a 12-h period. After several practise sessions, unstimulated and stimulated whole saliva samples were collected five times daily (at 8 a.m., 11 a.m., 2 p.m., 5 p.m., and 8 p.m.) from 30 healthy university students. Flow rate and total protein concentration were used as covariates, and gender as a between-subject factor in the MANOVA analysis. After this adjustment, there was significant within-subject variation in salivary IgA ($P < 0.001$), albumin ($P < 0.01$), amylase ($P < 0.05$), and total protein ($P < 0.001$) concentrations. Total protein correlated significantly with amylase albumin and IgA through different samplings. In addition, IgG correlated with albumin and lysozyme in the course of 12 h. On the whole, the correlations between variables remained stable during repeated samplings. In addition, rankings of subjects for the variables tended to be maintained across different samplings ($P < 0.001$). However, the observed within-subject variations in salivary IgA, albumin, amylase, and total protein concentrations suggest that these proteins are subject to short-term variation. □ *Saliva; salivary proteins*

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Many investigators have attempted to relate differences in salivary levels of antibacterial proteins to differences in oral health. However, the results have been inconsistent and difficult to interpret. In almost all studies, single point measurements of salivary antimicrobial factors have been correlated to clinical indices, such as DMF scores, which represent cumulative disease experience (1). Within-subject correlations among salivary antimicrobial protein levels over time can provide an indirect estimate of the extent to which analyses of these factors may influence the results of clinical investigations (2). It is also important to remember that many salivary antimicrobial factors interact with each other (3, 4). It seems that no single salivary defensive factor (except flow rate) affects oral health to a significant degree. This interaction may be one reason why it has been difficult to relate variations in salivary levels to oral ecology. It may be appropriate to consider salivary proteins as an integrated system (5).

It is well established that the flow rate and composition of saliva vary rhythmically over 24 h periods (6). The relative concentrations of the organic salivary constituents are known to depend on salivary flow rate (7), but their fluctuation during a single day has not been investigated. There are some published reports on temporal variations of salivary proteins (8–10), but specific data are often difficult to find. Little is also known of the correlations of salivary proteins and possible changes in these correlations during daytime (11). The present study was therefore made to investigate the correlations between lysozyme, IgA, IgG, IgM, albumin, amylase, and total protein concentrations in stimulated whole saliva in healthy adults over a 12-h period. In addition, we studied the within-subject variation of these variables and its effect on these correlations.

Our study hypotheses to be tested were that the time of collection of saliva may affect its flow rate and composition, and that certain correlations may exist between these variables during daytime.

Material and methods

The study was carried out at the Kuopio University Dental Clinic, Finland. A total of 30 healthy university students, 16 males and 14 females (mean ages: men 24.2 SD 2.9; women 21.7 SD 1.5 years; all 22.7 SD 2.8 years) were recruited. All subjects were accepted on a voluntary basis, and only healthy subjects with no history of significant medical conditions were included. Information about the subjects' health was recorded during an interview before collecting the first saliva samples. A further inclusion criterion was that baseline salivary flow rates should fall within the reference values used in our clinic for healthy adults; i.e. resting saliva >0.1 mL/min and stimulated saliva >0.7 mL/min. This criterion was fulfilled by all the subjects. The study protocol had been accepted by the ethics committee of the University of Kuopio.

The collection procedure was always carefully practised several times beforehand in order to reduce the bias in repeated measurements. In addition, the subjects were

given written instructions regarding saliva collections: they were told not to eat, drink, or smoke for 1 h before each sampling, but otherwise to act normally througout the day. The test subjects were instructed to eat breakfast at 6.30 a.m., and this was checked at the interview in the morning of the sampling day.

Saliva samples were collected in restful and quiet circumstances in our laboratory. Unstimulated and stimulated saliva samples were collected five times daily (at 8 a.m., 11 a.m., 2 p.m., 5 p.m., and 8 p.m.) from each subject. Unstimulated saliva was collected for a single 5-min period by the subject leaning forward and letting the saliva drain into a graduated sampling tube. Before collection, the mouth was emptied by initial swallow. Stimulated whole saliva was collected over a 3-min period, and saliva secreted during the first 30 s was discarded. Saliva flow was stimulated by chewing a piece of paraffin wax (1 g, Orion Diagnostica, Espoo, Finland) at a constant rate (about once a second). The amount of saliva collected was evaluated visually from graduated test tubes, and flow rates were calculated as ml/min.

Sampling tubes were placed in crushed ice immediately after collection. Stimulated saliva samples were used for analysis. Lysozyme was assessed using a modification of the lysoplate method (11), which is based on the capacity of lysozyme to lyse cell walls of *Micrococcus lysodeikticus*. Saliva was pipetted on the agarose gel plates before the sample was centrifuged. The diameters of clearance zones were measured after 24 h. The rest of the saliva was centrifuged ($1800 \times g$ 10 min) and the samples were stored at $-75°C$ until further analyzed. The total salivary immunoglobulin concentrations were then analysed from thawed samples using an enzyme immunoassay (ELISA) according to Lehtonen et al. (12). Albumin was assessed using a spectrophotometric method (13), total protein using the Lowry method (14) and amylase using an enzymatic colorimetric test kit (α-amylase EPS, Boehringer Mannheim, Mannheim, Germany).

Statistical analysis

Kolmogorov-Smirnov's test was used to check the normality of distributions. Repeated-measures design was used in the multivariate analysis of variance (MANOVA). These statistical tests require that data from repeated samplings correlate with each other, and this was tested by correlation plotting. Initial results were computed on the basis of observed concentrations. However, concentrations of salivary proteins can be subject to variation due to differences between the subjects in flow rates and total protein output (2). Since total protein concentration and flow rate seemed to correlate with the other variables in our data, we used MANOVA with covariates (flow rate and total protein) to test the within-subject variation of the repeated samplings.

To study the relationships between the variables at different time points, we used partial correlations. Partial correlation coefficients and the significance of the correlations between the variables were calculated with the aid of Pearson's formula. Flow rate and total protein concentration were controlled for.

Correlations within subjects across the samples were expressed as inter-item correlations (IIC) for each protein. These correlations give an idea of whether persons who show low or high values for a salivary protein remain at the low or high end of the range for each time point.

Results

Observed data

The means of the observed concentrations at different time points, 95% confidence intervals and group means are shown in Figs 1–4. A detailed description of the data for unstimulated and stimulated flow rates has been given elsewhere (15). Initial results were calculated on the basis of observed concentrations. In these data, the hourly variation of salivary IgA ($P < 0.001$), albumin ($P < 0.01$), amylase ($P < 0.001$), and total protein ($P < 0.001$) was statistically significant. Total protein correlated significantly with albumin ($r = 0.59$; $P < 0.001$ at 2 p.m.), amylase ($r = 0.73$; $P < 0.001$ at 2 p.m.), IgA ($r = 0.50$; $P < 0.01$ at 2 p.m.), and IgM ($r = 0.47$; $P < 0.01$ at 2 p.m.) levels. Stimulated flow rate correlated negatively with lysozyme ($r = -0.50$; $P < 0.01$ at 2 p.m.), IgA ($r = -0.67$; $P < 0.001$ at 2 p.m.), IgG ($r = -0.53$; $P < 0.01$ at 2 p.m.), and albumin ($r = -0.60$; $P < 0.001$ at 2 p.m.).

Within-subject variation and correlations across samples

The MANOVA analysis with covariates is presented in

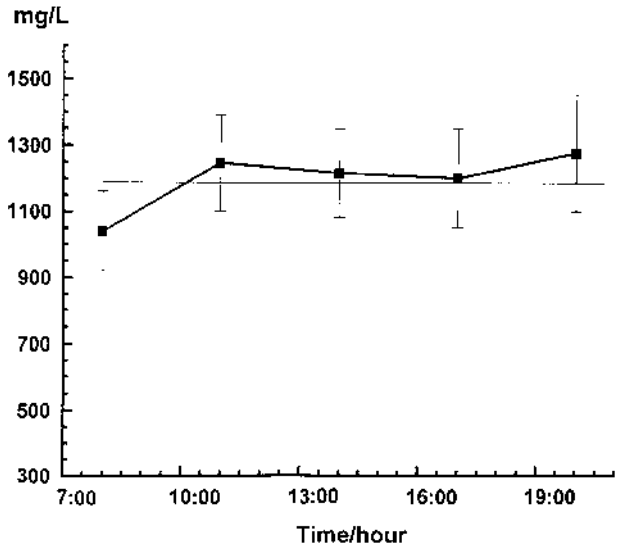

Fig. 1. Total protein concentrations and 95% confidence intervals over the 12-h period of sampling. Straight line represents the mean concentration of total protein (—■— protein; — group mean).

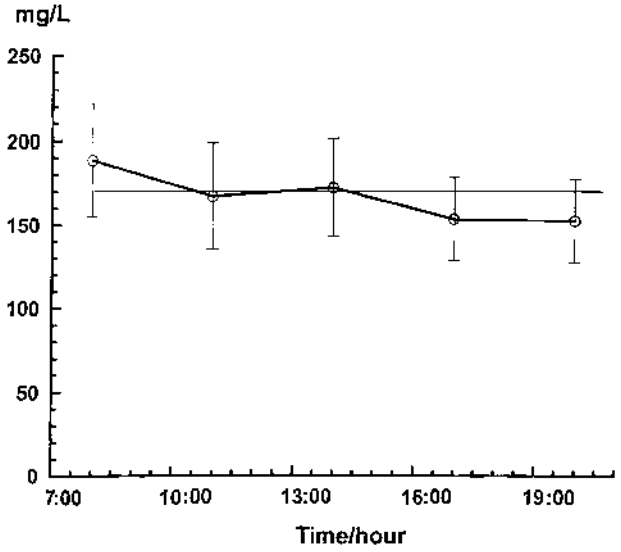

Fig. 2. Albumin concentrations and 95% confidence intervals in saliva over the 12-h period of sampling. Straight line represents the mean concentration of albumin (—○— albumin; — group mean).

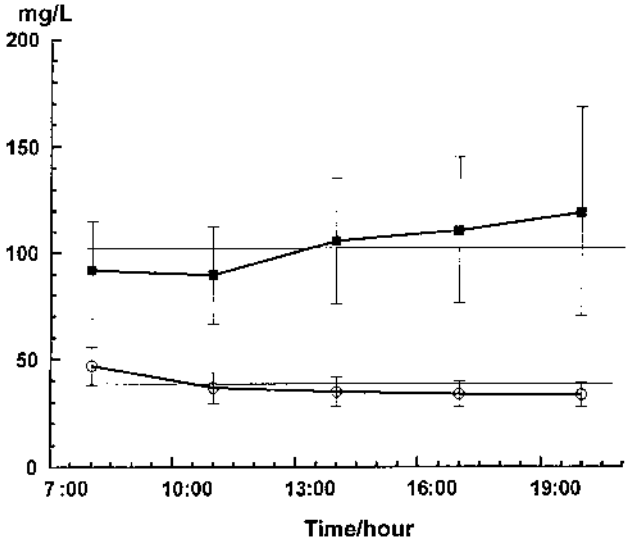

Fig. 4. Salivary lysozyme and IgA concentrations and 95% confidence intervals over the 12-h period of sampling. Straight lines represent the mean concentrations of variables (—■— lysozyme; —○— IgA; — group mean).

Table 1. Flow rate and total protein concentration were used as covariates and gender as a between-subject factor. After this adjustment there was still a significant within-subject variation in salivary IgA ($P < 0.001$), albumin ($P < 0.01$), amylase ($P < 0.05$), and total protein ($P < 0.001$) concentrations. Gender did not significantly affect the variation in any of these variables. The inter-item correlations, given in Table 1, were calculated from observed concentrations. There was a strong tendency for subjects to maintain a similar position relative to others across all samples, since inter-item correlations were significantly different from zero for all variables. The effect of the covariates on the whole observed variation is given in Table 2. The flow rate and total protein concentration affected significantly only the variation of amylase concentrations.

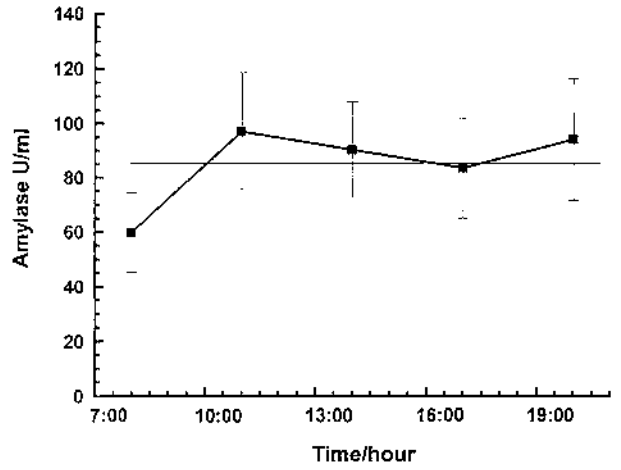

Fig. 3. Salivary amylase concentration and 95% confidence intervals over the 12-h period of sampling. Straight line represents the mean concentration of amylase (—■— amylase; — group mean).

Correlations between variables

Partial correlation coefficients and significances of the correlations between the variables in different samplings are presented in Table 3. Flow rate and total protein concentration were controlled for. Total protein correlated significantly with amylase, albumin, and IgA through different samplings. In addition, IgG correlated with albumin and lysozyme levels over the 12-h period. Amylase correlated negatively with all the other variables, but the correlation was statistically significant only with albumin in all the samplings.

Table 1. Multivariate analysis of variance (MANOVA) for repeated measures with covariates and inter-item correlations (IIC)

Parameter	F	*P*-value	IIC
Total protein[a]	7.5	$P < 0.001$	0.80***
Amylase	3.0	$P < 0.05$	0.78***
Albumin	4.1	$P < 0.01$	0.79***
Lysozyme	1.6	N.S.	0.77***
IgA	6.7	$P < 0.001$	0.80***
IgM	0.5	N.S.	0.88***
IgG	0.9	N.S.	0.81***

[a] Adjusted for flow rate only.
***$P < 0.001$.
Flow rate and total protein concentration were used as covariates and gender used as a between-subject factor in MANOVA.

Table 2. Analysis of covariates (flow rate and total protein concentration) for the study parameters

Dependent variable	Effect of F	Covariates *P* value
Amylase	173.3	<0.001
Albumin	2.81	N.S.
Lysozyme	0.9	N.S.
IgA	2.4	N.S.
IgM	0.3	N.S.
IgG	1.9	N.S.

MANOVA for repeated measures was used for analyzing the effect of covariates on the variation of repeated samplings.

Discussion

The main objective of this study was to investigate the associations between salivary albumin, amylase, total protein, lysozyme, IgA, IgM, and IgG in a number of healthy subjects over 12 h. The study also assessed the within-subject variation of these variables and its possible effect on these associations. This experiment was designed as a transverse type of study, rather than a longitudinal type, in which the rhythms of single subjects are followed over several days (16). The strengths of the present study are the use of a very homogeneous, healthy study group, and highly standardized collection methods. Other strengths include the use of more time points and more subjects than in most previous reports. Detailed data on flow rates and viscosities of the samples have been published earlier (15).

While amylase, together with proline-rich proteins, histatins, and mucins, is a major constituent of saliva, IgA, IgM, IgG, and lysozyme are minor constituents. In addition, while IgA and lysozyme are products of gland secretion, the major route of entry of albumin and IgG into the oral cavity is via crevicular fluid. However, some interesting relationships between salivary proteins can be seen in the present results. Amylase, which is thought to be an indicator of acinar cell function in salivary glands (17), was negatively correlated with the other salivary proteins, except total protein. Albumin, which is used as a marker for the degree of mucositis or inflammation in the oral cavity (18), correlated well with IgG levels and partly with IgA levels. Lysozyme and IgG also correlated with each

Table 3. Partial correlation coefficients (Pearson) and significances of the correlations (controlling for flow rate and total protein concentration)

Time		Amyl	Alb	Lys	IgA	IgM	IgG
8 a.m.	Prot[a]	0.79***	0.54**	0.12	0.64***	0.32	0.46*
	Amyl	1.0	−0.40	−0.18	−0.24	−0.33**	−0.55**
	Alb		1.00	0.40*	0.30	−0.06	0.43
	Lys			1.00	0.05	0.08	0.41*
	IgA				1.00	0.14	0.17
	IgM					1.00	0.18
	IgG						1.00
11 a.m.	Prot[a]	0.80***	0.55**	−0.38*	0.40*	0.27	0.25
	Amyl	1.00	−0.49	−0.30	−0.37	−0.23	−0.44
	Alb		1.00	0.30	0.71**	0.37	0.73***
	Lys			1.00	0.24	−0.13	0.43*
	IgA				1.00	0.33	0.40
	IgM					1.00	0.25
	IgG						1.00
2 p.m.	Prot[a]	0.79***	0.53**	0.04	0.39*	0.45*	0.33
	Amyl	1.00	−0.31	−0.28	−0.12	−0.27	−0.27
	Alb		1.00	0.49**	0.17	0.00	0.55**
	Lys			1.00	1.03	0.11	0.58
	IgA				1.00	−0.09	0.12
	IgM					1.00	0.14
	IgG						1.00
5 p.m.	Prot[a]	0.83***	0.63***	0.08	0.42*	0.47*	0.33
	Amyl	1.00	−0.50**	−0.28	−0.31	−0.42*	−0.40*
	Alb		1.00	0.31	0.55**	0.30	0.48*
	Lys			1.00	−0.6	0.07	0.65***
	IgA				1.00	0.20	0.17
	IgM					1.00	0.10
	IgG						1.00
8 p.m.	Prot[a]	0.87***	0.55**	0.18	0.39*	0.17	0.47*
	Amyl	1.00	−0.48*	−0.25	−0.45*	0.58***	−0.46*
	Alb		1.00	0.68***	0.49**	0.43*	0.68***
	Lys			1.00	0.23	0.22	0.60***
	IgA				1.00	0.14	0.40*
	IgM					1.00	0.11
	IgG						1.00

[a] Adjusted for flow rate only.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

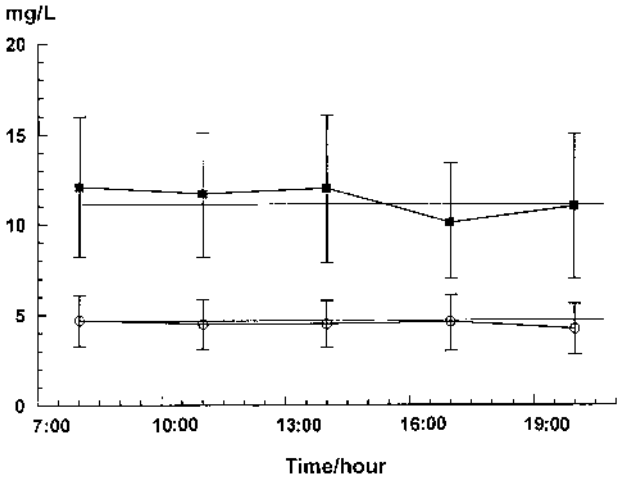

Fig. 5. Salivary IgG and IgM concentrations and 95% confidence intervals over the 12-h period of sampling. Straight lines represent the mean concentrations of variables (—■— IgG; —○— IgM; — group mean).

other, but we did not find any significant relationship between lysozyme and IgA. This is in agreement with findings in an earlier study by Rudney (5). On the other hand, in one previous study, lysozyme, lactoferrin, and IgA correlated closely with each other (11). On the whole, many of the correlations remained stable over different samplings, although some correlations were not stable during the 12-h period. For example, the IgA and albumin levels seemed to vary even when the effect of salivary flow rate and total protein concentrations were controlled for. The reason for such instability remains unclear.

An interesting observation was that the rankings of the subjects for total protein, amylase, albumin, lysozyme, IgA, IgM, and IgG tended to be stable across the samplings. This finding is also in agreement with that of Rudney et al. (2), who found that subjects remained in the same ranking position for total protein, lysozyme, lactoferrin, and salivary peroxidase even from one week to the next.

Our results suggest that there is a significant within-subject hourly variation in salivary IgA, total protein, amylase, and albumin levels. On the other hand, the hourly changes in lysozyme, IgM, and IgG concentrations were statistically not significant, so that the respective variation of these variables may not be equally important. However, lysozyme concentrations did show an increasing trend from the morning to the evening samples, and this too may be important to keep in mind when interpreting the various values. On the other hand, amylase concentration was strongly affected by changes in flow rate and total protein concentration.

There are many other published reports on the temporal variation of salivary proteins (8–10). Our results are in agreement with the findings of Jenzano et al. (8), who studied temporal variation of glandular kallikrein, amylase, and total protein in human whole saliva in 4 repeated samplings. They also found significant differences for total protein and amylase levels within days, and the lowest values in the morning. Our observations of ranges for salivary IgA levels in stimulated whole saliva are in agreement with earlier findings in stimulated parotid fluid, although the pattern of hourly variation was different (19). A negative correlation between stimulated flow rate and IgA concentrations has also been reported earlier (7). On the other hand, Bennet and Reade (20) did not find any significant relationship between the time of day and IgA concentration. Butler et al. (21) found some fluctuations in IgA in whole saliva, but the levels were still influenced more by the patient than by the time of sampling. We did not find statistically significant variation in lysozyme concentrations in repeated samplings, but the trend was an increase from the morning to the evening samples. However, Palenstein Helderman (22) found variations in lysozyme in whole saliva as well as in saliva from the labial sulcus. On the whole, the lysozyme concentrations of our subjects were higher than those reported earlier for stimulated whole saliva (1).

It is well documented that salivary albumin levels increase before the onset of mucositis and correlate with its severity (18). Salivary albumin levels have been used as a marker for the degree of mucositis (23, 24) and inflammation in salivary glands (25, 26). The levels of salivary albumin during a day in healthy adult patients have not been studied earlier, however. Butler et al. (21) found that albumin levels in whole saliva fluctuated in most of the elderly patients in their study. Our results suggest that there is a statistically significant hourly within-subject variation in salivary albumin levels even in healthy adults. The albumin levels in our study were higher than previously reported (18). In this study, we did not examine the oral or dental status of the subjects, so we do not know the degree of mucositis or gingival inflammation in the subjects. However, we have reason to believe that the oral health status of our study subjects was above average, because they were dental or medical students and very motivated to take care of their oral health. Therefore, it is important to note that salivary albumin is detectable in minor amounts even in subjects of this kind.

In our observations the salivary IgA, IgG, and albumin concentrations were highest in the morning. This can be due partly to the reduced flow rate in the morning, which was also observed in the present material (15). Another possible explanation could be the capacity of the salivary glands to store IgA and IgG, so that the concentration would be higher at the first sampling.

To conclude, it is important to acknowledge the existence of within-subject variation and its effect on salivary values. Observed within-subject variation in salivary IgA, albumin, amylase, and total protein concentrations suggests that these proteins are subject to short-term variation. Our results emphasize the need to standardize the collection of saliva and at least specify the time of collection of salivary samples. Nevertheless, in our study the correlations between variables remained stable across different samplings, and rankings of subjects

for total protein, amylase, albumin, lysozyme, IgA, IgM, and IgG tended to be maintained across the different samplings.

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