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Saliva in Prader–Willi syndrome: Quantitative and qualitative characteristics

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ABSTRACT

Objective: To evaluate salivary **flow** rates and assess whole salivary total protein, MUC7 and cystatin in individuals with Prader–Willi syndrome (PWS) compared with healthy controls.
Design: The participants were forty-eight individuals with PWS (mean age 20.2 ± 9.5) and an age- and sex-matched control group. Flow rates of unstimulated whole saliva (UWS), stimulated whole saliva (SWS), submandibular/sublingual (SS), and parotid saliva (PS) were recorded (ml/min) and unstimulated whole saliva used for further protein analysis. Total protein concentration was determined via the bicinchoninic acid method, and MUC7 and cystatin levels via a Dot Blot.
Results: Mean UWS (0.12 ± 0.11 vs. 0.32 ± 0.20, $p < 0.001$), SWS (0.41 ± 0.35 vs. 1.06 ± 0.63, $p < 0.001$) and SS (0.27 ± 0.19 vs. 0.50 ± 0.29, $p < 0.001$) salivary **flow** rates were **significantly** lower in PWS compared with controls. No **significant** difference was found in PS **flow** rate between the two groups. The mean total protein concentration (mg/ml) was 3.19 ± 3.04 in PWS compared with 1.32 ± 1.11 in controls ($p < 0.001$). Median concentration of MUC7 (mg/ml) was 1.29 (0.11–10.85) in the PWS group, and 0.39 (0.03–2.22) in the control group ($p < 0.001$). No **significant** difference was found in cystatin concentration between the groups. The output of proteins did not differ **significantly** between PWS and controls.
Conclusion: With the exception of parotid saliva, salivary **flow** rates were lower among individuals with PWS than in their matched controls. Saliva protein concentrations in UWS were high compared with the healthy group, although the protein output did not differ significantly.

1. Introduction

Prader–Willi syndrome (PWS) is a genetic disorder affecting multiple organ systems. The syndrome has characteristic phenotype including severe neonatal hypotonia, hyperphagia, obesity, short stature, hypogonadism and intellectual disability.[1–3] PWS affects males and females equally[4–7] and has traditionally been described as having two nutritional stages; poor feeding and failure to thrive in infancy followed by hyperphagia leading to obesity in later childhood.[3,8,9] The aetiology of the switch from poor feeding to hyperphagia is thought to be associated with abnormalities in the hypothalamic circuitry.[8] Obesity in PWS can be controlled by strict dietary restrictions.

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Most individuals with PWS have reduced secretion of growth hormone (GH) and are therefore treated with the hormone, thus stimulating growth and altering physical phenotype and body composition.[10–13] Necdin is important for the differentiation of central and peripheral sensory neurons and is congenitally absent in PWS.[14,15] Orofacial dysfunction is common in those with the syndrome.[16]

Dental caries, enamel defects and poor oral hygiene have been described in case reports,[17–22] although two recent surveys have identified more favourable oral health.[23,24] Severe tooth wear (both erosive wear and attrition) has been reported.[23,25]

Viscous saliva has been reported to be a diagnostic indicator of PWS in neonates[26] and is a consistent finding in PWS.[23,27,28] Decreased salivary flow rate[24,27,28] and increased amounts of salivary ions and proteins have been reported in individuals with the syndrome.[28] Unstimulated salivary flow rate in individuals with PWS has been identified as 30–38% of that in controls.[24,28] Saliva can be described as having five major functions that serve to maintain oral health: lubrication and protection, buffering action, maintenance of tooth integrity, antibacterial activity, and taste and digestion.[29,30] Mucin is essential for nearly all of these functions and cystatins are inhibitors of cystatin proteinases that may play a protective role during inflammation. The aims of the present study were to quantify the salivary flow rates of major salivary glands and to quantify MUC7 and cystatin in whole saliva in individuals with PWS and to compare findings with a healthy control group matched for age and gender.

2. Materials and methods

2.1. Participants

The study was part of a larger survey and was performed at the National Resource Centre for Oral Health in Rare Medical Conditions (TAKO-centre) and the Faculty of Dentistry, University of Oslo.[24] The study protocol was approved by the Regional Committee for Medical Research Ethics and informed consent was obtained from all participants. The study used a matched case–control design. Forty-eight individuals with a genetically confirmed diagnosis of PWS aged 6 years and older and a healthy control group without PWS participated in this part of the study. Demographic data and medical characteristics of the study population are summarised in Table 1. BMI criteria for the age group 6–18 years of age ($n = 23$) were age and gender adjusted.[31]

Table 1 – Characteristics of the study population.

	PWS ($n = 48$)	Control ($n = 48$)
Age (year) mean, range	20.2 (6–41)	20.7 (6–43)
	PWS *n* (%)	Control *n* (%)
Gender		
Female	23 (48)	23 (48)
Male	25 (52)	25 (52)
Genetic mechanisms		
Del 15	33 (69)	–
UPD 15	11 (23)	–
Meth+	4 (8)	–
Other conditions		
Diabetes	3 (6)	–
Epilepsy	3 (6)	–
Heart disease	1 (2)	–
Body mass index (BMI)[a]		
Obese (≥30)	18 (38)	–
Medication		
Psychopharmica[b]	11 (23)	–
Antihistamine[b]	6 (13)	–
Antiepilepticum[b]	2 (4)	
Proton-pump inhibitor[b]	1 (2)	

[a] BMI criteria was age- and gender adjusted for individuals aged 6–18 years according to the International Obesity Task Force (IONT).
[b] Medications having dry mouth as a known adverse effect.

2.2. Saliva collection

Saliva samples were collected between 10 a.m. and 1 p.m.[32] by a single examiner. All participants and/or their carers were advised in advance how to avoid stimulation of saliva during the hour immediately prior to the examination (avoid eating, drinking, brushing teeth or chewing gum). The participants rested in a quiet room for a few minutes prior to salivary sampling. Participants were asked to swallow immediately before the collecting period began. Samples were subsequently obtained by requesting the participants to tilt their head forward and expectorate all saliva into a cup every minute without swallowing. Unstimulated whole saliva (UWS) was collected for 5 min. Data on UWS flow rate has been previously reported (see the first report from this study).[24] Standard-size tasteless paraffin wax (1.5 g) was used as a stimulant for the collection of stimulated whole saliva (SWS). After 1-min of chewing and spontaneous swallowing, the 5-min collection period commenced. Parotid saliva (PS) was collected for 3 min, using acid candy as a stimulant, and with a modified Carlson–Crittenden cup[32] positioned over one of the Stensen's duct, usually the right one. Mixed submandibular/sublingual saliva (SS) was collected from the floor of the mouth for 3 min via gentle suction with a plastic pipette, using acid candy as a stimulant while blocking Stensen's ducts with parotid cotton rolls. Saliva was collected in plastic cups and weighed. Salivary volumes were calculated by weight, 1 g = 1 ml.[33] UWS was stored for a maximum of 90 min on dry ice, divided into aliquots and stored frozen (−80 °C) for later protein analysis. Due to viscous saliva in individuals with PWS, 200 μl tris-buffered saline (TBS) (Bio-Rad, Hercules, CA, USA) was added to all salivary samples and the test samples were standardised based on weight.

2.3. Total protein quantification

The total protein concentration in UWS was determined via the bicinchoninic acid (BCA) method,[34] using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) and bovine serum albumin (BSA) (2 mg/ml) as the protein standard. Aliquots of diluted standards and protein samples (25 μl) were pipetted into a 96 wells microplate (ELISA) and the assay was conducted according to the manufacturer's

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standard protocol. Total protein reactivity was expressed as absorbance at 562 nm by an ELISA reader (Asys Expert 96, Hitch GmbH, Eugendorf, Austria) and the results were presented as mg/ml.

2.4. *Quantification of salivary MUC7 and cystatin – Dot Blot analysis*

MUC7 and cystatin quantifications in UWS were performed in a Bio Dot Microfiltration Apparatus (Bio Rad, CA, USA). The weight of each individual sample was recorded. TBS (200 μl) was added to enable pipetting, and a calculated aliquot of each sample was further diluted with TBS to give a concentration of 50 μg protein/μl. Saliva samples (20 μl) with a starting concentration of 50 μg protein/μl were two-fold diluted and added to pre-soaked nitrocellulose membranes (BioRad). Purified Recombinant Human MUC7 (H00004589-Q01, Abnova, Taipei, Taiwan), ranging from 80 μg/ml to 10 μg/ml, and Human Cystatin SA protein (1201-P1 R&D Systems Minneapolis, MN, USA) ranging from 2.5 μg/ml to 0.3 μg/ml), were used as reference samples in a Dot Blot SNAP i.d. Protein Detection System (Millipore, Billerica, MA, USA). TBS was used as a blank negative control. Excess binding capacity of the nitrocellulose membranes were blocked for 1 h at room temperature, using 0.3% Non-Fat, Dry Milk (Bio-Rad) and 0.3% casein (Bio-Rad) respectively, in TBS with 0.1% Tween-20 (TTBS) (Bio-Rad). Expression of mucin was identified by MUC7 (H-150); sc-50433 rabbit polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) in a 1:100 dilution in TTBS, whereas expression of cystatin was identified by cystatin S/SA/SN; sc-73884 mouse monoclonal antibody (Santa Cruz) in a 1:100 dilution in TTBS. After incubation for 1 h at room temperature, the membranes were washed 3 times in TTBS before further incubation in a 1:1000 dilution of horseradish peroxidase (HRP) conjugated secondary antibodies (Goat anti Rabbit IgG, HRP cat. 4050-05, (MUC7) and Goat Anti-Mouse IgG, H + L chain specific, cat. 1031-05 (cystatin) from Southern Biotech, Birmingham, AL, USA). After incubation for 30 min, the membranes were washed 3 times in TTBS before visualisation using a freshly prepared 3,3-diaminobenzidine (DAB, Sigma–Aldrich, St. Louis, USA)-based horseradish peroxidase reaction product (0.3 g/l diaminobenzidine, 0.03% H_2O_2 and 0.03% $NiCl_2$ in 50 mM $(NH_4)HCO_3$).[35] Nonspecific cross reactivity or false positive signals were tested by applying only secondary antibodies to the membranes.

Band densities of the assumed MUC7 and cystatin were quantified using the Kodak Molecular Imaging System (Kodak Gel Logic 212, Carestream Health Rochester, NY, USA). Quantification of MUC7 and cystatin was performed using region of interest (ROI) tools. Standards were designated as ROIs and a standard mass curve generated to quantify unknown ROIs (mass-ng). Aliquots from one sample, used as a positive internal control, were included on all immunoblots (MUC7), and the inter-assay variation of this sample was calculated to 7%.

2.5. *Protein output*

The output of total protein and MUC7 was calculated in mg/min, while the output of cystatin was calculated in μg/min.

2.6. *Statistical analysis*

A two-sided independent samples t-test was used to compare UWS, SWS and SS flow rates and total protein concentrations between the PWS and control groups. A two-sided Mann–Whitney test was used to compare median PS flow rate and median MUC7 and cystatin concentration between the two groups. The Spearman correlation coefficient (r) was used to analyse the association between two continuous variables. In order to study the relationship between UWS and independent variables simultaneously in the study group, linear regression analyses was performed. The variables of age and medication became candidates for subsequent multivariate analysis. To analyse the relationship between salivary protein concentrations and several variables simultaneously, linear regression analysis was performed, with the following variables becoming candidates for subsequent multivariate analysis; age, gender and UWS flow rate. A significance level of 5% was used throughout. The statistical analysis was carried out using the statistical software program (SPSS© v. 18.0, SPSS Inc., Chicago, IL, USA).

3. Results

3.1. *Salivary flow rates*

Flow rates of UWS and SWS as well as SS and PS in the PWS and control group are presented in Table 2. Mean UWS, SWS and SS salivary flow rates were significantly lower in the PWS group. However, no statistical difference was found between median PS flow rates in the two groups. When multivariate linear regression analyses were performed within the PWS group the secretory rates of PS, SWS and SS were not significantly associated with obesity, genotype, age, gender or medication. UWS was significantly correlated with age ($p = 0.02$) in the PWS group but not in the control group ($p = 0.27$).

3.2. *Protein concentration and output in UWS*

Concentration and output of total protein, MUC7 and cystatin in UWS are presented in Table 3. The mean total protein concentration was significantly higher in the PWS compared with the control group ($p < 0.001$). MUC7 was increased about

Table 2 – Salivary flow rates in the PWS and healthy control group.

Saliva samples	PWS ($n = 48$)	Control ($n = 48$)	p-Value
UWS (ml/min)	0.12 ± 0.11	0.32 ± 0.20	<0.001[a]
SWS (ml/min)	0.41 ± 0.35	1.06 ± 0.63	<0.001[a]
PS (ml/min)	0.44 (0.04–2.56)[c]	0.53 (0.06–1.52)[c]	0.29[b]
SS (ml/min)	0.27 ± 0.19	0.50 ± 0.29	<0.001[a]

UWS, unstimulated whole saliva; SWS, chewing-stimulated whole saliva; SS, stimulated submandibular/sublingual saliva; PS, citric acid-stimulated parotid saliva.
[a] T-test.
[b] Mann–Whitney U-test.
[c] Median.

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Table 3 – Protein concentrations and outputs in unstimulated whole saliva in the PWS and healthy control group.

	PWS ($n = 48$)	Control ($n = 48$)	p-Value
Concentration			
Total protein (mg/ml)	3.19 ± 3.04	1.32 ± 1.11	<0.001[a]
MUC7 (mg/ml)	1.29 (0.11–10.85)[c]	0.39 (0.03–2.22)[c]	<0.001[b]
Cystatin (μg/ml)	65.3 (5.8–2301.4)[c]	48.3 (2.4–830.4)[c]	0.08[b]
Output			
Total protein (mg/min)	0.38 ± 0.36	0.42 ± 0.36	0.59[a]
MUC7 (mg/min)	0.16 (0.01–1.30)[c]	0.12 (0.01–0.71)[c]	0.61[b]
Cystatin (μg/min)	7.8 (0.7–276.2)[c]	15.3 (0.8–265.7)[c]	0.19[b]

[a] T-test.
[b] Mann–Whitney U-test.
[c] Median.

Table 4 – Results from multivariate linear regression analysis on data from the PWS group.

Dependent variables	Independent variables	B	95% CI	p-Value
Total protein	Gender	−0.26	−2.02–1.50	0.766
	Age	0.10	0.00–0.21	0.059
	UWS	−13.34	−22.91–(−3.78)	0.007
MUC7	Gender	−0.14	−0.83–0.55	0.689
	Age	0.03	−0.01–0.07	0.189
	UWS	−2.03	−5.75–1.69	0.277
Cystatin	Gender	0.89	0.26–1.52	0.007
	Age	0.06	0.02–0.10	0.002
	UWS	−5.40	−8.82–(−1.97)	0.003

UWS, unstimulated whole saliva.

three-fold in the PWS group compared with the control group ($p < 0.001$). MUC7 constitutes approximately 40% of the total protein in the PWS group and 30% in the control group. The majority of cystatins in whole saliva, cystatin S/SA/SN (S-like cystatins) were analysed in the present study. The cystatin concentration, however, was not elevated to a statistically significant level ($p = 0.08$). The output of proteins did not differ significantly between the two groups. Bivariate correlations were computed between total protein and genotype, obesity, age, gender, medications and UWS flow rate in the study group. The same independent variables were included in bivariate correlations with MUC7 and cystatin. In the multivariate models, the flow rate of UWS was significantly associated with total protein and cystatin, but not with MUC7. Age and gender were also associated with cystatin (Table 4).

4. Discussion

This study demonstrated that the clinically observed viscous saliva in individuals with Prader–Willi syndrome contains higher levels of total protein and MUC7 than the healthy controls. The observed high concentration of cystatin in the PWS group did not reach statistical significance. Due to reduced salivary flow rates in individuals with PWS, there was no difference between the two groups in terms of the amount of protein secreted per minute. Whole saliva flow rate has been evaluated previously,[28] but to our knowledge this is the first study to evaluate the PS and SS flow rate in PWS. Whole saliva was used to investigate total protein, mucin and cystatin concentrations because, although non-salivary components, such as gingival crevicular fluid and nasal secretions are present, whole saliva largely derives from secretions from all salivary glands, and thus represents the total of the various salivary components.

The salivary proteome is a complex protein mixture resulting from activity from all salivary glands with contribution of other components from the oral environment, making exact analysis difficult. However, the determined concentrations of total protein and cystatin in our healthy control group are consistent with previously reported values.[36–40] Few reports have been published on the concentration of mucins in saliva. Due to differences in collection protocols, techniques, and the source of mucin used (whole saliva or glandular saliva), reported values of mucins have varied widely and are difficult to compare. MUC7 is one of two mucous glycoproteins in the secretions of submandibular, sublingual glands and minor salivary glands.[41] Mucins have a multifunctional role in the oral cavity as they lubricate oral surfaces, provide a protective barrier between hard and soft tissues and external environment, and facilitate mastication and speech.[41] MUC7 is also considered to be a component of the host defence system because it binds to a large number of oral bacteria.[42,43] Our goal was to quantify the high-molecular-weight MUC5B and the low-molecular-weight MUC7, however, we failed to find primary antibodies towards MUC5B giving reliable results in Dot Blot.

The variability in protein concentration, total protein and MUC7 between individuals was quite large and corresponded with the variability reported in other studies.[44,45] The wide range in MUC7 concentration made some methodological limitations. Due to high protein concentration in some individual samples, several dilution steps were introduced

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to ensure that we estimated the concentration of MUC7 in the linear area or where true dilution of MUC7 was observed. For some individuals, however, further dilution should have been done as we might have under-estimated the concentration of mucin. MUC7 is a glycosylated protein, and although the antibody used in this study is raised against amino acids 1–75 mapping at the unglycosylated N-terminus of MUC7, unspecific binding to, i.e. carbohydrate/glycosylated sites cannot be excluded.

The concentration of cystatin did not differ significantly between the PWS and the healthy group. Salivary cystatin has been found to inhibit human lysosomal proteinases that may play protective roles during inflammation,[46] also in the oral cavity. The concentration of cystatin was significantly correlated to age, gender (higher concentrations in males) and whole saliva secretion. The majority of cystatins in whole saliva, cystatin S/SA/SN (S-like cystatins) were identified in the present study.

Due to mental status and young age, some participants may have had problems following instructions and the data may possibly deviate somewhat from true values. However, all study participants were well prepared by means of picture aided information prior to the examination and cooperated sufficiently for saliva collection to be carried out.

To our knowledge, this is the first study evaluating stimulated secretion from parotid glands in PWS and comparing it with a healthy group. Citrus-flavoured candy was used for stimulation to meet cooperation challenges. This may explain the somewhat higher median parotid gland flow rate in healthy individuals in this study compared with previous studies.[47,48] A single examiner collected saliva from one gland, usually the right parotid gland. If collection was unsuccessful from this site, the left gland was used. High correlation between flow rates from the left and right parotid glands has been reported[48] and bias due to collection from left or right was therefore not expected. In the present study no association was observed between stimulated parotid flow rate and obesity, genotype, age, gender or medication (having dry mouth as a known side effect) in the study group. The parotid gland has a very low secretory rate during resting (unstimulated) conditions compared to reflex stimulation (taste, mastication) and a normal reflex stimulation of taste may account for the small difference in parotid flow rate between the PWS and healthy group in the present study. The mean SS flow rates were also somewhat higher in the healthy group in the present study than suggested in previous reports.[47] However, due to different stimulants, the results are difficult to compare directly.

The reduced whole salivary flow rates (unstimulated and stimulated) found in the PWS group is in accordance with previously reported findings.[28] In the present study, mean UWS flow rate increases with age among individuals with PWS. This contrasts sharply with the findings from the control group. A possible explanation for this may be the unusually low water intake and drinking behaviour observed in individuals with PWS. Infants with PWS seem to dislike and have an unusually small intake of water.[49] This behaviour persists for many, but some individuals with PWS successfully increase their fluid intake and also accept pure water as they grow older. Previous studies have demonstrated a relationship between whole body hydration, reduced saliva flow rate and increased total protein concentration of saliva.[50–52] Individuals with PWS are less sensitive to thirst and are particularly at risk of dehydration in high temperatures.[53] In individuals with PWS, therefore, there is a possible association between hypohydration, diminished salivary flow rate and increased total protein concentration.

Both salivary flow rate and salivary composition are controlled by the autonomic nervous system. Abnormalities in feeding[8,54] altered temperature regulation,[55] increased pain threshold,[56,57] impaired vomiting reflex,[58] and diminished salivation[28] described in individuals with PWS suggest dysfunction of the autonomic nervous system. The Ndn gene, encoding the protein necdin, is deleted in individuals with PWS.[14,15] Necdin is important for differentiation and normal development of the autonomic nervous system. Reduced innervation of submandibular and parotid glands has been demonstrated in mice,[59] and this provides a plausible explanation for deficiencies of salivary gland fluid secretion. Although protein concentration was elevated in the present study, output did not differ from that found in the control group. This suggests that a comparable level of protein is secreted into reduced water content in individuals with PWS. Parasympathetic and sympathetic autonomic nerves work together in controlling and influencing different cells to stimulate salivary gland secretion.[60] The parasympathetic impulses usually stimulate most of the fluid secretion into saliva while sympathetic nerves release noradrenalin which triggers a greater release of proteins, primarily from acinar cells, but also from ductal cells in the glands. It has been demonstrated that the primary parasympathetic salivary centres form connections with the lateral hypothalamus where the regulation of feeding, drinking and body temperature occurs.[60] However, further research is needed to investigate the observation of normal protein output into reduced salivary fluid volume.

5. Conclusion

Whole saliva volume was low in PWS, and both UWS- and SWS-flow rates were approximately 40% of that found in the healthy group. Reflex stimulation from the parotid glands in response to citric acid seemed to be intact and within the normal range. Although the total protein and MUC7 concentrations in UWS were higher in the PWS group, the output of all examined proteins was comparable between PWS and control, indicating that the fluid and protein secretion mechanisms are differently affected by the condition.

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Competing interest

The authors declare that they have no competing interests.

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Ethical approval

The study was approved by the Regional Committee for Medical Research Ethics, East-Norway (REK-1). Ref nr. 1.2006.14.

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