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Parotid salivary sodium levels of Sjögren's syndrome patients suggest B-cell mediated epithelial sodium channel disruption

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ABSTRACT

Patients with primary Sjögren's syndrome (SS) suffer widely from lack of saliva production. Here we investigate potential mechanisms underpinning changes in SS patient saliva composition. Sodium concentration was significantly higher in all saliva samples collected: unstimulated submandibular/sublingual (SmSI) saliva ($p < 0.0001$), stimulated SmSI saliva ($p = 0.002$) and stimulated parotid (PG) ($p < 0.0001$) saliva, compared to non-SS sicca controls. Chloride, phosphate and potassium ion concentrations, α -amylase activity and total protein content correlations were less consistently changed between SS and non-SS saliva types. Stimulated PG salivary sodium levels correlated with the degree of CD45⁺ lymphocytic cell infiltrate in the parotid glands ($r = 0.69$, $p < 0.001$), and even more strongly so with infiltrating CD20⁺ B cells ($r = 0.73$, $p < 0.0001$). CD3⁺ T cells were only moderately correlated with salivary sodium ($r = 0.23$, $p = 0.015$). In non-SS control or focus score (FS) negative SS PG tissue, the epithelial sodium channel (ENaC), responsible for sodium transport out of saliva, was localised to the apical membrane of luminal striated duct cells. In PG tissue from FS+ SS patients, apical ENaC expression appeared absent. We hypothesise that B cell-related proinflammatory cytokines in SS salivary glands may dysregulate sodium transport channels in SS.

Introduction

The average human produces around 0.5-1.5L of saliva per day (1). This multi-faceted, finely-tuned fluid contains a mixture of mucous proteins (in particular mucins 5B and 7), digestive enzymes (α -amylase, lysozymes), IgA antibodies, buffering compounds and electrolytic ions (2, 3). Each component plays a critical role in the maintenance

of oral health. Mucous proteins provide a non-adhesive coating for the oral epithelium and facilitate swallowing. Digestive enzymes initiate the process of digestion of complex carbohydrates, whilst salivary antibodies prime the oral mucosa for the attack of unwelcome microbial visitors (2, 3). Bicarbonate in saliva provides an essential buffering property to saliva, maintaining a close to neutral pH, (pH5.5-6.5) and an unfavourable environment for bacteria (2, 3). High bicarbonate ion concentrations together with high potassium ion concentration in saliva, relative to plasma, also function to neutralise acidic foods in the mouth and protect tooth enamel from demineralisation. The complex mixture in charged ions also ensures that sufficient calcium ions, critical for continued mineralisation of teeth, are physically present in the immediate environment surrounding the teeth. Proper final saliva ion balance is achieved through the modification of the primary saliva initially produced and secreted by acinar cells. The luminal cells of the striated duct, equipped with an array of ion transporter channels, perform this function, using the gradient of ion charges to transport chloride and sodium ions out of saliva and into striated duct cells, and bicarbonate and potassium in the reverse direction (4).

A hallmark clinical feature of patients with the autoimmune disease primary Sjögren's syndrome (SS) is severely reduced saliva production, frequently referred to via the Latin term 'sicca', meaning dried or withered (5, 6). SGs of SS patients often contain lymphocytic infiltration rich in hyperactive B cells, CD4⁺ and CD8⁺ T cells, and dendritic cells (7, 8). Infiltrates are associated with the striated ducts and form periductal foci (defined as clusters of more than ≥ 50 lymphocytes), sometimes displaying further architectural patterning

Competing interests: none declared.

comprising follicular dendritic cell networks and germinal centers. Lymphocytes also invade the striated ducts, in a symbiotic relationship that appears to stimulate proliferation of both epithelial cells and B cells (lymphoepithelial lesions, LELs (9)). Five to ten percent of SS patients develop mucosa-associated lymphoid tissue (MALT) lymphomas, the origin of which is proposed to be B cells resident in LELs in the parotid SG (5, 10).

The volume of saliva itself in pSS has been well established to be decreased, compared to non-SS sicca controls, although this does not appear to be related to the degree of lymphocytic infiltration (11-14). Protein content in saliva in pSS, for example α -amylase and mucins, appears to vary in composition (12). In terms of buffering, two studies suggested that pH values of saliva from pSS patients (5.78 ± 0.42) are lower than healthy controls (6.19 ± 0.55), suggesting abnormalities in bicarbonate buffering capabilities (15, 16).

What previous data has consistently suggested, however, is a noticeable change in electrolyte content in the saliva of SS patients. Across the board, levels of salivary sodium, measured in a panel of saliva types (unstimulated or stimulated, whole or gland specific) and compared against varying patient control groups (non-SS sicca patients, healthy individuals or those with connective tissue disease) are increased in the saliva of patients with SS (summarised in Table I (11, 12, 16-19)). This is in stark contrast to the other salivary electrolytes. Occasional significant differences between SS and respective control groups in concentrations of potassium, chloride and phosphate were detected, but not consistently across saliva types and studies (Table I (11, 12, 16-19)). Several studies have also utilised the concentrations of sodium detected in saliva to calculate a cut-off point, enabling discrimination between SS and non-SS. Asashima *et al.* for example set a threshold of sodium in unstimulated whole saliva to distinguish between SS and non-SS-connective tissue disease patients (17). Additionally, elevated levels of sodium in parotid SG specific saliva was found to be associ-

ated with SS patients with a focus score >2 and with presence of serum autoantibodies, by Pederson *et al.* (12). The merging of submandibular/sublingual salivary flow rates, and parotid gland saliva sodium and chloride levels allowed Kalk *et al.* to distinguish between SS and non-SS patients with a sensitivity of 0.85 and specificity of 0.96 (20). In summary, whilst there seems to be general agreement that sodium levels are consistently increased in the saliva of SS patients, its use as a clinical biomarker for pSS remains to be widely implemented. Any biological insight into the origin of this defect is still wanting, although considering the role of ductal cells in sodium resorption, a likely explanation may involve abnormalities in this SG cell subset in pSS (17). In order to attempt to find an explanation for the increased sodium levels in saliva of SS patients, we first validated previous findings of increased sodium levels in the saliva of SS patients. We examined sodium, potassium, chloride and phosphate concentrations in stimulated, unstimulated and parotid gland specific saliva. For patient groupings, we employ the most recent ACR-EULAR criteria to define SS patients. In keeping with our advancement in understanding of parotid SG pathology in pSS, we examine the correlation of salivary sodium levels with the patient-matched extent of CD45⁺, CD20⁺ and CD3⁺ cell infiltration, and LEL severity. We hypothesise that epithelial sodium channels, potentially internalised in the SG ductal epithelial cells due to local B-cell based inflammation, lay foundations for the increased sodium in saliva of SS patients.

Methods

Patients

Parotid gland biopsies obtained from consecutive patients with sicca complaints were taken during routine biopsy for pSS diagnosis work-up trajectory between 2013 and 2016. Institutional review board (IRB) approval was obtained (METc2013.066), and informed consent was provided by patients involved in this cohort. SS patients retrospectively fulfilled the 2016 ACR-EULAR classification criteria (n=47) (23).

Non-SS sicca patients experienced dry mouth but did not fulfil the ACR-EULAR criteria (n=65). The non-SS sicca group could be further divided into those patients with sicca complaints following medication use (n=8), as a consequence of metabolic disease (n=16) and from other causes (n=41). Other causes include tobacco or alcohol use, nerve damage or chronic sialadenitis from non-autoimmune origin, or partial fulfilment of ACR-EULAR criteria. The general characteristics of each patient group are outlined in Table II, including proportion of each group fulfilling components of the 2016 ACR-EULAR SS criteria.

Saliva collection

Unstimulated gland-specific saliva was collected over a five-minute period, stimulated saliva over a ten-minute period. Parotid gland saliva was obtained through the use of modified "Lashley cups" ("Carlson-Crittenden cups"). The submandibular/sublingual saliva is obtained from the floor of the mouth by aspiration with a syringe while simultaneously blocking the openings of the parotid glands with the Lashley cups. To obtain stimulation, a citric acid solution (2% w/v) was applied to the lateral edges of the tongue.

Determination of salivary sodium, potassium, phosphate and chloride

Flame photometry (for sodium and potassium determination), titration with silver ions (for chloride ion determination) and spectrophotometry (for phosphate and total protein content) were used (11). Amylase was measured by the method described by Pierre, Tung and Nadj (1976) (21). When sufficient saliva for a complete sialochemical analysis could not be obtained, preference was given to the values of Na⁺, K⁺ and amylase. Patients were asked to abstain from eating, drinking, smoking or using oral hygiene products (brushing teeth or using mouthwash) for at least one hour before collection of saliva.

CD45⁺ area and CD3⁺ / CD20⁺ cell number calculation

Parotid gland biopsies were formalin fixed, paraffin embedded and sectioned

Table I. Summary of studies on salivary ion concentrations.

Study	Ben-Aryeh 1981	Stuchell 1984	Sreebny 1996	Kalk 2001	Pederson 2005	Asashima 2013
<i>n</i> pSS group	22	15 [^]	26 ^{^^}	33	11 or 12	71
pSS criteria	NA	NA	NA	AECG 1996	AECG 2002	AECG 2002
Comparison groups	Non-SS sicca (<i>n</i> =14), healthy individuals (<i>n</i> =15)	Healthy individuals (<i>n</i> =12)	Patients with reduced or normal salivary flow (<i>n</i> =23)	Non-SS sicca (<i>n</i> =42), healthy controls (<i>n</i> =36)	Age-matched healthy controls (<i>n</i> =20)	Non-SS CTD (<i>n</i> =54), healthy individuals (<i>n</i> =75)
Saliva examined	UWS	SPS	UWS	SPS, SSS,	UWS, SWS, UPS, SPS	UWS
Saliva component and <i>p</i> values						
Sodium	<0.001 ^a	<0.01	<0.01	<0.05 ^a , <0.05 ^a .	0.008, 0.020, 0.002, 0.034.	<0.05 ^d , >0.05 ^e .
Chloride	ND	<0.01	ND	<0.05 ^b , <0.05 ^b .	0.003, 0.037, 0.588, 0.307	<0.05 ^d , >0.05 ^e .
Potassium	<0.02 ^a	ND	ND	>0.05, >0.05.	0.536, 0.533, 0.209, 0.689.	<0.05 ^d , >0.05 ^e .
Calcium	ND	ND	ND	>0.05, >0.05.	0.285, 0.588, 0.887. 0.008	ND.
Phosphate <i>p</i>	ND	<0.01	ND	>0.05, <0.05 ^c .	0.961, 0.606, 0.066, 0.577	ND
Other	-	-	-	ROC analysis sodium as SS predictor	SPS, UPS, sodium and chloride correlation with FS.	ROC analysis Sodium cut-off levels

UWS: unstimulated whole saliva; SWS(P): stimulated whole saliva (paraffin-chewing); UPS: unstimulated parotid saliva; SPS(CA): stimulated parotid saliva (citric acid); SSS: stimulated submandibular/sublingual gland saliva (citric acid).

p-values are presented respectively with order of saliva types examined.

FS: focus score. [^]with grade 4 lymphocytic infiltration in labial salivary glands. ^{^^}No official diagnosis. 'pSS' patients grouped based on low stimulated/unstimulated salivary flow, plus complaints of dry mouth and dry eyes.

^aSignificant comparing pSS to both non-SS and healthy individuals control groups. ^bSignificant comparing pSS to healthy individuals. ^cSignificant compared to non-SS individuals. ^dSignificant compared to non-SS-CTD and healthy individuals. ^eWhen comparing pSS to sSS.

LELs: lymphoepithelial lesions; ND: no data. For sake of clarity, results pertaining to sSS have been omitted from this table. CTD: connective tissue disease; ROC: receiver operation characteristic.

at 3 μm thickness. Slides were immunohistochemically stained for CD45, CD3 and CD20 using an automated staining platform (Benchmark XT, Ventana Medical Systems, Inc.), following the manufacturer's protocol, with anti-CD45, anti-CD3 and anti-CD20 antibodies as previously published (22). Relative area of CD45⁺ infiltrate compared to the total glandular area was calculated, and percentages of numbers of CD3⁺ and CD20⁺ cells compared to the total number of cells were evaluated, both using QuPath version 0.1.2.

Lymphoepithelial lesion severity score calculation

A lymphoepithelial lesion (LEL) is considered to be represented by hyperplastic epithelial cells of the striated ducts, in which lymphocytes are present (23). We have previously published a scoring system to allow grading of LELs, according to the following criteria and the publication of van

Table II. Clinical characteristics of patient groups.

	Non-SS sicca subgroups				
	SS	Non-SS sicca total	Metabolic	Medication	Other
<i>n</i>	47	65	16	8	41
Age (years)	53 (±14)	49 (±13)	47 (±11)	56 (±15)	48 (±13)
Females	45 (96)	55 (85)	11 (69)	7 (88)	28 (90)
Anti-SSA/Ro antibodies	36 (77)	3 (5)	0 (0)	1 (13)	1 (3)
Focus score >1	17 (36)	2 (3)	0 (0)	0 (0)	2 (5)
UWSF ≤0.1 mL/min	26 (55)	25 (39)	9 (56)	0 (0)	14 (45)
Schirmer's test ≤5mm/5min	39 (83)	35 (54)	11 (69)	3 (38)	17 (55)
OSS ≥5	20 (43)	7 (11)	1 (6)	0 (0)	6 (19)
ACR EULAR ≥4	47 (100)	0 (0)	0 (0)	0 (0)	0 (0)

Data are expressed as number (%), or mean (±SD).

UWSF: unstimulated whole saliva flow; OSS: ocular staining score.

Ginkel (9, 22, 24). Briefly, Stage 0 SDs contained lymphocytes but no epithelial hyperplasia. Stages 1-3 contained lymphocytic ductal infiltration, with <50% epithelial hyperplasia, 50-100% hyperplasia, and full ductal occlusion, respectively.

Immunohistochemical staining for ENaC

3 μm sections of parotid salivary gland

tissue from pSS or non-SS patients were dewaxed and rehydrated through a series of alcohols. Antigen retrieval was performed using 10mM Sodium Citrate buffer (pH 6.0), containing 0.5 % Tween detergent. Expression of the epithelial sodium transporter channel (ENaC) was detected using the ThermoFisher Multivision Polymer detection system kit, in combination with a rabbit-anti-human polyclonal, anti-

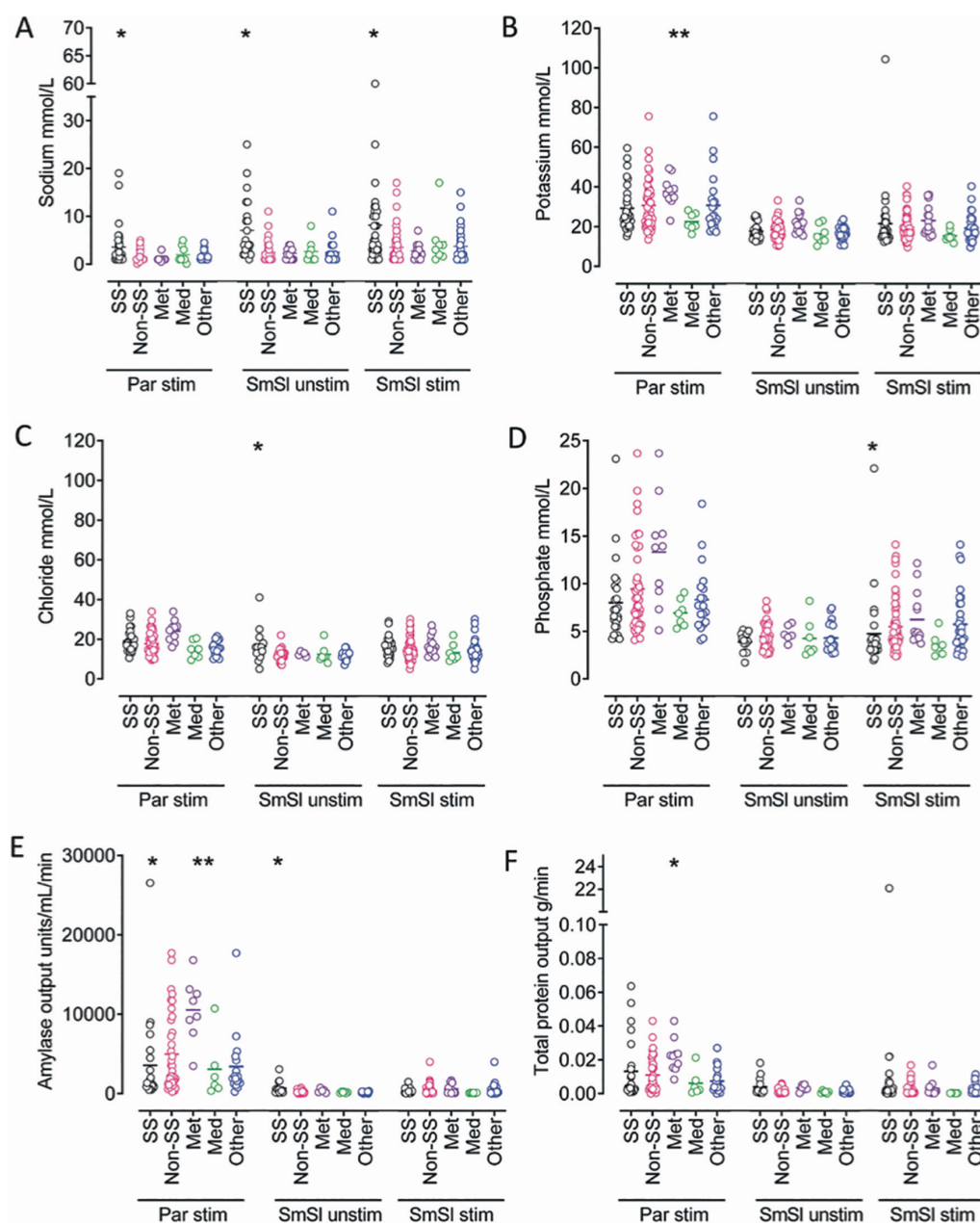
Fig. 1. Sodium concentration is increased in stimulated parotid saliva, stimulated submandibular/sublingual saliva and unstimulated submandibular/sublingual saliva in pSS patients compared to non-SS controls.

A: Sodium concentration in stimulated parotid saliva, unstimulated submandibular/sublingual saliva and stimulated submandibular/sublingual saliva, in SS, non-SS, patients with metabolic disease (Met), patients with medication induced hyposalivation (Med) and patients with other causes of hyposalivation (Other).

B: chloride, **C:** phosphate, **D:** potassium **E:** amylase output and **F:** total protein output in the same saliva types and patient groups as **A**. Each data point represents a separate patient.

* $p < 0.05$ between SS group and non-SS.

** $p < 0.001$ between metabolic disease and SS group. Bars represent mean.



ENaC antibody, at 1:100 dilution. Tissue sections were counterstained with haematoxylin, before mounting and visualising using the Olympus BX50 microscope.

Statistical analysis

All data was tested for normality before further statistical analysis using Shapiro-Wilk testing.

For testing for statistical differences between two groups, normally distributed data was analysed using independent sample t-tests. For all non-normally distributed data, correlations were analysed using Spearman's rho testing.

Results

Sodium concentration is consistently higher in saliva from SS patients compared to non-SS controls

Sodium concentration in stimulated parotid saliva, and both unstimulated and stimulated submandibular/sublingual saliva was significantly higher in SS patients than non-SS controls (2.5-fold, 2.9-fold and 2.3-fold higher means, respectively; Fig. 1A). Significant increases or decreases in chloride and phosphate concentrations were also detected in some saliva types between pSS patients and non-SS patients (Fig.

1B-C), although sodium remains the only ion significantly different in all three saliva types tested. No significant difference in potassium ion concentration across all patient groups or saliva types was observed (Fig. 1D). Examining the non-SS subgroups individually, a significant difference in potassium concentration was also detected between the metabolic disease group and SS group (Fig. 1D).

Amylase output, but not total protein output, was significantly reduced in stimulated parotid saliva from pSS patients compared to non-SS controls (Fig. 1E-F). In contrast, amylase output

was increased in stimulated submandibular saliva compared to controls (Fig. 1E). We also observed a significant increase in total protein and amylase output content between patients with metabolic diseases, compared to non-SS controls (Fig. 1F).

Parotid salivary sodium concentration in pSS patients correlates with degree of parotid gland lymphocytic infiltration, and most significantly with CD20⁺ B cells

Concentration of sodium in stimulated parotid saliva from pSS patients was significantly correlated with the relative area of CD45⁺ lymphocytic infiltration of matched parotid salivary gland biopsies ($r=0.688$, $p<0.001$; Fig. 2A). A similar association was found with the FS ($r=0.809$, $p<0.001$; data not shown). Stimulated parotid gland concentrations of chloride, potassium and phosphate ions, amylase output and total protein output did not correlate significantly with degree of CD45⁺ parotid gland infiltration (Fig. 2B-F), nor FS (data not shown). The amount of α -amylase in the parotid saliva of pSS patients does not correlate to the sodium of the gland (Fig. 2G).

Infiltration of the SGs of SS patients is predominantly composed of B cells, particularly in more advanced diseases stages (8). There was a significant correlation between percentage of CD20⁺ B cells (relative to all cells) in SS patient SGs, and the sodium concentration in stimulated parotid gland SG saliva ($r=0.73$, $p<0.0001$; Fig. 3A). This correlation although still significant, was much weaker with CD3⁺ T cells ($r=0.23$, $p=0.015$; Fig. 3B). Lymphoepithelial lesions (LELs) represent facets of SG biology in SS whereby lymphocytes, and specifically B cells, invade the striated ducts. Both B cells and epithelium are highly proliferative in LELs. Considering that the striated ducts are the sites of sodium resorption ordinarily, we probed the relationship between LELs and salivary sodium levels. The maximum parotid SG LEL severity score was significantly correlated with salivary sodium concentration, although less so than percentage

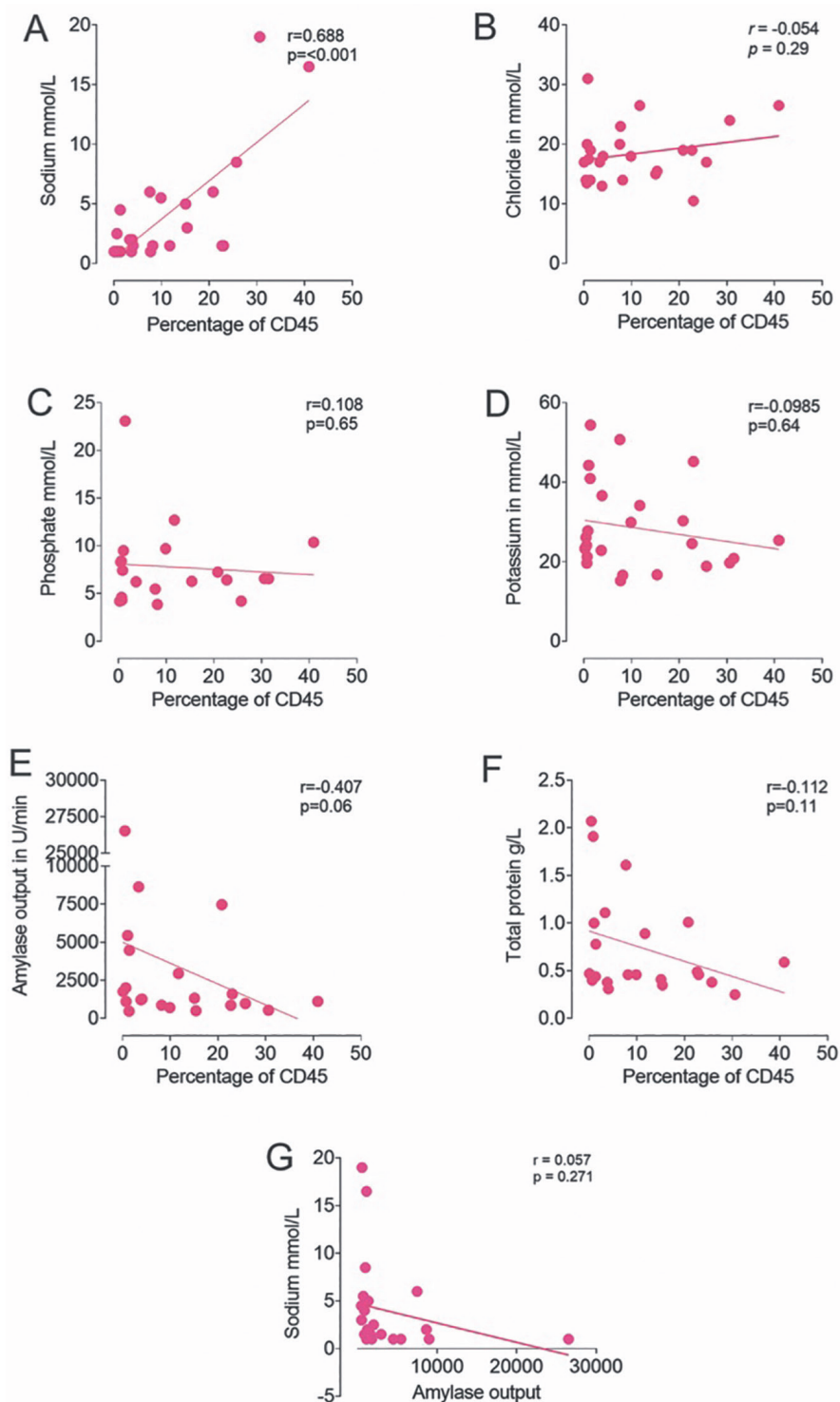


Fig. 2. Salivary sodium concentration is correlated with CD45⁺ infiltration degree in SS patient parotid salivary glands.

A: Correlation of sodium concentration with CD45⁺ cells as percentage of all parotid gland parenchyma (%percentage of CD45⁺). **B:** Correlation of chloride ion concentration with CD45⁺ percentage area. **C:** Correlation of phosphate ion concentration with CD45⁺ percentage area. **D:** Correlation of potassium ion concentration with CD45⁺ percentage area. **E:** Correlation of amylase output with CD45⁺ percentage area. **F:** Correlation of total protein output with CD45⁺ percentage area. Each data point represents a separate patient. **G:** Correlation of sodium concentration with amylase output.

area of CD20⁺ B cells ($r=0.46$, $p=0.02$; Fig. 3C).

Representation of six parameters of

the parotid saliva and salivary gland in SS patients together in the form of a heatmap, namely stimulated parotid

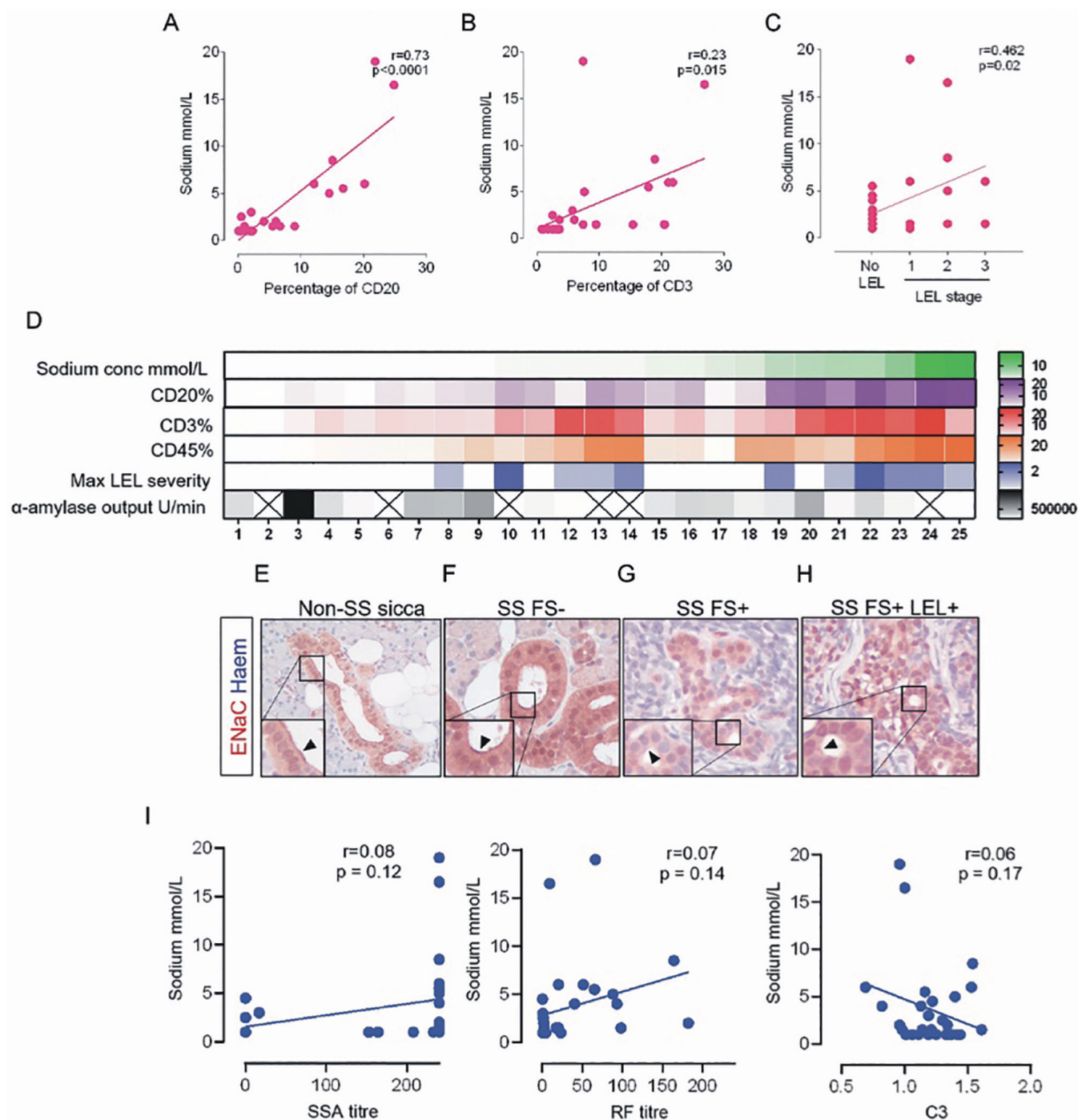


Fig. 3. Parotid salivary gland B cell infiltration is associated with increased salivary sodium levels.

A: Correlation of sodium in parotid gland specific saliva with CD20% of total cells. **B:** Correlation of sodium in parotid gland specific saliva with CD3% of total cells. **C:** Correlation of sodium in parotid gland specific saliva with maximum LEL severity score. **D:** Patient matched heatmaps of sodium concentration in stimulated parotid gland saliva (mmol/L), CD20% area, CD3% area, CD45% area, maximum LEL severity score and α -amylase output (U/min). Legends for each parameter are given to the right of each respective heatmap. **E-H:** Immunohistochemical staining for the epithelial sodium transporter channel ENaC. **E:** non-SS parotid gland tissue. **F:** SS tissue with a negative focus score. **G:** SS tissue with a positive focus score. **H:** SS tissue with a positive focus score and presence of a LEL. **I:** Correlation of parotid gland saliva with SSA and RF autoantibody titres, and serum levels of C3.

saliva sodium concentration, CD20%, CD3%, CD45%, maximum LEL severity score and α -amylase output, underscores the correlation between B-cells and elevated sodium levels (Fig. 3D-H).

Epithelial sodium channel protein expression in striated duct epithelial cells in close proximity to lymphocytic infiltration in pSS patient parotid salivary glands is absent
Sodium transport into striated ductal

epithelial cells, necessary in order to modulate the osmotic balance and flow of critical electrolytes, is facilitated by epithelial sodium channels (ENaCs). ENaCs are usually localised to the apical membrane of luminal epi-

thelial cells of the striated ducts in the salivary glands, as shown for a non-SS sicca patient (Fig. 3E). In tissue from a pSS patient with a focus score of 0, ENaC apical localisation was still observed in the apical membrane of the luminal striated duct cells (Fig. 3F). In striated ducts of a pSS patient with a positive focus score (*i.e.* FS \geq 1.0), however, ENaC presence at the apical membrane of the luminal striated duct cells seemed to be absent (Fig. 3G). The ductal cells still displayed weak immunopositivity, suggestive of cytoplasmic ENaC localisation (Fig. 3G). Ducts distant to infiltration displayed mostly normal ENaC expression (data not shown). A similar phenotype of loss of apical membrane expression of ENaC was also observed in the epithelial cells of a LEL (Fig. 3H).

Salivary sodium levels do not correlate significantly with systemic disease activity

No significant correlations were found between parotid gland saliva sodium levels and SSA or RF autoantibody titres, or levels of the complement C3 protein (Fig. 3I).

Discussion

Reduced saliva production is one of the first symptoms leading clinicians towards a suspicion of SS, often triggering the arrangement of biopsies and the examination of the SGs for presence of lymphocytic infiltration. Alongside reduced saliva production, our data and work of others has also demonstrated that salivary sodium levels are significantly increased in SS patient saliva, compared to a variety of control groups (11, 12, 16-19). Salivary changes in other electrolytes were more variable, as shown previously, and confirmed in this study (11, 12, 16-19). As a logical extension of this work, several groups attempted to use salivary sodium levels as an easy read out for SS. Pedersen *et al.* generated a multi-factorial matrix via the combination of reduced unstimulated and stimulated parotid saliva flow data, and parotid sodium levels, the combination of which was more likely to be found in SS patients with minor SG focus scores of at least 2, and

presence of serum autoantibodies (12). Although our study showed robust significant differences between the SS group and non-SS at the group level across three saliva types (*i.e.* consistently increase in sodium concentration in SS patients), the standard deviation within each group and generally lower sodium concentrations detected makes deriving a reliable cut-off value challenging. Other inflammatory diseases of the salivary glands, for example juvenile parotitis, sialadenitis, and inflammation following radiation therapy have all been suggested to show increased salivary sodium values (25). We presume these two reasons combined explain why sodium thresholds do not appear to have been implicated into daily clinical practice by authors who did determine cut-off value to distinguish SS broadly from non-SS. We suggest that salivary sodium values may be the most useful in combination with other clinical parameters suggestive of SS, for example SSA autoantibody positivity, and reduced saliva and tear production, for the distinguishing of SS from non-SS patients.

To obtain more insight into this rise in sodium levels in SS patients, we examined histopathological parameters of the parotid glands in SS. Our data is the first study to specifically correlate parotid salivary sodium levels with the amount of infiltrate, and more specifically with B cells, in the parotid SG. Bearing in mind this correlation, it is feasible that parotid gland saliva sodium levels could be employed as a surrogate for salivary gland biopsy. Reflection of B cell predominance as inferred from sodium levels in SS patients may also be clinically useful in terms of predicting likelihood of MALT lymphoma development in the parotid SG, from which 5-10% of SS patients suffer.

Sodium is secreted initially by acinar cells, in conjunction with water, and subsequently resorbed by ductal cells. In principle there are thus at least two non-mutually exclusive feasible explanations for this alteration in SS: either an increased secretion or reduced resorption. Increased sodium secretion by acinar cells in SS may still be re-

sponsible for the increased sodium levels we observe in SS saliva. This is not something we have addressed in this study. Resorption of sodium by ENaC channels expressed on luminal ductal cells however, we have probed. ENaC is also expressed at other sites in the body, such as bronchial epithelial cells and intestines, where several studies have been performed regarding their role in inflammatory diseases (26-29). Studies in the context of lungs examining sodium resorption from the alveolar and airway lumens through their respective epithelial cells into the interstitium, in pathologies such as asthma, acute respiratory distress syndrome (ARDS) and acute lung injury, may provide a hint to a potential mechanism in SS (26). One of the key cytokines implicated in ARDS, TGF β has been demonstrated to downregulate ENaC expression at mRNA and protein level in primary cultures of rat and human alveolar epithelial cells, and also to induce its internalisation from the luminal cell surface (27). Within the context of acute lung injury, TGF β has similarly been reported to decrease expression of the alpha subunit of ENaC expression at mRNA and protein levels in human alveolar type II cells, an effect mediated by activation of the MAPK and ERK1/2 pathways(27). IL-1 β would seem similarly to reduce ENaC expression in acute lung injury, mediated by the activation of p38 MAPK and independent of TGF β (28). Incubation of polarised human bronchial epithelial cells with IL-4, a cytokine associated with asthma, also decreased activity of sodium channels, an effect also mimicked by IL-13, and conversely blocked by an antagonist of IL4Ra (30). In *in vitro* cell culture of epithelial cells from the colon, application of IFN γ and TNF- α was demonstrated to reduce ENaC mRNA levels (29). These data collectively, and the fact that other inflammatory situations of the SG also induce an increase in salivary sodium levels, strongly suggest that cytokines influence the expression and localisation of ENaC in various tissues and conditions (25).

With regards to SS, the potential complexity of IFN γ and TNF- α in reduced

ENaC expression is interesting, considering that both are overexpressed in the SGs of patients with SS. Our data show a strong positive correlation between the proportion of CD20⁺ B cells in the SG in SS patients, and the level of sodium in the saliva. Expression of ENaC appeared to be aberrant in FS+ SS patients both with and without lymphoepithelial lesions (LELs), implying that a soluble factor secreted by B cells is a likely candidate for dysregulation of ENaC expression, and not *per se* the physical invasion of the striated ducts by B cells, or the epithelial hyperplasia. The maximum LEL severity score was also correlated with sodium concentration, a likely surrogate readout for the number of B cells close to the ducts, considering that more severe LELs are associated with more intraepithelial B cells (9). The potential involvement of B cells in sodium dysbalance is also supported by the observation that striated ducts distal to inflammatory foci do not display dysregulated ENaCs. Potential candidate cytokines secreted by B cells and already linked to ENaC dysregulation include IFN γ and TNF- α , although a role for other cytokines highly expressed in SS, including IL-6, cannot be excluded.

The extent of lymphocytic infiltration of the SGs in SS is not well correlated with salivary flow (11-14). The correlation of salivary sodium levels with the level of infiltrate, and in particular CD20⁺ B cells, associated with later SS SG pathogenesis and not earlier events, suggests that dysbalanced saliva electrolytes represents a later phenotype (31). Reduction in saliva production in SS patients, is likely to be the consequence of a separate mechanism, appearing earlier in the chronological timeline

Translating this back to the clinic, the measurement of salivary sodium levels may represent a proxy parameter, together with additional clinical readouts such as autoantibody titre, for distinguishing SS patients from non-SS. Indeed, salivary sodium levels do not correlate with autoantibody titres, suggesting that the two measures may need to be used in tandem. Salivary sodium levels are also likely to reflect extent

of B cell invasion of the parotid salivary glands in SS patients. Collection of whole unstimulated saliva, as a replacement for biopsies, is an extremely attractive prospect in terms of technical ease, and therefore costs and feasibility. Although not allowing examination of the architecture of the salivary glands on a histological level, sialochemistry may allow indirect assessment of major salivary gland health, and optimise the diagnostic workflow of peripheral rheumatology centres, with regards to SS.

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