

DMBT1, S100A7 and SPINK4 in Relation to Dental Caries Activity and Salivary Flow Rate in Adults

A Case-Control Study

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Abstract

Saliva delivers a network of innate defense proteins to the tooth surface, but several recently characterized salivary proteins have not been examined in relation to caries activity. This study assessed whether unstimulated salivary deleted in malignant brain tumors 1 (DMBT1), psoriasin (S100A7) and serine peptidase inhibitor Kazal type 4 (SPINK4) differ according to caries activity in adults and how they relate to salivary flow rate and pH. In this case-control study, 88 adults aged 18–35 years were allocated to caries-free ($n = 28$), low or inactive caries ($n = 30$) and high active caries ($n = 30$) groups based on caries experience, lesion severity and clinical lesion activity. Unstimulated whole saliva was collected; flow rate, pH and total protein were recorded, and the three proteins were measured by enzyme-linked immunosorbent assay and expressed as raw concentration, per milligram of total protein and as output per minute. Flow rate and pH declined across the groups, whereas total protein rose. DMBT1 and S100A7 concentrations increased with caries activity and correlated positively with caries experience and active lesion count and negatively with flow rate. After normalization to total protein, the DMBT1 difference disappeared, whereas S100A7 retained a weaker ordered trend. SPINK4 concentration did not differ, although its protein-normalized level and output were lower in the caries groups. S100A7 best discriminated high active caries from caries-free status (area under the curve 0.854) and remained associated with this group after adjustment for age, sex, plaque and sugar intake (odds ratio 1.60 per ng/mL), including in penalized models also adjusted for flow rate. The salivary innate response to active caries appears protein specific. S100A7 is a candidate activity marker warranting prospective validation, raw DMBT1 largely reflects reduced salivary volume, and the lower SPINK4 values are exploratory because they depend on flow- and protein-based derivations.

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Introduction

Dental caries remains the most prevalent chronic disease of humankind, with untreated

lesions in permanent teeth affecting roughly one third of the world's population [1]. The disease is now understood as a dysbiotic, sugar-

driven process in which the balance between demineralization and remineralization at the tooth surface is governed by the composition

of the biofilm and by host factors, foremost among them saliva [2]. Saliva buffers plaque acids, clears fermentable substrate, supplies calcium and phosphate, and coats the enamel with a pellicle whose protein constituents both mediate and restrict bacterial colonization [3]. A substantial part of this protective capacity resides in a network of innate defense proteins, including lysozyme, lactoferrin, peroxidases, histatins, defensins and the cathelicidin LL-37, which act by direct microbial killing, aggregation, iron sequestration and immunomodulation [4].

The relationship between these proteins and caries activity is far from uniform. Studies in Iraqi populations have reported lower salivary LL-37 in vitamin D-deficient women with higher caries experience, and an inverse correlation between LL-37 and the number of decayed teeth [5], whereas beta-defensin 2 tended to rise with caries in pregnant women [6], and both lactoperoxidase and lactoferrin were markedly elevated in preschool children with severe early childhood caries [7]. Elevated secretory IgA, histatin-5 and lactoperoxidase have likewise been described in adolescents with high caries intensity [8]. These divergent patterns suggest that some salivary defense molecules fall as protection is lost, while others increase as a compensatory or inflammatory response to an active lesion burden. Distinguishing between these two behaviors is important if salivary proteins are to be used as indicators of disease activity rather than merely of exposure.

Three proteins with well-defined innate functions outside the mouth have received little attention in cariology. Deleted in malignant brain tumors 1 (DMBT1), identical to salivary agglutinin and lung gp-340, is a scavenger receptor cysteine-rich glycoprotein that binds and agglutinates *Streptococcus mutans* and many other bacteria [9,10]. Paradoxically, when adsorbed to the enamel pellicle it also serves as an adhesion receptor, and a size variant of the protein has been associated with higher caries experience in children [11]. Psoriasin (S100A7) is a zinc-sequestering S100 protein first identified as the principal *Escherichia coli*-killing factor of human skin [12] and later shown to be the dominant antimicrobial protein of the healthy human tongue, secreted onto the oral mucosal surface [13]; its expression in oral epithelium rises with inflammatory severity [14]. Serine peptidase inhibitor Kazal type 4 (SPINK4) is a small, secreted protease inhibitor that supports the intestinal mucus barrier, is induced by microbiota-derived lipoproteins and changes in parallel with intestinal inflammatory activity [15]; it is abundantly expressed by goblet cells [16]. Although DMBT1 and S100A7 are established components of oral innate

defense [10,13] and SPINK4 belongs to a family of mucosal protease inhibitors, the salivary concentrations of these proteins in relation to caries have not, to our knowledge, been reported.

The present study therefore measured DMBT1, S100A7 and SPINK4 in unstimulated whole saliva from adults stratified by caries activity, and related these proteins to conventional caries indices, salivary flow rate and pH. Because salivary protein concentrations are strongly influenced by flow, each protein was also expressed relative to total protein and as secretion output per minute. The aim was to determine whether any of the three proteins tracks caries activity independently of salivary volume and could serve as a candidate biomarker. We hypothesized that proteins induced by the lesion-associated host response would rise with caries activity independently of salivary flow, whereas proteins that depend on glandular or mucosal secretory capacity would fall. This aim was largely achieved: S100A7 tracked caries activity independently of salivary flow rate, DMBT1 largely reflected salivary volume, and the apparent reduction in SPINK4 depended on how the protein was expressed.

Material and Methods

Study design and ethical approval

This case-control study was conducted between 1 November 2025 and 1 April 2026 at the outpatient dental clinics of Samarra, Salah ad Din Governorate, Iraq, and is reported in accordance with the STROBE recommendations for observational studies [17]. The protocol was approved by the Research Ethics Committee of the College of Education for Pure Sciences, University of Samarra (approval no. EDUSamarraH01 Bio-9, dated 1 October 2025), and the investigation was conducted in accordance with the Declaration of Helsinki. The purpose, procedures and non-invasive nature of the study were explained to every participant, and written informed consent was obtained before enrolment. Each participant received a serial code that was used on all forms and sample tubes to preserve confidentiality.

Participants

Adults of both sexes aged 18–35 years attending the clinics were screened consecutively, and eligible individuals were enrolled into each group until the target number was reached, with frequency matching by sex to minimize potential confounding, so that men and women were equally represented within each group (14 men and 14 women in Group 1; 15 men and 15 women in each of Groups 2 and 3). Of 118 individuals screened, 30 were excluded because they did not meet the inclusion criteria or met one or more of the pre-specified exclusion criteria, and their data were not

analyzed; 88 participants (74.6%) were included. Eligible participants had at least 20 natural teeth, were able to provide an adequate saliva sample, and could be unequivocally classified into one of the three study groups. Exclusion criteria were severe gingivitis or periodontitis, any form of tobacco use, pregnancy or lactation, fixed or removable orthodontic appliances or removable prostheses, use of xerogenic medication (antihistamines, antidepressants, diuretics), systemic antibiotics or anti-inflammatory drugs within the preceding three months, professional fluoride application within the preceding three months, antiseptic mouthwash within the preceding two weeks, systemic disease known to affect salivary secretion or composition (diabetes mellitus, autoimmune disease, Sjögren syndrome), and any history of head and neck radiotherapy. Participants were allocated to three groups based on the clinical examination described below. Group 1 (caries-free, $n = 28$) comprised individuals with DMFT = 0 and no active lesion. Group 2 (low or inactive caries, $n = 30$) comprised individuals with DMFT 1–4 whose lesions were all classified as inactive (arrested) according to the Nyvad criteria described below. Group 3 (high active caries, $n = 30$) comprised individuals with DMFT ≥ 5 and at least two active lesions scored 3–6 on the International Caries Detection and Assessment System (ICDAS). Individuals whose clinical profile did not meet any group definition, such as DMFT 1–4 with active lesions or DMFT ≥ 5 without active lesions, were not enrolled and are counted among the 30 excluded individuals described above. No formal a priori sample size calculation was performed; the sample reflects the number of eligible participants enrolled during the study period. A sensitivity calculation for a three-group comparison (two-sided $\alpha = 0.05$, power 0.80, $N = 88$) showed that the study could detect effects of $f \geq 0.34$, which lie between medium and large, so smaller differences may have gone undetected.

Data collection

A structured form recorded age, sex, educational level, residence, daily tooth-brushing frequency, use of fluoride toothpaste, daily frequency of between-meal sugar intake and time since the last dental visit, together with the clinical and laboratory variables listed below.

Saliva collection, flow rate and pH

Unstimulated whole saliva was collected between 09:00 and 11:00 to limit circadian variation, following the protocol of Navazesh and Kumar [18] as applied in previous Iraqi salivary studies [19,20]. Participants refrained from eating, drinking and tooth-brushing for at least 90 minutes beforehand. Seated comfortably with the head tilted slightly forward, and instructed not to chew, swallow or speak, each

participant allowed saliva to pool in the mouth and drain passively into a sterile graduated tube for exactly five minutes. Flow rate (mL/min) was calculated as the collected volume divided by five. Salivary pH was measured immediately after collection with a digital pH meter: the electrode was immersed in the sample for approximately 30 seconds until the reading stabilized, and the value was recorded without delay to avoid post-collection drift. The meter was calibrated daily before use with standard buffer solutions of pH 4.01 and 7.01 according to the manufacturer's instructions. Saliva was collected before the dental examination to prevent contamination with blood or gingival fluid, and any sample with visible blood was discarded.

Clinical examination

The oral examination was performed after saliva collection, as specified in the study protocol, to avoid contamination of the sample with blood from gingival probing. A single calibrated examiner used a plane mouth mirror and a WHO periodontal probe under adequate clinical illumination, following standard infection-control procedures. Caries experience was recorded as DMFT and DMFS according to WHO criteria [21]. Lesion severity was scored with ICDAS, and the highest ICDAS code and the number of lesions in the codes relevant to the group definitions were recorded for each participant [22]. Lesion activity was assessed with the Nyvad criteria [23], which classify lesions as active or inactive (arrested) based on surface appearance, tactile texture and location relative to plaque-stagnation areas; radiographs were not taken. Oral hygiene and gingival health were assessed with the plaque index and gingival index of Löe and Silness [24,25], and these indices were recorded for use as potential confounders together with age, sex and sugar intake frequency. Before data collection, the examiner was calibrated in caries diagnosis and lesion-activity assessment, with a pre-specified minimum Cohen's kappa of 0.80 for intra-examiner agreement.

Sample processing

Coded tubes were placed immediately in an insulated box with ice packs and transported to the laboratory. Within two hours of collection, samples were centrifuged at 3,000 rpm for 15 minutes to remove cells and debris. The clear supernatant was divided into three Eppendorf aliquots and stored at -20°C until analysis. Each aliquot was thawed only once, and repeated freeze-thaw cycles were avoided.

Quantification of DMBT1, S100A7 and SPINK4

Salivary DMBT1, S100A7 (psoriasin) and SPINK4 were quantified with commercially available sandwich enzyme-linked immunosorbent assay (ELISA) kits (SunLong Biotech Co., Ltd., Hangzhou, Zhejiang, China; catalogue

numbers SL5148Hu, SL4697Hu and SL5135Hu, respectively) according to the manufacturer's instructions. According to the manufacturer, the S100A7 kit (SL4697Hu) has a measurement range of 21.2–1,300 pg/mL and a sensitivity of 4.2 pg/mL, the SPINK4 kit (SL5135Hu) has a range of 0.1–40 ng/mL and a sensitivity of 0.01 ng/mL, and the DMBT1 kit (SL5148Hu) has a range of 31.2–2,000 pg/mL and a sensitivity of 6.1 pg/mL. The manufacturer reports intra- and inter-assay coefficients of variation below 10% and 12%, respectively, for all three kits. For all three assays, 10 μL of saliva was added to 40 μL of sample diluent, giving a baseline dilution of 1:5. Standards and diluted samples were added to antibody-coated wells, followed by the enzyme conjugate, with the prescribed incubation and washing steps; chromogenic substrate was then added, the reaction was stopped, and absorbance was read at 450 nm on a microplate reader. Concentrations were interpolated from the standard curve and multiplied by the total dilution factor. Samples whose readings exceeded the highest standard were further diluted and re-assayed, and the result was multiplied by the corresponding total dilution factor. For S100A7, the upper limit of the standard curve (1,300 pg/mL, i.e. 1.3 ng/mL) at the baseline 1:5 dilution corresponds to approximately 6.5 ng/mL in undiluted saliva, so samples above this level were further diluted to a total dilution of 1:10 to 1:20 before re-assay, extending the measurable range to approximately 13–26 ng/mL. For DMBT1, samples exceeding the highest standard (2,000 pg/mL, i.e. 2.0 ng/mL, corresponding to approximately 10 ng/mL in undiluted saliva at the 1:5 dilution) required additional dilution before re-assay according to the manufacturer's instructions; however, the final dilution factor for each sample was not fully available in the run records (see Limitations). All S100A7 concentrations are expressed in ng/mL. Because published data on salivary SPINK4 were lacking, a pilot run on six to eight samples was performed before the main assays to confirm that all three proteins were detectable in saliva, to select the appropriate sample dilution and to verify that sample values fell within the range of the standard curve. All standards and samples were assayed in duplicate, and the mean of the two readings was used in the analysis. Duplicate readings were accepted when their agreement fell within the limits specified by the manufacturer; samples with unacceptable disagreement or with readings outside the standard curve were re-assayed. All samples were labelled with serial codes that did not reveal the participant's identity or clinical group, and the ELISA runs and reading of the results were performed without knowledge of group allocation; the group codes were linked to the

laboratory results only after all measurements had been completed and the results finalized.

Total protein and derived indices

Total salivary protein was measured by the Bradford dye-binding method with bovine serum albumin as standard [26]. Each protein was expressed in three ways: as the raw ELISA concentration (ng/mL); as the concentration normalized to total protein (ng per mg of total protein), to reduce the influence of inter-individual differences in saliva concentration; and as secretion output (ng/min), calculated as concentration multiplied by flow rate, which represents the quantity of protein actually delivered to the mouth per minute.

Statistical analysis

Data were entered into IBM SPSS Statistics version 31 (IBM Corp., Armonk, NY, USA), which was used for all analyses including the Firth penalized regression models, and were checked for missing values and entry errors. Normality was assessed with the Shapiro–Wilk test. Normally distributed variables are presented as mean $\pm$ standard deviation and were compared across the three groups with one-way analysis of variance followed by Tukey's post hoc test; non-normal and ordinal variables are presented as median (interquartile range) and were compared with the Kruskal–Wallis test followed by Dunn's post hoc test with Holm correction. Categorical variables were compared with the chi-square test. The Jonckheere–Terpstra test was used to examine graded changes across the ordered groups. Because nine biomarker variables were compared, their P values were also interpreted against a Bonferroni-adjusted threshold of 0.0056 and results significant only at the nominal level are treated as exploratory. Spearman's rank correlation described the relationships between each protein and DMFT, number of active lesions, salivary flow rate and pH. Because ratio and product variables are mathematically coupled to their denominators and multipliers [27], conclusions were not based on the derived indices alone: the influence of salivary volume was examined by adding flow rate to the regression models, and the derived indices were analyzed in separate sensitivity models. Receiver operating characteristic (ROC) analysis assessed the ability of each protein to discriminate Group 3 from Group 1; the area under the curve (AUC) with a bootstrap 95% confidence interval (1,000 resamples) was reported, and the optimal cut-off was identified with the Youden index. Multivariable logistic regression, with each protein entered in a separate model adjusted for age, sex, plaque index and sugar intake frequency, estimated the odds ratio for high active caries per unit increase in the protein. Because these models contained 30 events and five

predictors, below the commonly recommended ten events per variable [28], they were repeated with Firth's penalized likelihood [29] with the same covariates (Model 1) and with salivary flow rate added as a further covariate (Model 2). In a final sensitivity analysis, the protein-normalized and output values of each protein were entered in place of the raw concentration in Firth models with the Model 1 covariates. Statistical significance was set at $P < 0.05$.

Results

Characteristics of the study groups

Of 118 individuals screened, 30 (25.4%) were excluded because they did not meet the inclusion criteria, met one or more exclusion criteria, or had an intermediate clinical profile that fitted none of the group definitions, and 88 (74.6%) were enrolled: 28 in Group 1, 30 in Group 2 and 30 in Group 3. The groups were comparable in age and, by design, identical in sex distribution (Table 1). Behavioral risk indicators differed as expected: participants with high active caries brushed less frequently and consumed sugar between meals more often than the other two groups, and plaque index rose stepwise across the groups ($P = 0.006$); ordered trends were significant for tooth-brushing frequency (Jonckheere–Terpstra $P = 0.005$) and sugar intake frequency ($P = 0.002$). Gingival index did not differ significantly, consistent with the exclusion of participants with marked periodontal inflammation. Educational level, residence, use of fluoride toothpaste and time since the last dental visit were recorded to describe the sample and did not differ significantly between the groups (data not shown).

Caries indices and physical properties of saliva

Caries experience separated the groups as intended, with median DMFT of 0, 3 and 7 and median DMFS of 0, 4 and 15 in Groups 1, 2 and 3 respectively; active lesions were confined to Group 3, with a median of four per participant (Table 2). Unstimulated salivary flow rate fell from a median of 0.47 mL/min in caries-free participants to 0.41 mL/min in the low-caries group and 0.36 mL/min in the high active caries group ($P = 0.003$), and salivary pH declined in parallel from 7.07 ± 0.23 to 6.79 ± 0.24 and 6.74 ± 0.29 ($P < 0.001$). In contrast, total salivary protein rose across the groups, from 1.09 ± 0.27 mg/mL to 1.25 ± 0.21 and 1.37 ± 0.42 mg/mL ($P = 0.005$), a pattern consistent with concentration of the secretion as its volume decreased.

Salivary DMBT1, S100A7 and SPINK4

Raw concentrations of DMBT1 and S100A7 increased progressively with caries activity (Table 3). Median DMBT1 rose from 37.6 ng/mL in Group 1 to 42.8 ng/mL in Group 2 and 48.4 ng/mL in Group 3 ($P = 0.009$), and median

S100A7 from 7.8 to 9.9 and 11.5 ng/mL ($P < 0.001$). SPINK4 concentration did not differ between the groups ($P = 0.247$). The Jonckheere–Terpstra test confirmed ordered increases for DMBT1 ($P = 0.002$) and S100A7 ($P < 0.001$) but not for SPINK4 ($P = 0.232$). In pairwise comparisons, S100A7 was higher in both caries groups than in caries-free participants, whereas DMBT1 differed only between the caries-free and high active caries groups (Table 3).

Normalization altered this picture in an informative way. Once expressed per milligram of total protein, DMBT1 no longer differed between the groups ($P = 0.398$; trend $P = 0.241$), and its secretion output per minute was likewise similar ($P = 0.293$; trend $P = 0.608$), indicating that the rise in raw DMBT1 concentration was largely attributable to the reduced salivary volume in the caries groups. S100A7 showed a weaker gradient after normalization to total protein (medians 6.9, 8.3 and 9.4 ng/mg; $P = 0.073$) and in output per minute ($P = 0.099$); neither difference was significant overall or in pairwise comparisons, but the ordered trend was significant for both ($P = 0.020$ and $P = 0.032$). SPINK4 showed a different pattern: its raw concentration was unchanged, but its protein-normalized level (medians 1.71, 1.28 and 1.07 ng/mg; $P = 0.031$; trend $P = 0.024$) and its secretion output (0.92, 0.51 and 0.62 ng/min; $P = 0.01$; trend $P = 0.011$) were lower in the two caries groups than in caries-free participants. In pairwise comparisons, SPINK4 output was lower in both caries groups than in the caries-free group, whereas no pairwise difference in the normalized value remained significant after Holm correction. The output did not decline monotonically, being lowest in the low or inactive caries group, and because the raw concentration did not differ, these differences largely mirror the lower flow rate and higher total protein of the caries groups. Of the nine Kruskal–Wallis biomarker comparisons, only raw S100A7 remained significant at the Bonferroni-adjusted threshold, so the SPINK4 differences are regarded as exploratory.

Correlations with caries indices and salivary properties

Across the whole sample, DMBT1 and S100A7 correlated positively with DMFT ($r = 0.353$ and 0.474 respectively, both $P < 0.001$) and with the number of active lesions ($r = 0.277$, $P = 0.009$ and $r = 0.371$, $P < 0.001$), and negatively with salivary flow rate ($r = -0.306$, $P = 0.004$ and $r = -0.313$, $P = 0.003$) (Table 4). S100A7 additionally correlated negatively with salivary pH ($r = -0.315$, $P = 0.003$), whereas DMBT1 did not. SPINK4 was unrelated to the caries indices or pH but correlated positively with flow rate ($r = 0.272$, $P = 0.01$).

Discriminatory performance and adjusted associations

In ROC analysis comparing high active caries with caries-free status (Table 5), S100A7 achieved an AUC of 0.854 (95% CI 0.742–0.94); at a cut-off of ≥ 10.2 ng/mL its sensitivity was 70% and specificity 89.3%. DMBT1 gave an AUC of 0.73 (95% CI 0.578–0.856) with 70.0% sensitivity and 71.4% specificity at ≥ 42.40 ng/mL, while SPINK4 showed only weak discrimination (AUC 0.607, 95% CI 0.509–0.746).

In conventional maximum-likelihood models adjusted for age, sex, plaque index and sugar intake frequency (Table 6), S100A7 remained independently associated with high active caries, with an adjusted odds ratio of 1.596 per ng/mL (95% CI 1.148–2.218, $P = 0.005$). DMBT1 was not independently associated (OR 1.029, 95% CI 0.99–1.071, $P = 0.149$). SPINK4 showed an inverse association, each 1 ng/mL increase being associated with a 57% reduction in the odds of high active caries (OR 0.431, 95% CI 0.187–0.994, $P = 0.048$).

Sensitivity analyses

With Firth's penalized likelihood (Table 7), S100A7 remained associated with high active caries (OR 1.455 per ng/mL, 95% CI 1.106–1.914, $P = 0.007$), and the association persisted after additional adjustment for salivary flow rate (OR 1.382, 95% CI 1.047–1.826, $P = 0.023$). DMBT1 was not associated with high active caries in either model. The inverse association of SPINK4 was attenuated and no longer significant (Model 1: OR 0.502, 95% CI 0.232–1.083, $P = 0.079$; Model 2: OR 0.553, 95% CI 0.231–1.326, $P = 0.184$). When the derived expressions were substituted for the raw concentrations (Table 8), neither the protein-normalized nor the output values of DMBT1 or S100A7 were associated with high active caries, whereas protein-normalized SPINK4 (OR 0.324, 95% CI 0.132–0.793, $P = 0.014$) and SPINK4 output (OR 0.097, 95% CI 0.015–0.628, $P = 0.014$) were inversely associated.

Discussion

To our knowledge, this is the first report of salivary DMBT1, S100A7 and SPINK4 in relation to caries activity in adults. Three findings stand out. First, S100A7 rose with caries activity, correlated with lesion burden, retained its association after adjustment for behavioral confounders and salivary flow rate, and discriminated active from caries-free individuals with good accuracy. Second, DMBT1 appeared elevated in caries only as a raw concentration; once salivary volume was accounted for, the difference disappeared. Third, SPINK4 concentration was unchanged, and its lower normalized and output values in caries, together with an inverse association that did not withstand

penalized estimation, should be regarded as exploratory. Together these results show that the innate salivary response to caries is protein-specific and that the way a salivary protein is expressed, as concentration, as a fraction of total protein or as output per minute, can change its apparent relationship with disease. The consistent decrease in unstimulated flow rate and pH with increasing caries activity, accompanied by a rise in total protein, is in keeping with the established role of salivary volume in the defense of the tooth surface [3]. Reduced flow prolongs sugar clearance and weakens buffering, and it also concentrates proteins whose secretion is not volume dependent. This last effect is the most plausible explanation for the DMBT1 findings. DMBT1 is secreted predominantly by the serous cells of the submandibular and minor glands and by ductal epithelium [10], and its output per minute did not differ between the groups. The higher raw concentration in caries therefore reflects less dilution rather than increased production. The absolute DMBT1 values measured here (medians 38–48 ng/mL) are lower than might be expected for a major constituent of salivary agglutinin, which may reflect the epitope recognized by the kit antibodies, the incompletely documented final dilution factor or incomplete recovery of this large, heavily glycosylated and self-aggregating protein after centrifugation; relative differences between the groups are therefore more informative than absolute concentrations. Whether this concentration effect has biological consequences is uncertain: DMBT1 in solution agglutinates *S. mutans* and promotes its clearance, but DMBT1 adsorbed to the pellicle promotes adhesion of the same organism [9,11], so an altered ratio of fluid-phase to surface-bound protein in a low-flow mouth could conceivably favor colonization. The positive correlation between DMBT1 and DMFT observed here is consistent with the association of the gp-340 size variant I with higher caries experience in Swedish children [11], although we did not genotype or size-fractionate the protein.

S100A7 behaved differently. Its raw concentration rose stepwise, and although normalization to total protein attenuated the gradient, which remained significant only in the ordered trend test, the protein remained associated with active caries in penalized models, including after adjustment for salivary flow rate, and gave the highest AUC of the three markers. However, when S100A7 was expressed per milligram of total protein or as output, its association with active caries was no longer significant, so part of the raw-concentration signal is shared with the overall protein concentration of saliva; confirmation in larger samples is needed before S100A7 can be regarded as independent

of salivary composition. S100A7 is a product of stratified oral epithelium rather than of the salivary glands, is secreted onto the mucosal surface [13], and is up regulated by inflammatory cytokines, by bacterial contact and by breach of the epithelial barrier [12,14]. An active carious lesion, with its associated plaque accumulation, acidic microenvironment and low-grade mucosal irritation, is a plausible stimulus for such induction. The negative correlation between S100A7 and salivary pH is consistent with this interpretation. Because gingival index did not differ between the groups and participants with marked periodontal inflammation were excluded, gingival inflammation alone is unlikely to explain the S100A7 gradient, although plaque-associated mucosal irritation cannot be excluded. In this respect S100A7 resembles lactoperoxidase, lactoferrin, histatin-5 and secretory IgA, which have been found elevated in caries-active children and adolescents [7,8], and beta-defensin 2, which tended to rise with caries in pregnant women [6]; these proteins appear to increase as a host response to the lesion burden. It contrasts with LL-37, which was lower in caries-active Iraqi women and correlated inversely with decayed teeth [5, 30], and which has been reported both higher and lower in early childhood caries [31]. *S. mutans* strains from caries-active individuals are more resistant to host antimicrobial peptides than those from caries-free individuals [32], which may explain why a rising S100A7 level fails to arrest disease.

SPINK4 is the least studied of the three proteins, and its result depends most on how it is expressed. Its raw concentration was similar in all groups, whereas its output per minute and its share of total protein were lower in caries. Because output is the product of concentration and flow rate, and the normalized value is the ratio of concentration to total protein, an unchanged concentration combined with lower flow and higher total protein will produce this pattern even without any change in SPINK4 production [27]. The non-monotonic output, lowest in the low or inactive caries group, and the loss of significance after correction for multiple comparisons point in the same direction. The inverse association of raw SPINK4 with active caries in the conventional logistic model ($P = 0.048$) was not retained with Firth's penalized likelihood ($P = 0.079$) or after additional adjustment for flow rate ($P = 0.184$), whereas significant inverse associations persisted only for the derived expressions, which carry the flow and protein terms. The SPINK4 signal therefore rests largely on these derivations. If a genuine reduction exists, it would be biologically plausible: in the gut, SPINK4 is secreted by goblet cells, stabilizes the mucus layer, promotes goblet cell differentiation and is induced by

microbial diacylated lipoproteins, and circulating SPINK4 distinguishes patients with inflammatory bowel disease from healthy controls and tracks disease activity [15]. By analogy, reduced salivary SPINK4 could reflect diminished mucosal barrier function, and its protease-inhibitory activity might limit the degradation of other salivary defense proteins. These possibilities are speculative, and the SPINK4 findings should be treated as hypothesis-generating until confirmed in larger samples with models that account for flow rate directly.

Several limitations should be acknowledged. The case-control design establishes association, not causation, and cannot determine whether the changes in S100A7 precede or follow lesion activity. The sample was modest and was adequately powered only for relatively large effects; the logistic models had fewer than ten events per variable, although penalized estimation gave consistent results for S100A7, and the ROC cut-offs were derived and evaluated in the same data, so the reported AUC values and odds ratios are likely to be optimistic. The group definitions contrasted clearly separated clinical profiles and did not enroll individuals with intermediate profiles, which sharpens the comparison but inflates correlation coefficients and limits the applicability of the cut-offs to unselected populations. Participants were recruited from a single city and were young adults without periodontal disease, so the findings may not generalize to children, older adults or people with periodontitis, in whom S100A7 is likely to be influenced by gingival inflammation. Caries activity was assessed clinically, without radiographs, by a single examiner; although calibration followed a pre-specified threshold of Cohen's kappa ≥ 0.80 , the kappa value achieved was not recorded and therefore cannot be reported. Samples were stored at -20°C rather than -80°C and for variable periods, the relative centrifugal force ($\times g$) was not recorded because the rotor radius was not documented, and the ELISA kits have not been specifically validated for saliva, so absolute concentrations should be compared with other studies cautiously. In particular, the final total dilution factor applied to DMBT1 samples was not fully documented in the available run records. Because the upper limit of the DMBT1 standard curve (2.0 ng/mL) at the baseline 1:5 dilution covers original concentrations only up to about 10 ng/mL, and observed concentrations exceeded this level in many samples, most samples probably required additional dilution; in the absence of documented final dilution factors and of dilution-linearity verification, absolute DMBT1 values should be interpreted with caution, although between-group comparisons are less affected provided that the same procedure was

applied to all groups. Salivary microbiology was not characterized, so the relationship between the three proteins and *S. mutans* or *Lactobacillus* counts remains to be established. Finally, ELISA measures total immunoreactive protein and does not distinguish isoforms, glycoforms or zinc-bound and zinc-free S100A7, which differ in antimicrobial activity [12].

Within these limits, the study supports three conclusions. Salivary S100A7 increases with caries activity in adults and merits evaluation as a marker of active disease in prospective studies. Raw DMBT1 concentration is a poor indicator of caries activity because it is dominated by salivary flow; normalized or output-based measures are preferable. The lower SPINK4 values in caries depend on flow- and protein-based derivations and require confirmation before any mechanistic interpretation. Future work should follow caries-free individuals longitudinally to test whether a rise in S100A7 predicts lesion onset, should relate the three proteins to the salivary microbiome and to pellicle composition, and should examine whether S100A7 or SPINK4 levels change after caries control, including after interventions that reduce the plaque bacterial load, such as antibacterial mouthwashes [33]. Combining S100A7 with flow rate and pH in a multivariable risk score and validating the cut-off identified here in an independent sample, would be the logical next step toward clinical application.

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Author Contributions

Mohammed Ahmed Mustafa conceived and designed the study, performed the clinical examinations, sample collection and laboratory analyses, analyzed the data and wrote the manuscript. All authors reviewed and approved the final manuscript.

Ethical Approval and Consent

The study protocol was approved by the Research Ethics Committee of the College of Education for Pure Sciences, University of Samarra (approval no. EDUSamarraH01 Bio-9, dated 1 October 2025). Written informed consent was obtained from all participants after the purpose and procedures of the study had been explained. The study was conducted in accordance with the Declaration of Helsinki.

Conflict of Interest

The authors declare no financial or personal conflicts of interest, and no direct academic competition, relevant to the content of this article.

Data Availability

The data supporting the findings of this study are available from the corresponding author on reasonable request.

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Table 1. Demographic and behavioral characteristics of the study groups.

Variable	Caries-free (n = 28)	Low/inactive caries (n = 30)	High active caries (n = 30)	P-value	Test
Age (years)	25.46 ± 3.7	25.27 ± 4.49	26.7 ± 4.58	0.38	One-way ANOVA
Sex, female/male, n (%)	14 (50)/14 (50)	15 (50)/15 (50)	15 (50)/15 (50)	1	Chi-square
Tooth-brushing frequency (times/day)	2 (1–2)	2 (1–2)	1 (1–1.75)	0.014	Kruskal–Wallis
Sugar intake frequency (times/day)	2 (1–2)	2 (2–3)	3 (2–3)	0.009	Kruskal–Wallis
Plaque index	0.84 ± 0.38	1.02 ± 0.38	1.15 ± 0.3	0.006	One-way ANOVA
Gingival index	0.72 ± 0.24	0.81 ± 0.26	0.82 ± 0.29	0.275	One-way ANOVA

Values are mean ± standard deviation for normally distributed variables and median (interquartile range) for non-normal or ordinal variables; sex is shown as number (percentage). Plaque and gingival indices according to Löe and Silness. P-values refer to the overall comparison of the three groups.

Table 2. Caries indices and physical properties of unstimulated whole saliva.

Variable	Caries-free (n = 28)	Low/inactive caries (n = 30)	High active caries (n = 30)	P-value	Test
DMFT	0	3 (1.25–4)	7 (7–9.75)	<0.001	Kruskal–Wallis
DMFS	0	4 (3–6.75)	15 (12–17.75)	<0.001	Kruskal–Wallis
Number of active lesions	0	0	4 (3–4)	<0.001	Kruskal–Wallis
Salivary flow rate (mL/min)	0.47 (0.4–0.59)	0.41 (0.31–0.44)	0.36 (0.31–0.47)	0.003	Kruskal–Wallis
Salivary pH	7.07 ± 0.23	6.79 ± 0.24	6.74 ± 0.29	<0.001	One-way ANOVA
Total protein (mg/mL)	1.09 ± 0.27	1.25 ± 0.21	1.37 ± 0.42	0.005	One-way ANOVA

DMFT, decayed, missing and filled teeth; DMFS, decayed, missing and filled surfaces. Active lesions were scored 3–6 on the International Caries Detection and Assessment System. P values refer to the overall comparison of the three groups; differences were considered significant at $P < 0.05$.

Table 3. Salivary DMBT1, S100A7 and SPINK4 as raw concentration, protein-normalised concentration and secretion output.

Biomarker	Caries-free (n = 28)	Low/inactive caries (n = 30)	High active caries (n = 30)	P-value
DMBT1 (ng/mL)	37.6 (26.32–45.02) ^a	42.75 (30.4–54.12) ^{ab}	48.35 (40.57–63.7) ^b	0.009
S100A7 (ng/mL)	7.77 (5.77–9.38) ^a	9.89 (7.87–11.69) ^b	11.46 (9.61–16.3) ^b	<0.001
SPINK4 (ng/mL)	1.71 (1.48–2.4) ^a	1.42 (1.1–2.27) ^a	1.52 (1.32–2.15) ^a	0.247
DMBT1/total protein (ng/mg)	35.22 (25.79–42.46) ^a	34.33 (23.62–47.24) ^a	40.84 (29.23–51.05) ^a	0.398
DMBT1 output (ng/min)	17.99 (12.68–25.27) ^a	13.83 (11.29–24.48) ^a	19.05 (15.14–25.93) ^a	0.293
S100A7/total protein (ng/mg)	6.88 (4.95–9.21) ^a	8.29 (5.98–10.52) ^a	9.43 (6.59–13.17) ^a	0.073
S100A7 output (ng/min)	3.37 (2.64–4.64) ^a	3.71 (2.96–4.79) ^a	4.83 (3.2–6.14) ^a	0.099
SPINK4/total protein (ng/mg) [†]	1.71 (1.39–2.07) ^a	1.28 (0.86–1.9) ^a	1.07 (0.89–2.05) ^a	0.031
SPINK4 output (ng/min)	0.92 (0.62–1.27) ^a	0.51 (0.39–1) ^b	0.62 (0.41–0.91) ^b	0.01

Values are median (interquartile range); all comparisons by Kruskal–Wallis test; Bonferroni-adjusted threshold for the nine biomarker comparisons, $P < 0.0056$. Within a row, values sharing a superscript letter do not differ significantly (Dunn's test with Holm correction). [†]Kruskal–Wallis $P < 0.05$, but no pairwise comparison remained significant after Holm correction. Normalized concentration = biomarker concentration (ng/mL) ÷ total protein (mg/mL). Secretion output = biomarker concentration (ng/mL) × salivary flow rate (mL/min).

Table 4. Spearman correlation coefficients between salivary biomarkers, caries indices and salivary properties (n = 88).

Biomarker	DMFT, r (P)	Active lesions, r (P)	Salivary flow rate, r (P)	Salivary pH, r (P)
DMBT1	0.353 (<0.001)	0.277 (0.009)	-0.306 (0.004)	-0.172 (0.109)
S100A7	0.474 (<0.001)	0.371 (<0.001)	-0.313 (0.003)	-0.315 (0.003)
SPINK4	-0.108 (0.317)	-0.076 (0.484)	0.272 (0.01)	0.086 (0.425)

Values are Spearman's r with the P value in parentheses. DMFT, decayed, missing and filled teeth.

Table 5. Receiver operating characteristic analysis for discrimination of high active caries (Group 3) from caries-free status (Group 1).

Biomarker	AUC	95% CI	Optimal cut-off (ng/mL)	Sensitivity	Specificity
DMBT1	0.73	0.578–0.856	≥ 42.4	70%	71.4%
S100A7	0.854	0.742–0.94	≥ 10.2	70%	89.3%
SPINK4	0.607	0.509–0.746	≤ 1.54	56.7%	67.9%

AUC, area under the curve; CI, confidence interval (bootstrap, 1,000 resamples). Optimal cut-offs were determined by the Youden index.

Table 6. Adjusted logistic regression for high active caries (Group 3 versus Group 1).

Biomarker	Adjusted odds ratio	95% CI	P-value
DMBT1	1.029	0.99–1.071	0.149
S100A7	1.596	1.148–2.218	0.005
SPINK4	0.431	0.187–0.994	0.048

Each biomarker was entered in a separate model adjusted for age, sex, plaque index and sugar intake frequency. Odds ratios are per 1 ng/mL increase in biomarker concentration. CI, confidence interval. Estimates from conventional maximum likelihood; penalized and flow-adjusted estimates are given in Table 7.

Table 7. Firth penalized logistic regression for high active caries (Group 3 versus Group 1).

Biomarker	Model	Adjusted OR (95% CI)	P-value
DMBT1	Model 1	1.024 (0.987–1.062)	0.206
DMBT1	Model 2	1.015 (0.975–1.056)	0.479
S100A7	Model 1	1.455 (1.106–1.914)	0.007
S100A7	Model 2	1.382 (1.047–1.826)	0.023
SPINK4	Model 1	0.502 (0.232–1.083)	0.079
SPINK4	Model 2	0.553 (0.231–1.326)	0.184

Model 1, adjusted for age, sex, plaque index and sugar intake frequency; Model 2, Model 1 plus salivary flow rate. Each biomarker was entered in a separate model. Odds ratios are per 1 ng/mL increase in biomarker concentration. Values in bold are significant at P < 0.05. CI, confidence interval; OR, odds ratio.

Table 8. Sensitivity analysis with protein-normalized and output expressions of each biomarker (Firth penalized logistic regression, Group 3 versus Group 1).

Alternative expression	Adjusted OR	95% CI	P-value
DMBT1/total protein (ng/mg)	0.986	0.959–1.014	0.341
DMBT1 output (ng/min)	0.964	0.896–1.037	0.327
S100A7/total protein (ng/mg)	1.079	0.943–1.234	0.268
S100A7 output (ng/min)	1.167	0.861–1.581	0.321
SPINK4/total protein (ng/mg)	0.324	0.132–0.793	0.014
SPINK4 output (ng/min)	0.097	0.015–0.628	0.014

Each expression was entered in a separate model adjusted for age, sex, plaque index and sugar intake frequency (Model 1 co-variates). Odds ratios are per one-unit increase. Output = biomarker concentration × salivary flow rate. Because these expressions are mathematically coupled to salivary flow rate and total protein, they should be interpreted alongside Table 7. Values in bold are significant at $P < 0.05$.