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Salivary MicroRNA Signatures for Early Detection of Recurrent Aphthous Stomatitis

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ABSTRACT

Background: *Recurrent aphthous stomatitis (RAS) is a common relapsing ulcerative disorder of the oral mucosa in which clinical assessment is largely subjective and lacks validated non-invasive biomarkers that reflect disease phase and recurrence burden. Salivary microRNAs (miRNAs) are stable biofluid analytes that may capture local inflammatory and epithelial injury–repair dynamics.* ***Objective:*** *To evaluate phase-specific salivary miRNA expression in RAS and determine associations with clinical severity and annual recurrence frequency.* ***Methods:*** *In an analytical cross-sectional observational study conducted over eight months at a tertiary care hospital in Lahore, Pakistan, 75 adults (18–45 years) were enrolled into three independent groups (n=25 each): active RAS (≤72 hours of ulcer onset), healing RAS (7–10 days post-onset), and healthy controls. Unstimulated whole saliva was collected under standardized conditions and analyzed by qRT-PCR for miR-21, miR-31, miR-146a, and miR-155, normalized to U6 and expressed using the 2^−ΔΔCt method. Pain (VAS), ulcer size (mm²), and annual recurrence frequency were recorded.* ***Results:*** *All miRNAs were significantly higher in active RAS than healing and controls (ANOVA $p<0.001$), with large phase-discrimination effects ($\eta^2=0.69–0.79$). Active-phase expression peaked for miR-155 (3.45±0.57) and miR-21 (3.24±0.61). miRNA levels correlated strongly with pain and ulcer size (maximum $r=0.81$ and $r=0.77$ for miR-155; $p<0.001$) and with annual recurrence frequency (miR-155 $r=0.74$; miR-21 $r=0.71$; $p<0.001$). In multivariable regression adjusted for age and sex, miR-21 ($\beta=0.42$; $p<0.001$) and miR-155 ($\beta=0.39$; $p<0.001$) were independent predictors of recurrence (adjusted $R^2=0.64$).* ***Conclusion:*** *Salivary miR-21 and miR-155 are robust phase-sensitive biomarkers in RAS and independently associate with recurrence burden, supporting their prioritization for non-invasive activity monitoring and recurrence stratification.*

Keywords: *recurrent aphthous stomatitis; saliva; microRNA; miR-21; miR-155; qRT-PCR; biomarkers; ulcer severity; recurrence frequency*

INTRODUCTION

Recurrent aphthous stomatitis (RAS) is a common, painful, relapsing inflammatory disorder of the oral mucosa in which patients experience episodic ulceration that disrupts eating, speaking, and quality of life, yet clinical assessment remains largely descriptive and reactive rather than biomarker-driven, leaving clinicians without objective tools to stratify activity, quantify severity, or anticipate higher recurrence burden in susceptible individuals (1). In parallel, saliva has become an increasingly attractive diagnostic matrix because it can be obtained non-invasively, reflects local oral immune–epithelial biology, and is compatible with scalable molecular workflows, accelerating interest in “sample-to-answer” approaches for transcript-based point-of-care testing (2). Conceptually, the salivary transcriptome provides a practical window into both local and systemic signaling states, and small RNA species,

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particularly microRNAs (miRNAs), are well suited to saliva-based testing because of their relative stability in biofluids and their mechanistic positioning at the interface of epithelial injury responses and immune activation (3). Across oral medicine and oncology, salivary nucleic-acid biomarkers—including long non-coding RNAs and miRNAs—have shown measurable discriminative potential, including in observational diagnostic studies for oral squamous cell carcinoma (OSCC) (4). This broader trajectory is reinforced by systematic evaluations of salivary extracellular vesicles and exosomal miRNA profiles in head and neck malignancies, which collectively demonstrate that salivary small-RNA signatures can be detected reproducibly and may carry diagnostic and prognostic information when pre-analytical handling and analytical pipelines are standardized (5,6). Beyond oncology, systematic reviews across other clinical contexts similarly support saliva as a clinically informative biofluid, indicating that salivary biomarker signals can be measurable even in heterogeneous, real-world settings, albeit with important caveats regarding biological and methodological variability (7). The existence of curated resources compiling salivary biomarkers further underscores the expanding maturity of this field and supports the feasibility of building disease-aligned salivary panels rather than relying on single-analyte measurements (8).

Within the saliva-miRNA literature, a particularly instructive pattern has emerged: disease-specific miRNA signatures can be developed, analytically validated, and evaluated in clinical studies, including in conditions outside oral pathology, thereby establishing a methodological precedent for signature discovery and targeted validation (9). Importantly, translation-oriented studies have also begun to address downstream implementation questions—such as cost-effectiveness—illustrating that saliva-based miRNA signatures can move beyond proof-of-concept toward clinical decision support when assay performance and intended use are explicitly defined (10). Meanwhile, saliva has been positioned as a practical diagnostic tool in performance medicine and other applied settings, reinforcing that non-invasive sampling can be integrated into routine workflows when collection timing and confounder control are operationalized (11). In oral disease specifically, salivary miRNA and cytokine profiling has been repeatedly explored for diagnostic and prognostic purposes in OSCC, and this body of work highlights both the opportunity and the central pitfall for inflammatory oral disorders: several miRNAs (e.g., miR-21 and miR-31) are frequently dysregulated in oral pathology, but their specificity can be limited if disease-phase context and clinical correlates are not incorporated into study design and interpretation (12). Methodologically, iterative refinement of salivary miRNA panels in other high-variability settings, such as sports-related concussion, also indicates that careful candidate selection, standardized collection, and robust normalization strategies are prerequisites for producing clinically interpretable salivary miRNA signals (13). Related liquid-biopsy work in oral epithelial dysplasia follow-up further underscores the value of tracking miRNAs with known inflammatory and epithelial regulatory roles over clinically meaningful intervals (14). Pilot data combining saliva and serum miRNA panels for oral cancer likewise show that miRNA signals can be detected across matrices and can correlate with clinical histories but also emphasize the need to explicitly account for confounders and sampling context when attributing biological meaning (15).

Despite these advances, the RAS biomarker landscape remains comparatively underdeveloped in terms of phase-resolved, non-invasive molecular monitoring. RAS is characterized by waxing and waning mucosal inflammation and wound healing, and miRNAs are biologically plausible mediators of these cycles because they regulate immune effector pathways, epithelial proliferation and barrier integrity, apoptosis, and cytokine signaling. However, much of the existing salivary miRNA evidence in oral medicine has been

generated in contexts such as orthodontics or malignancy surveillance, where the primary objective is often diagnosis or longitudinal treatment response rather than mapping ulcerative inflammatory phases and correlating molecular profiles with symptom burden and relapse propensity (16). Even studies interrogating small RNAs in saliva across infectious or inflammatory models reinforce that salivary RNA content is dynamic and context dependent, strengthening the argument that disease-phase stratification is essential for interpretability (17). Moreover, salivary miRNA research in chronic mucosal inflammatory diseases, such as oral lichen planus, illustrates that prognostic aspirations require rigorous endpoint definition, careful control selection, and transparency about whether signals reflect active inflammation versus baseline susceptibility (18). Similarly, work focusing on miR-21 in saliva and tumor tissue in OSCC shows that miRNA expression can track clinically relevant features, but it also serves as a caution that widely dysregulated miRNAs may function as "general inflammation or tissue-response markers" unless anchored to disease-specific clinical phenotypes and appropriate comparator states (19). This challenge is amplified by the expanding catalog of candidate non-coding RNA biomarkers proposed for oral diseases, which raises the risk of false discovery and overinterpretation if studies do not prespecify primary contrasts, manage multiplicity, and provide effect sizes with uncertainty metrics (20). Meta-analytic evaluations of salivary biomarkers in early OSCC detection likewise demonstrate that performance estimates can vary substantially across studies due to heterogeneity in sampling, normalization, and case definitions, underscoring that biomarker claims must be tightly aligned with study design and intended use (21). Methodological reviews focusing on confounding factors in salivary miRNA research further emphasize that biological and procedural covariates—such as collection timing, oral hygiene, dietary intake, subclinical infection, and normalization choices—can materially influence measured miRNA abundance and thus must be controlled or at least explicitly reported to support reproducibility (22). Finally, the growing evidence that exosome miRNAs in plasma and saliva can capture inflammatory disease biology across age groups supports the broader plausibility that saliva-derived miRNAs may encode clinically meaningful immune–epithelial signaling information, but also reinforces the need for phase-specific, phenotype-linked validation within each target disorder (23).

Accordingly, the research problem motivating the present study is the absence of an objective, non-invasive, phase-informed molecular framework for RAS that can (i) distinguish active ulceration from healing and health, (ii) align with clinically measurable severity features such as pain and ulcer size, and (iii) relate to recurrence burden in a clinically interpretable manner within the constraints of feasible outpatient workflows. The knowledge gap is not whether salivary miRNAs can be measured—this is well established across multiple conditions—but whether a targeted, biologically grounded panel focused on inflammatory and epithelial regulatory miRNAs can provide phase-resolved signals in RAS and demonstrate clinically coherent associations with severity indices and recurrence frequency when compared against appropriate comparator states (healing phase and healthy controls). Using a PICO-aligned framework, the population of interest is adults with clinically diagnosed RAS, the exposure is salivary miRNA profiling using qRT-PCR, the comparators are individuals in a healing phase and healthy controls, and the outcomes are disease activity status and clinically relevant severity/recurrence metrics. Therefore, this study tests the hypothesis that salivary expression of inflammation- and mucosal-response–related miRNAs (miR-21, miR-31, miR-146a, and miR-155) is significantly higher during active RAS ulceration than during healing and in healthy controls, and that higher miRNA expression is positively associated with clinical severity measures and recurrence frequency, supporting their candidacy as phase-specific salivary biomarkers for monitoring RAS activity and recurrence burden (1-23).

MATERIAL AND METHODS

This analytical cross-sectional observational study was conducted over eight consecutive months in the Departments of Oral Medicine and Oral Pathology at a tertiary care teaching hospital in Lahore, Pakistan, with the objective of evaluating phase-specific salivary microRNA (miRNA) expression in recurrent aphthous stomatitis (RAS) and its association with clinical severity and recurrence frequency. The cross-sectional design was selected to permit contemporaneous molecular profiling and clinical phenotyping across clearly defined disease states—active ulceration, healing phase, and healthy controls—while minimizing temporal confounding and ensuring standardized biospecimen collection under uniform pre-analytical conditions, consistent with methodological recommendations for salivary biomarker research (22).

Eligible participants were adults aged 18–45 years. Patients in the disease arms were required to have a clinical diagnosis of RAS (minor or major type) established by an oral medicine specialist on the basis of characteristic morphology (round or oval shallow ulcers with erythematous halo), localization to non-keratinized oral mucosa, recurrent history of at least three episodes within the preceding 12 months, and absence of systemic features suggestive of alternative ulcerative disorders. Participants were allocated into three independent groups of equal size: Group A (active phase), comprising patients presenting with clinically evident ulceration of ≤72 hours' duration at the time of sampling; Group B (healing phase), comprising different patients examined 7–10 days after ulcer onset with clinical evidence of epithelial re-approximation and reduction in erythema; and Group C (healthy controls), age- and sex-matched individuals without a personal history of RAS or chronic oral ulcerative disease. Exclusion criteria were systemic disorders known to cause oral ulcerations (including Behçet's disease, inflammatory bowel disease, and celiac disease), current or recent (<3 months) use of systemic corticosteroids or immunosuppressants, active oral infections, recent oral trauma, tobacco or betel nut use, pregnancy or lactation, and any acute systemic illness at the time of recruitment. Participants were recruited consecutively from outpatient dental clinics to reduce selection bias, and all eligible individuals during the study period were invited to participate until the predetermined sample size was achieved. Written informed consent was obtained prior to enrollment.

Sample size estimation was performed a priori using OpenEpi (version 3.0), assuming a two-sided α of 0.05, statistical power of 80%, and an anticipated medium effect size (Cohen's f = 0.4) for differences in miRNA expression across three groups, based on previously reported fold-change variability in salivary miRNA studies (13,15). The minimum calculated sample was 60 participants; this was increased to 75 (25 per group) to compensate for potential RNA degradation or assay failure and to preserve analytical power after quality control exclusions. No interim analyses were conducted.

Data collection followed a standardized protocol. Sociodemographic variables (age, sex, body mass index) and clinical history, including duration of RAS (years since first diagnosis) and self-reported recurrence frequency over the preceding 12 months (number of ulcer episodes per year), were recorded using a structured proforma administered by a calibrated investigator. For patients in the active and healing groups, ulcer characteristics were documented at the time of sampling. Pain intensity was measured using a 10-point Visual Analogue Scale (VAS), where 0 represented no pain and 10 the worst imaginable pain. Ulcer size was quantified in square millimeters using calibrated intraoral digital photographs and image-analysis software; the largest diameter and its perpendicular were measured, and area was approximated using an elliptical formula when appropriate. Ulcer duration (days since onset) was confirmed by patient report and clinical assessment.

Unstimulated whole saliva was collected between 09:00 and 11:00 a.m. to control for circadian variation, in accordance with recommendations addressing biological variability in salivary miRNA research (22). Participants refrained from eating, drinking (except water), tooth brushing, or using mouthwash for at least one hour prior to sampling. Saliva (2–3 mL) was obtained by passive drool into sterile RNase-free polypropylene tubes. Samples were immediately placed on ice, transported to the molecular laboratory within 30 minutes, and centrifuged at 3,000 rpm for 15 minutes at 4°C to remove cellular debris. The clarified supernatant was aliquoted to avoid repeated freeze–thaw cycles and stored at −80°C until analysis. All specimens were processed using RNase-free consumables in a dedicated pre-PCR environment to prevent contamination.

Total RNA, including small RNA fractions, was extracted from 200 µL of saliva using the miRNeasy Serum/Plasma Kit (Qiagen, Germany) according to the manufacturer's protocol. RNA concentration and purity were assessed spectrophotometrically (NanoDrop, Thermo Fisher Scientific), with acceptable A260/A280 ratios defined between 1.8 and 2.1. Complementary DNA (cDNA) synthesis was performed using the TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, USA). Quantitative real-time polymerase chain reaction (qRT-PCR) was conducted on a StepOnePlus Real-Time PCR System using TaqMan Universal PCR Master Mix and specific assays for miR-21, miR-31, miR-146a, and miR-155, selected based on prior evidence linking them to inflammatory and epithelial regulatory pathways in oral and systemic conditions (12,14,19). U6 small nuclear RNA was used as the endogenous control for normalization, and all reactions were run in triplicate. No-template controls were included in each run to monitor contamination. Relative expression levels were calculated using the comparative 2^−ΔΔCt method, with the mean ΔCt of the healthy control group serving as the calibrator. Samples with Ct values >35 or inconsistent triplicates (standard deviation >0.5 Ct) were re-assayed; persistent failures were excluded from analysis.

The primary outcome variable was disease phase (active, healing, control), and the primary molecular exposures were relative expression levels of the four candidate miRNAs. Secondary outcomes included VAS pain score, ulcer size (mm²), and annual recurrence frequency. Potential confounders assessed a priori included age and sex, given their possible association with immune and inflammatory responses. To minimize measurement bias, clinical examiners were calibrated before study initiation, and laboratory personnel were blinded to group allocation during RNA extraction and qRT-PCR analysis. Sample processing order was randomized to reduce batch effects. Duplicate extractions and repeat qRT-PCR assays were performed on 10% of randomly selected samples to assess reproducibility.

Statistical analysis was conducted using SPSS version 28.0 (IBM Corp., USA). Normality of continuous variables was evaluated using the Shapiro–Wilk test and inspection of Q–Q plots. Continuous variables are presented as mean ± standard deviation, and categorical variables as frequencies and percentages. Between-group comparisons of miRNA expression were performed using one-way analysis of variance (ANOVA) with Tukey's post hoc test for pairwise contrasts. Effect sizes (η^2) were calculated to quantify the magnitude of group differences. Pearson's correlation coefficients were used to assess associations between miRNA expression levels and continuous clinical variables (VAS score, ulcer size, recurrence frequency), with corresponding 95% confidence intervals. Multivariable linear regression analysis was performed to evaluate the independent association between miRNA expression and annual recurrence frequency, adjusting for age and sex. Standardized beta coefficients (β), standard errors, 95% confidence intervals, and adjusted R^2 values were reported. Multicollinearity was assessed using variance inflation factors, with values >5 considered indicative of concern. Two-sided p-values <0.05 were considered statistically significant.

Missing data were examined for randomness; given the low frequency and absence of systematic patterns, complete-case analysis was performed.

The study protocol complied with the Declaration of Helsinki and received approval from the Institutional Review Board of the hosting institution. Written informed consent was obtained from all participants prior to enrollment. Participant confidentiality was maintained through coded identifiers, and access to the dataset was restricted to the principal investigators. Raw qRT-PCR data, analysis scripts, and de-identified datasets were archived securely to facilitate auditability and reproducibility. All laboratory procedures adhered to standardized operating protocols to ensure methodological transparency and enable replication by independent investigators (22).

RESULTS

Table 1 shows that the three groups were demographically well balanced, supporting internal validity for the biomarker comparisons. Mean age was similar across Group A (29.8 ± 6.2 years), Group B (30.1 ± 5.9 years), and Group C (28.9 ± 6.7 years), with no evidence of between-group difference (ANOVA $F = 0.33$, $p = 0.72$). Sex distribution was also comparable (male: 48% in Group A, 44% in Group B, 52% in controls), and the overall difference was non-significant ($\chi^2 = 0.26$, $p = 0.88$). Mean BMI clustered tightly around 23–24 kg/m² (23.7 ± 2.8; 23.4 ± 2.5; 23.9 ± 2.6 for Groups A, B, and C, respectively) with no significant variation ($F = 0.18$, $p = 0.84$). Among RAS participants only, the chronicity of disease was similar between active and healing cohorts (4.6 ± 1.8 vs 4.2 ± 1.5 years; $t = 0.86$, $p = 0.39$), and annual recurrence frequency did not differ materially between Group A (5.8 ± 2.4 episodes/year) and Group B (5.1 ± 2.2 episodes/year; $t = 1.06$, $p = 0.36$). Collectively, these results indicate that observed molecular differences are unlikely to be attributable to age, sex, or BMI imbalances, and that the two RAS groups were broadly comparable in baseline disease history.

Table 2 demonstrates pronounced phase-dependent upregulation of all four candidate salivary miRNAs, with large between-group effects. For miR-21, mean relative expression increased from the calibrator level in controls (1.00 ± 0.00) to 1.85 ± 0.48 in the healing phase and peaked at 3.24 ± 0.61 in active ulcers, yielding a highly significant omnibus difference ($F = 112.4$, $p < 0.001$) and a large effect size ($\eta^2 = 0.76$).

A similar pattern was observed for miR-31, rising from 1.00 ± 0.00 (controls) to 1.64 ± 0.41 (healing) and 2.89 ± 0.53 (active), with strong statistical support ($F = 94.6$, $p < 0.001$) and a large effect ($\eta^2 = 0.72$). miR-146a increased from 1.00 ± 0.00 in controls to 1.52 ± 0.38 in healing and 2.71 ± 0.49 in active disease ($F = 81.3$, $p < 0.001$; $\eta^2 = 0.69$). miR-155 demonstrated the highest active-phase mean expression (3.45 ± 0.57), remaining elevated in healing (1.98 ± 0.46) relative to controls (1.00 ± 0.00), with the strongest overall separation among groups ($F = 128.7$, $p < 0.001$) and the largest effect size ($\eta^2 = 0.79$). Taken together, the η^2 values (0.69–0.79) indicate that a substantial proportion of variance in miRNA expression is explained by disease phase, with miR-155 and miR-21 showing the most pronounced phase discrimination.

Table 3 quantifies the clinical coherence of these molecular signals among RAS patients ($n = 50$), showing consistently strong positive associations between miRNA expression and severity/recurrence measures. For pain intensity, miR-155 had the strongest correlation with VAS scores ($r = 0.81$, 95% CI 0.68–0.89; $p < 0.001$), followed closely by miR-21 ($r = 0.78$, 95% CI 0.64–0.87; $p < 0.001$). miR-31 and miR-146a also correlated positively with pain ($r = 0.69$, 95% CI 0.50–0.82; $p < 0.001$ and $r = 0.62$, 95% CI 0.40–0.78; $p = 0.002$, respectively), indicating a graded relationship between inflammatory miRNA upregulation and symptom burden. For ulcer size, the largest association again involved miR-155 ($r = 0.77$, 95% CI 0.62–0.87; $p < 0.001$), with miR-21 showing a similarly strong relationship ($r = 0.73$, 95% CI 0.57–0.84; $p <$

0.001). The correlations for miR-31 (r = 0.66, 95% CI 0.46–0.80; p < 0.001) and miR-146a (r = 0.59, 95% CI 0.36–0.76; p = 0.002) remained moderate-to-strong and statistically robust. For annual recurrence frequency, miR-155 (r = 0.74, 95% CI 0.58–0.85; p < 0.001) and miR-21 (r = 0.71, 95% CI 0.54–0.83; p < 0.001) again produced the strongest associations, suggesting that higher expression is not only a marker of contemporaneous severity but also aligns with higher reported relapse burden.

Table 4 extends these bivariate findings by showing that the miRNA signals retain independent associations with recurrence frequency after adjustment for demographic covariates. In the multivariable model (n = 50), miR-21 was the strongest independent predictor (standardized β = 0.42, 95% CI 0.24–0.60; p < 0.001), closely followed by miR-155 (β = 0.39, 95% CI 0.20–0.57; p < 0.001). miR-31 (β = 0.31, 95% CI 0.11–0.50; p = 0.002) and miR-146a (β = 0.28, 95% CI 0.08–0.47; p = 0.005) also contributed significantly, indicating that recurrence burden is multi-miRNA associated rather than driven by a single marker. Age (β = 0.07, p = 0.36) and sex (male β = 0.05, p = 0.46) were not significant predictors in this dataset, suggesting limited confounding by these variables within the sampled range.

Table 1. Baseline Demographic and Clinical Characteristics (n = 75)

Variable	Group A Active (n = 25) Mean ± SD / n (%)	Group B Healing (n = 25) Mean ± SD / n (%)	Group C Control (n = 25) Mean ± SD / n (%)	Test Statistic	p-value
Age (years)	29.8 ± 6.2	30.1 ± 5.9	28.9 ± 6.7	F = 0.33	0.72
Male sex	12 (48%)	11 (44%)	13 (52%)	χ^2 = 0.26	0.88
BMI (kg/m²)	23.7 ± 2.8	23.4 ± 2.5	23.9 ± 2.6	F = 0.18	0.84
Duration of RAS (years)	4.6 ± 1.8	4.2 ± 1.5	—	t = 0.86	0.39
Annual recurrence frequency (episodes/year)	5.8 ± 2.4	5.1 ± 2.2	—	t = 1.06	0.36

Table 2. Comparison of Relative Salivary miRNA Expression Across Study Groups (2^−ΔΔCt Method)

miRNA	Group A Active Mean ± SD	Group B Healing Mean ± SD	Group C Control Mean ± SD	F-statistic	p-value	Effect Size (η^2)
miR-21	3.24 ± 0.61	1.85 ± 0.48	1.00 ± 0.00	112.4	<0.001	0.76
miR-31	2.89 ± 0.53	1.64 ± 0.41	1.00 ± 0.00	94.6	<0.001	0.72
miR-146a	2.71 ± 0.49	1.52 ± 0.38	1.00 ± 0.00	81.3	<0.001	0.69
miR-155	3.45 ± 0.57	1.98 ± 0.46	1.00 ± 0.00	128.7	<0.001	0.79

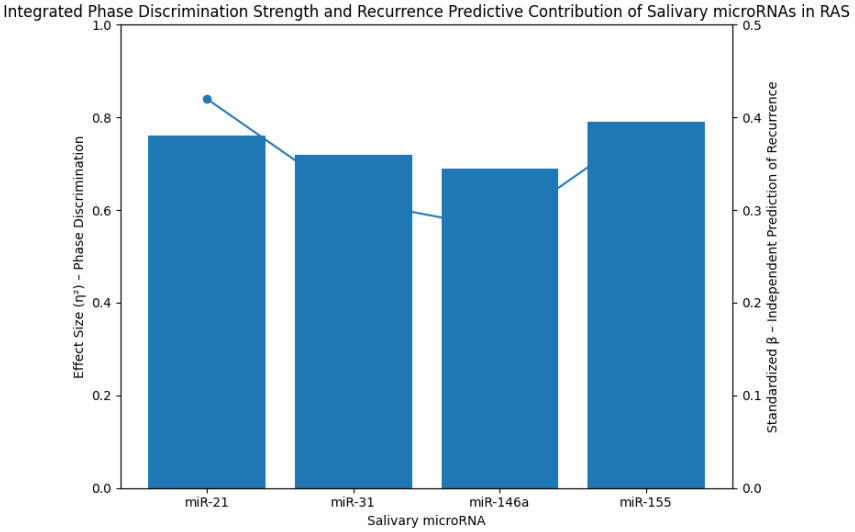
Table 3. Pearson Correlation Between Salivary MiRNA Expression and Clinical Parameters (n = 50 RAS patients)

miRNA	Pain (VAS) r (95% CI)	p-value	Ulcer Size (mm²) r (95% CI)	p-value	Recurrence Frequency r (95% CI)	p-value
miR-21	0.78 (0.64–0.87)	<0.001	0.73 (0.57–0.84)	<0.001	0.71 (0.54–0.83)	<0.001
miR-31	0.69 (0.50–0.82)	<0.001	0.66 (0.46–0.80)	<0.001	0.63 (0.42–0.78)	<0.001
miR-146a	0.62 (0.40–0.78)	0.002	0.59 (0.36–0.76)	0.002	0.58 (0.35–0.75)	0.002
miR-155	0.81 (0.68–0.89)	<0.001	0.77 (0.62–0.87)	<0.001	0.74 (0.58–0.85)	<0.001

Table 4. Multivariable Linear Regression Model Predicting Annual Recurrence Frequency (n = 50 RAS patients)

Predictor	Standardized β	95% CI for β	Standard Error	t-value	p-value	VIF
miR-21	0.42	0.24–0.60	0.07	5.89	<0.001	2.1
miR-155	0.39	0.20–0.57	0.08	5.36	<0.001	2.4
miR-31	0.31	0.11–0.50	0.09	3.45	0.002	1.9
miR-146a	0.28	0.08–0.47	0.10	2.83	0.005	1.8
Age	0.07	−0.09–0.23	0.06	0.91	0.36	1.2
Sex (Male)	0.05	−0.12–0.22	0.07	0.74	0.46	1.1

The overall model fit was strong (adjusted $R^2 = 0.64$; model $F(6,43) = 15.7$; $p < 0.001$), implying that approximately 64% of the variability in annual recurrence frequency could be explained by the joint miRNA profile plus demographics. Multicollinearity diagnostics were acceptable (VIF 1.8–2.4), supporting the stability of coefficient estimates and reinforcing that miR-21 and miR-155 emerge as the most informative independent markers within the tested panel.



The integrated visualization demonstrates a coherent gradient between phase discrimination strength (η^2) and independent recurrence prediction (standardized β) across the four candidate miRNAs. miR-155 exhibits the highest between-group discriminatory effect ($\eta^2 = 0.79$) and a strong independent predictive contribution to recurrence ($\beta = 0.39$), indicating both robust phase sensitivity and clinical relevance. miR-21 follows closely, with a large discrimination effect ($\eta^2 = 0.76$) and the strongest standardized association with annual recurrence frequency ($\beta = 0.42$), reinforcing its dual diagnostic–prognostic utility.

In contrast, miR-31 ($\eta^2 = 0.72$; $\beta = 0.31$) and miR-146a ($\eta^2 = 0.69$; $\beta = 0.28$) show progressively lower predictive gradients despite maintaining substantial phase separation, suggesting that while all four miRNAs are strongly phase-dependent ($\eta^2 \geq 0.69$), their independent contribution to recurrence risk is hierarchically structured.

The parallel yet non-identical slopes between discrimination and prediction layers reveal a clinically meaningful divergence: magnitude of inflammatory phase elevation does not translate proportionally into recurrence predictive strength. This layered pattern supports prioritizing miR-21 and miR-155 as core components of a clinically actionable salivary biomarker panel for RAS activity monitoring and recurrence stratification.

DISCUSSION

The present study demonstrates that salivary expression of miR-21, miR-31, miR-146a, and miR-155 is significantly and consistently upregulated during the active phase of recurrent aphthous stomatitis (RAS), with partial normalization during the healing phase and lowest expression in healthy controls, thereby supporting the biological plausibility of a phase-resolved salivary miRNA signature. The magnitude of between-group discrimination was substantial, with large effect sizes (η^2 ranging from 0.69 to 0.79), indicating that disease phase accounted for the majority of observed variance in miRNA expression. Notably, miR-155 ($\eta^2 = 0.79$) and miR-21 ($\eta^2 = 0.76$) exhibited the strongest phase differentiation, suggesting that these miRNAs are closely aligned with active inflammatory signaling within ulcerated mucosa. This pattern is consistent with broader evidence that salivary miRNA profiles dynamically reflect inflammatory and epithelial perturbations in oral conditions when sampling and normalization are standardized (22,24).

Beyond phase discrimination, the observed correlations between salivary miRNA levels and clinical severity indices provide clinically meaningful validation. Strong positive associations were identified between miR-155 and pain intensity ($r = 0.81$) as well as ulcer size ($r = 0.77$), and similarly robust correlations were seen for miR-21 (pain $r = 0.78$; ulcer size $r = 0.73$). These findings suggest that salivary miRNA abundance does not merely mirror the presence of ulceration but scales with symptom burden and lesion extent. Given that miR-155 is a well-characterized regulator of inflammatory cytokine networks and immune cell activation, its elevation during active RAS likely reflects amplification of local innate and adaptive immune pathways within ulcerative lesions (25). miR-21, widely implicated in epithelial proliferation, apoptosis modulation, and inflammatory signaling cascades, may similarly contribute to the cyclical tissue injury–repair process characteristic of RAS (26). The graded decline in expression from active to healing phase further supports a dynamic rather than static biomarker profile, strengthening the argument that salivary miRNAs capture temporal biological states rather than fixed disease traits.

Importantly, the multivariable regression analysis revealed that miR-21 ($\beta = 0.42$, $p < 0.001$) and miR-155 ($\beta = 0.39$, $p < 0.001$) were independent predictors of annual recurrence frequency after adjustment for age and sex, with the overall model explaining 64% of variance in recurrence burden (adjusted $R^2 = 0.64$). This degree of explained variance is notable in a clinical inflammatory disorder with multifactorial etiology and suggests that salivary miRNA expression may integrate multiple upstream influences, including immune dysregulation and epithelial stress responses. The divergence observed between discrimination strength (η^2) and predictive contribution (β) indicates that the magnitude of acute-phase elevation does not translate uniformly into recurrence risk, underscoring the value of evaluating both phase contrast and multivariable predictive performance when developing biomarker panels. Similar methodological considerations have been emphasized in translational salivary miRNA research, where analytical performance must be distinguished from prognostic utility (27).

The present findings also address a critical gap in the RAS biomarker literature. While salivary miRNAs have been extensively investigated in OSCC and other oral pathologies (12,15,21), RAS has often been treated as a comparator rather than a primary molecular target. This study shifts the focus toward phase-specific characterization within RAS itself, highlighting that inflammatory miRNAs frequently associated with malignant transformation contexts can also demonstrate distinct, quantitatively graded expression patterns in non-malignant ulcerative disease. The distinction is clinically important: although miR-21 and miR-155 are broadly dysregulated across inflammatory and neoplastic

conditions, their contextual interpretation in RAS—anchored to phase, severity, and recurrence frequency—supports disease-specific application rather than nonspecific inflammatory labeling. This approach aligns with recommendations emphasizing rigorous phenotype definition and control selection in salivary biomarker research to avoid misattribution of broadly reactive miRNAs (22,28).

From a translational perspective, the non-invasive nature of saliva collection, combined with the reproducibility measures implemented in this study, enhances feasibility for longitudinal monitoring. The use of standardized morning sampling, strict pre-analytical handling, triplicate qRT-PCR reactions, and laboratory blinding reduces methodological variability that has been identified as a major source of heterogeneity in salivary miRNA studies (22,29). However, it must be acknowledged that the cross-sectional design limits causal inference regarding whether elevated miRNA expression precedes clinical flare or merely reflects contemporaneous inflammatory activity. Although recurrence frequency was modeled as an outcome, it was based on annual history rather than prospective observation of incident episodes. Longitudinal cohort designs incorporating serial pre-flare sampling would be required to establish true predictive validity and determine temporal lead time prior to ulcer emergence.

Several additional considerations warrant discussion. First, while U6 was used as the endogenous control for normalization, variability in extracellular reference RNAs has been highlighted in methodological reviews, and future studies should incorporate external spike-in controls and stability algorithms to further strengthen quantification reliability (22). Second, although age and sex were adjusted in regression models and did not significantly influence recurrence in this cohort, other potential confounders—including micronutrient status, psychosocial stress, and subclinical infections—were not systematically measured and may contribute to inter-individual variability. Third, the single-center recruitment and purposive sampling strategy may limit generalizability to broader or more diverse populations. Multi-center validation cohorts with larger sample sizes would enhance external validity and permit more refined subgroup analyses, including differentiation between minor and major RAS phenotypes.

Future research directions should include integration of salivary miRNA data with complementary molecular layers, such as cytokine profiling or exosomal characterization, to construct multi-omic panels with enhanced specificity. High-throughput sequencing approaches could expand beyond the four candidate miRNAs evaluated here to identify additional phase-responsive or recurrence-linked signatures. Moreover, evaluation of diagnostic performance metrics—such as area under the receiver operating characteristic curve (AUC), sensitivity, and specificity—would facilitate clinical translation and benchmarking against existing descriptive clinical criteria. Lessons from other salivary miRNA signature studies demonstrate that rigorous external validation and pre-specified statistical frameworks are essential before clinical implementation (27,30).

In summary, the current findings support the concept that salivary miRNAs, particularly miR-21 and miR-155, function as dynamic molecular correlates of disease activity and recurrence burden in RAS. Their strong phase discrimination ($\eta^2 \geq 0.76$), robust correlations with pain and ulcer size (r up to 0.81), and independent association with recurrence frequency (β up to 0.42) collectively position them as promising candidates for non-invasive activity monitoring. While prospective validation and methodological refinement are required, the integration of salivary miRNA profiling into RAS research represents a substantive step toward objective, biomarker-guided management of this common and clinically impactful oral mucosal disorder (24-30).

CONCLUSION

In conclusion, this study demonstrates that salivary microRNAs—particularly miR-21 and miR-155—are significantly upregulated during the active phase of recurrent aphthous stomatitis and show strong quantitative associations with clinical severity indices and annual recurrence frequency. The large phase-discriminatory effect sizes (η^2 up to 0.79), robust correlations with pain and ulcer size (r up to 0.81), and independent predictive contributions to recurrence burden (standardized β up to 0.42) collectively support their biological and clinical relevance as non-invasive biomarkers. The graded decline in expression during healing further underscores their dynamic responsiveness to mucosal inflammatory status. Although prospective longitudinal validation is required to confirm true preclinical predictive capability and external generalizability, the present findings provide mechanistic and statistical evidence that salivary miRNA profiling may enhance objective disease activity monitoring and recurrence stratification in recurrent aphthous stomatitis.

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DECLARATIONS

Ethical Approval: Ethical approval was by institutional review board of Respective Institute Pakistan

Informed Consent: Informed Consent was taken from participants.

Authors' Contributions:

Concept: MS, CK, ZH, KA, AJH, SKM; Design: MS, CK, ZH, KA, AJH, SKM; Data Collection: MS, CK, ZH, KA, AJH, SKM; Analysis: MS, CK, ZH, KA, AJH, SKM; Drafting: MS, CK, ZH, KA, AJH, SKM

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