ORIGINAL ARTICLE

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ASSESSING THE APPLICABILITY OF SALIVA FOR GENE EXPRESSION STUDIES IN ADVANCED PERIODONTITIS

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Abstract. *Periodontitis is a common chronic inflammatory disease that affects the supporting structures of the teeth and can lead to tooth loss if left untreated. Beyond its local impact, the condition is increasingly recognized as a component of systemic inflammation, involving activation of immune pathways and cytokine networks. Understanding these molecular processes requires reliable biological material that reflects both local and systemic immune activity. So far, most gene expression studies in periodontitis have been performed using peripheral blood, which provides information about systemic immune status but is obtained through an invasive procedure. In this study, we aimed to evaluate whether saliva could serve as a convenient and non-invasive alternative for RNA-based functional analyses of immune system-related genes in patients with advanced periodontitis. Saliva samples were collected from twelve patients diagnosed with severe disease. Total RNA was extracted, and its concentration and purity were measured spectrophotometrically. Despite natural variability among individuals, all samples yielded RNA suitable for further gene expression evaluation. Our findings suggest that saliva can provide RNA of adequate yield and purity to analyze the expression of immune-related genes in periodontitis. Given its easy and non-invasive collection, saliva represents a promising biological source for molecular investigations of inflammatory processes and may support broader diagnostic and research applications in health sciences.*

Key words: *saliva, quality, applicability, gene expression*

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INTRODUCTION

Periodontitis is a chronic inflammatory condition that affects the teeth and, in its advanced stage, their adjacent tissues. There were several studies through the years that have been performed to study epidemiological [1, 2] and genetic [3, 4] characteristics of periodontitis in a Bulgarian population. So far, there have been no studies on gene expression in periodontitis, nor have there been studies using saliva for RNA extraction.

Gene expression analysis is a fundamental approach for understanding cellular activity, disease mechanisms, and biomarker discovery. By measuring RNA transcription levels, researchers can determine which genes are active under specific biological conditions. Standard methods – including qPCR/RT-qPCR, RNA-seq, microarrays, and digital PCR – typically rely on high-quality RNA extracted from biological samples, such as blood, tissue, cerebrospinal fluid, urine, or cultured cells. Blood and tissue remain the most commonly used materials due to their reliable

RNA yield and direct biological relevance. However, they require invasive sampling, limiting their use in large population studies or in conditions requiring repeated monitoring. Saliva has gained attention for a long time as a promising non-invasive alternative. It contains epithelial cells, immune cells, extracellular vesicles, and cell-free RNA, reflecting both local and systemic physiological states [5, 6].

Despite its advantages, saliva presents analytical challenges – high variability, low RNA amounts, RNA degradation due to RNases and microbial enzymes, and heterogeneous cell composition. These issues raise concerns about data reproducibility and assay sensitivity [7, 8]. Nevertheless, saliva is particularly relevant in studies of oral disease, including periodontitis, a chronic inflammatory condition with strong local immune involvement [9].

This study evaluates whether saliva provides RNA of sufficient quality and quantity for immune-related gene expression profiling in patients with advanced periodontitis. The NanoString nCounter platform was used due to its robustness and precision with low-quantity and/or partially degraded RNA.

MATERIALS AND METHODS

Sample collection and RNA isolation

Saliva samples were collected from twelve individuals, including ten patients with advanced periodontitis and two healthy controls without periodontal pathology. Saliva samples were obtained between 10:00 AM and 12:00 PM to minimize circadian variability. Participants were instructed to refrain from eating and from toothbrushing for at least 1 hour prior to sampling, in order to reduce potential contamination from interfering substances. Before collection, each participant rinsed the oral cavity with water and waited a minimum of 10 minutes.

Unstimulated whole saliva was collected using the passive drool technique into sterile collection tubes. Participants were asked to allow saliva to pool naturally in the mouth and to transfer it into the tube periodically over a 5-minute interval, with the head slightly tilted forward to facilitate flow. The target collection volume was approximately 3-4 mL.

Total RNA was isolated using a commercially available kit that includes a preservation buffer (RNA shield buffer) that stabilized RNA in the human saliva (Quick-DNA/RNA Miniprep Plus Kit, cat. # D7003, Zymo research, CA, US). The kit comprises purification columns capable of isolating up to 100 µg of high-quality RNA via a silica membrane in ≥ 50 µl (elution volume). In addition to the columns, the kit contains a protective agent (RNA shield buffer) that maintains total RNA integrity, preventing artifact gene induction or loss of gene expression during sample handling, thereby enabling reliable gene activity analysis. The kit could isolate RNA more than 17 nucleotides (nt) according to the manufacturer's specifications [10].

Following collection, saliva samples were mixed with the RNA shield buffer and were kept at 4 °C until the completion of the corresponding dental procedure. Samples were then transported in a cooled container to the Department of Medical Genetics for subsequent RNA isolation. All samples were collected within a 48–72-hour timeframe to minimize storage duration prior to processing. Total RNA was extracted using standard protocols suitable for saliva, and RNA concentration and purity were assessed using a spectrophotometer (NanoDrop, ThermoFisher Scientific, MA, US).

NanoString gene expression analysis

Gene expression profiling was performed using the NanoString platform (Bruker, WA, US) and using the Human Inflammation Panel (cat. #XT-CSO-HIN2-12). To account for inter-sample variability, data were normalized to the geometric mean of six stable housekeeping genes: *CLTC, GAPDH, GUSB, HPRT1, PGK1*, and *TUBB* [11]. Genes with a mean normalized count below 20 were excluded to minimize noise from low-abundance transcripts. The final dataset included 89 genes associated with inflammatory and immune pathways. Differential expression was evaluated by comparing periodontitis patients to healthy controls, with upregulation and downregulation patterns assessed to characterize the inflammatory profile in saliva.

RESULTS

A total of twelve saliva samples obtained from patients with advanced periodontitis were analyzed to assess RNA yield and purity as key determinants for the applicability of saliva in immune-related gene expression studies. RNA concentration varied substantially between samples, ranging from 20 to 98.5 ng/µL, indicating heterogeneous cellular content in the collected saliva. Despite this variability, the samples demonstrated concentrations above 20 ng/µL, which is generally considered sufficient for downstream qPCR-based gene expression assays (Figure 1).

Despite inter-individual differences, likely influenced by factors such as smoking or systemic comorbidities, most samples approached the recommended purity range for high-quality RNA.

Age of the patients	Stage of periodontitis	RNA concentration (ng/μL)	A 260	A 280	r 260/280	260/230
49	III	50.6	1.266	0.743	1.7	0.8
50	III	40.0	1.012	0.622	1.62	0.5
55	III	22	0.55	0.371	1.48	0.34
40	III	20	0.523	0.336	1.56	0.55
45	III	40	1.022	0.676	1.51	0.54
45	III	21	0.533	0.323	1.65	0.21
44	III	82	2.052	1.425	1.44	0.48
61	III	25	0.626	0.342	1.83	0.89
62	III	21.5	0.54	0.33	1.66	0.37
47	IV	53.4	1.34	0.81	1.65	0.7
33	no periodontitis	26.5	0.67	0.4	1.66	0.42
29	no periodontitis	98.5	2.46	1.74	1.42	0.48

Fig. 1. RNA concentrations and ratio indicators according to age and stage of periodontitis

Assessment of RNA purity using the A260/A280 ratio showed that most samples fell within an acceptable range for transcriptomic applications. The majority clustered between 1.50 and 1.70, with one sample reaching 1.83, reflecting relatively low protein contamination. Three samples (1.42-1.48) exhibited borderline purity, but still within workable limits. The A260/230 ratio was considerably lower across all samples, ranging between 0.21 and 0.89, which is considered normal for saliva samples because of organic substance contamination. There was no correlation between the patients' age or the stage of periodontitis and the quality indicators or the concentration of the isolated RNA.

The subsequent NanoString expression profiling was performed on all the RNA from all 12 saliva samples. After the normalization to the geometric mean of six stable housekeeping genes (*CLTC, GAPDH, GUSB, HPRT1, PGK1*, and *TUBB*), the transcripts showed the following quality parameters: mean percentage of probes above the threshold (95.55%), mean mRNA normalization factor (1.01), and mean mRNA content normalization factor (4.57) (Figure 2).

After the exclusion of the low-abundance transcripts (normalized transcript counts below 20), 89 genes altogether were found to be associated with inflammatory and immune pathways. The analysis revealed a pronounced pro-inflammatory signature in patients with advanced periodontitis, with 75% of genes upregulated and 25% downregulated relative to healthy controls, reflecting broad activation of inflammatory pathways in saliva from affected individuals. The successful execution of the RNA expression analysis by the NanoString platform confirmed the applicability of saliva as a source for RNA despite the commented imperfections.

Sample No	% probes above the threshold	mRNA positive normalization factor	mRNA content normalization factor
1	87.36	1.04	0.64
2	99.63	0.94	3.24
3	98.88	0.95	5.03
4	99.26	0.93	11.56
5	95.17	1.14	0.25
6	95.54	0.95	0.29
7	99.63	1.26	0.98
8	99.26	0.95	4.13
9	99.26	1.04	16.66
10	98.88	1.04	1.49
11	98.88	0.89	5.33
12	98.88	0.97	5.28

Fig. 2. Quality control parameters of the samples reported by NanoString software

DISCUSSION

The assessment of the RNA purity revealed that the majority of the analyzed samples covered the requirements for purity. Three RNA samples were borderline, suggesting partial protein carryover. The ratio A 260/230 was lower across the samples, but this is due to the contamination with polysaccharides, other organic compounds, and salts in saliva. Such values are typical for saliva-derived RNA due to the presence of mucins, food remnants, and microbial components. Although suboptimal, these ratios do not preclude gene expression analysis, as previous studies have demonstrated that low A260/230 values primarily affect spectrophotometric estimates rather than downstream amplification efficiency [12].

There is no clear linear relationship between RNA concentration and purity indicators, suggesting that higher RNA yield does not necessarily correlate with increased contamination. Boxplot analyses further confirmed the wide variability in concentration and consistently low A260/230 ratios, highlighting the inherent complexity of saliva as a biological matrix. Overall, the results demonstrate that saliva samples from patients with advanced periodontitis can provide sufficient RNA quantity and acceptable purity for immune-related gene expression studies. However, the uniformly low A260/230 values underline the necessity for optimized extraction and cleanup protocols to ensure reliable and reproducible downstream analysis.

The findings demonstrate that saliva, while challenging as a biological matrix, can yield RNA of adequate purity for gene expression studies when properly handled and stabilized. Previous work has shown that human mRNAs – both full-length and partially degraded – can be recovered from saliva, though stability is limited and degradation proceeds rapidly in unstabilized samples [13]. The wide variation in RNA concentration reflects known influences, such as epithelial shedding, oral microbiota, enzymatic degradation, smoking, and individual physiological differences. Indeed, in some transcriptome studies, the majority of RNA isolated from saliva has turned out to be microbial rather than human [7].

The NanoString nCounter platform proved suitable for saliva-based transcriptomics due to its sensitivity, direct probe-based detection, and compatibility with partially degraded RNA. Unlike qPCR or RNA-seq, NanoString does not require reverse transcription or amplification, reducing bias and making it well-suited for low-input and heterogeneous samples [14].

The observed upregulation of 75% of tested genes highlights strong immune activation in advanced periodontitis, consistent with the disease's known inflammatory profile. These results align with previous findings that salivary transcriptomes can distinguish patients with periodontitis from healthy individuals, albeit with limited overlap at the individual gene level compared to tissue-based profiles [9]. These data support the idea that saliva accurately reflects local immune responses and may serve as a surrogate for tissue-based expression profiling in periodontal disease. The presence of detectable and biologically relevant expression patterns strengthens the case for saliva's applicability for population studies, longitudinal monitoring, and non-invasive diagnostics [9].

Limitations of the study

This study represents a pilot investigation, and as such, the sample size – particularly of the control group - is limited. Future work will include a larger and more balanced control cohort to improve statistical power and generalizability. Standardization remains essential: RNA stabilization buffers, rapid processing, and controlled collection protocols critically influence yield and integrity [15]. Broader validation in larger cohorts is also necessary to determine the robustness of saliva-based gene expression biomarkers across diverse populations and disease severities. The variability between individuals – in salivary flow, oral microbiome composition, and environmental/ extrinsic factors – may result in inconsistent sample quality and limit reproducibility [16].

Despite these limitations, saliva-based transcriptomic profiling holds significant clinical promise. Non-invasive collection facilitates repeated monitoring and longitudinal studies, enabling early detection of periodontitis and other inflammatory diseases.

CONCLUSION

This study demonstrates that saliva can provide RNA of sufficient quantity and acceptable purity for immune-related gene expression analysis, even in the context of its inherent variability and degradation-prone nature. Using the NanoString nCounter system, reliable expression patterns were obtained, reflecting the inflammatory status of patients with advanced periodontitis.

Saliva represents a practical, non-invasive, and highly accessible biological material for molecular research in chronic inflammatory conditions. Its use may expand diagnostic opportunities, facilitate large-scale or longitudinal studies, and support broader applications in precision health and oral disease monitoring.

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Ethical statement: *This study has been performed in accordance with the ethical standards as laid down in the Declaration of Helsinki.*

Informed Consent from Participants: *Informed consent was obtained from all participants included in the study.*

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