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Saliva-based cell-free DNA and cell-free mitochondrial DNA in head and neck cancers have promising screening and early detection role

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Abstract

Background: Cell-free DNA (cfDNA) and cell-free mitochondrial DNA (cf-mtDNA) have been postulated as potential diagnostic and prognostic biomarkers for different human malignancies. Early detection of head and neck malignancies is fundamental for optimal patient management. This study, therefore, aimed to assess the utility of saliva-based liquid biopsy as a noninvasive source of cfDNA and cf-mtDNA for detecting head and neck cancer (HNSCC).

Methods: One hundred thirty-three patients diagnosed with either oral leukoplakia (OLK) or HNSCC were compared with 137 healthy volunteers. An unstimulated whole saliva sample was collected from each participant. The absolute copy numbers of salivary cf-mtDNA and cfDNA were quantified using Multiplex Quantitative PCR. Two diagnostic indices based on the investigated molecules were assessed for their ability to differentiate between different diagnostic categories.

Results: The median scores of cfDNA and cf-mtDNA were statistically significantly higher among HNSCC patients ($p < 0.05$), revealing area under the curve values of 0.758 and 0.826, respectively. The associated accuracy for this test in discriminating HNSCC from other diagnostic categories was 77.37% for the cfDNA-based index and 80.5% for the cf-mtDNA-based index. The median score of cfDNA was statistically significantly higher for patients with severe epithelial dysplasia (OED) compared to those with epithelial keratosis with no OED and mild OED. However, there was no significant difference between controls and OLK individuals.

Conclusion: cfDNA and cf-mtDNA showed potential for use as precision medicine tools to detect HNSCC. Further multi-centre prospective studies are warranted to assess the prognostic utility of these molecules.

KEYWORDS
cell-free DNA, cell-free mitochondrial DNA, head and neck cancer, liquid biopsy, precision medicine

1 | INTRODUCTION

Head and neck squamous cell carcinomata (HNSCC) are aggressive, life-threatening malignancies involving the mucosal epithelium of the oral cavity, pharynx, and larynx.[1] While HNSCC is one of the most deadly human malignancies, its 5-year survival rates have slightly improved over the past 15 years.[2] Early diagnosis of HNSCC substantially affects survival.[1] Surveillance, Epidemiology, and End Results registry reported 5-year survival rates of up to 85% in cases with localised HNSCC compared to less than 40% for those with distant metastasis.[2]

Early diagnosis of HNSCC in some cases is challenging, whereby a histopathological assessment of surgical biopsies is the gold standard for HNSCC diagnosis.[1] However, surgical biopsy has several limitations for being invasive, not always feasible, requires specialised training, and does not detect intra-tumoural, temporal and metastatic heterogeneities, which in turn negatively affects the assessment accuracy.[3] Moreover, symptom recognition and appropriate referral of malignant and potentially malignant lesions are not usually predictable as they solely depend on an individual's knowledge and competency.[4] A previous study reported that HNSCC patients had two to three consultations with their general dental practitioners before referral to receive a final diagnosis.[5] Likewise, it has been shown that the overall diagnostic accuracy among general dentists in detecting squamous cell carcinoma (SCC) and oral leukoplakia (OLK) was less than 50%.[4] Therefore, a striking need to develop a tool that helps general practitioners detect and triage patients with HNSCC is warranted.[6] Attempts were undertaken using various tools, such as oral brush biopsy, which showed promising results.[6,7] However, the necessity of certain levels of training, as well as the related expenses, may hinder their applications.[6,7] In recent years, liquid biopsy, as a reliable tool in precision medicine, has gained an expanding interest in terms of cancer detection and treatment planning.[3,8] This approach is defined as sampling and analysing non-solid biological materials like blood, urine, and saliva, and it represents a rapid and minimally/non-invasive alternative to tissue biopsies.[3] A recent comparative next-generation sequencing study demonstrated a good patient-level concordance of certain nucleic acid biomarkers between tissue and liquid biopsies, highlighting the potential utility of liquid biopsies in cancer diagnostics.[9]

Circulating cell-free DNA (cfDNA) is degraded double-strand DNA fragments from cells of the whole body.[8] The haematopoietic system is responsible for releasing cfDNA in the plasma of healthy individuals,[10,11] while the presence of certain physiological or pathological conditions, such as pregnancy or malignancy, releases additional cfDNA into body fluids.[11] Thus, the detection of cfDNA provides a unique tool to identify abnormalities in a minimally or non-invasive manner.[11,12] In cancer patients, tumour cells contribute to cfDNA by releasing circulating-tumour DNA (ctDNA), where tumour genetic aberrations like mutations and epigenetic alterations can be detected in cfDNA using next-generation sequencing.[8] In attempts to evaluate potential applications of the cfDNA as a diagnostic biomarker in cancer, several quantitative-based studies found that the cfDNA concentration in various body fluids (blood and urine) is higher among cancer patients compared to healthy individuals.[13,14]

Though cfDNA has genomic DNA origin, there is another contributor to cfDNAs with its genome, mitochondria.[12] Cell-free mitochondrial DNA (cf-mtDNA) are short sections of mitochondrial DNA released by cells undergoing stress or pathological changes.[12] Previous studies found that cf-mtDNA is a more efficient marker than cfDNA in detecting cancer patients,[12,15] while the specificity of quantifying cf-mtDNA was up to 97% in identifying patients with urologic malignancies.[15]

Literature included limited, and in some instances contradicted, information about the profile and the diagnostic utilities of cfDNA and cf-mtDNA in HNSCC. Hereof, the primary aim of this study was to investigate the diagnostic utility of the quantity of these molecules in discriminating HNSCC from healthy individuals. The second principal aim was to assess the influence of various demographic, clinical, and microscopic variables on the quantity of saliva-based cfDNA and cf-mtDNA.

2 | MATERIALS AND METHODS

2.1 | Study design and participants

This quantitative correlational cross-section study was approved by the Human Ethics Committee at Damascus University (Ref: 1065-2019) and conducted between January 2019 and December 2020 at the Faculty of Dentistry of Damascus University and Al-Mowasah Hospital (Damascus, Syria). The study abided by STARD 2015 guidelines for Reporting Diagnostic Accuracy Studies and was carried out following the Declaration of Helsinki.

The participants were divided into two major categories, pathological (HNSCC and OLK) and healthy controls. All participants aged more than 18 years and signed informed consent. For selecting the pathological group, the following criteria were used (1) patients clinically differentially diagnosed with OLK or suggestive HNSCC by an oral medicine/oral surgery specialist, (2) no cancer history (other than the potential malignancy for HNSCC patients), (3) no history of chemotherapy or radiotherapy, (4) no history of immunotherapy or autoimmune diseases, and (5) no current contraindication for undergoing surgical biopsies. Surgical biopsies from the pathological participants were assessed microscopically by two senior pathologists to confirm the diagnosis of lesions.[16] Cases with oral epithelial dysplasia (OED) were categorised based on World Health Organization (WHO) classifications.[16] For healthy controls, the following inclusion criteria were applied (1) patients visited dental clinics at Damascus University for routine dental procedures not related to oral soft tissue problem, (2) no history of oral potentially malignant disorders, (3) no history of malignancies, (4) no history of chemotherapy or radiotherapy, and (5) no history of immunotherapy or autoimmune diseases. Participants with chronic medical conditions and currently under medication were excluded. Individuals with a history of organ transplant and female participants who are pregnant were also excluded.

Relevant demographic and social data were collected from each participant. Individuals who currently smoke, or who had smoked in the last year, an average of 10 cigarettes per day were considered smokers.

2.2 | Saliva collection

All participants were asked to rinse their mouths with sterile water and to refrain from eating, drinking, smoking, or brushing their teeth at least 2 h before sample collection. The participants were then asked to relax for 5 min and to swallow saliva in their mouths.[17] Unstimulated whole saliva was then collected for 5–10 min (until a fill line was reached) using a saliva collection kit (PAXgene Saliva Collector, Cat, # 769040, Qiagen). This kit contains stabilising agents to protect salivary DNA from degradation over transportation time. All samples were stored at 4°C and processed in a laboratory within 24 h.

2.3 | Saliva DNA extraction and qPCR amplification

To extract DNA from the saliva samples, each sample was centrifuged at 1600 rpm for 10 min at 4°C to remove cells and for another 10 min at 2600 rpm to remove cellular debris. The supernatant was carefully aspirated and stored at −80°C until DNA extraction. DNeasy Blood and Tissue Kit (Cat, # 69504, Qiagen) was applied to extract DNA from the processed saliva supernatant according to its manufacturer's instructions. Total DNA from each sample was quantified using Qubit dsDNA HS and BR Assay Kit (Cat, # Q32850, Invitrogen) and Qubit 4.0 Fluorometer (Cat, # Q33238, Invitrogen). The final eluate was collected and stored at −80°C until further analysis.

Quantification of cfDNA and cf-mtDNA was performed using multiplex qPCR assays. Human-specific primers were used for the detection of the beta-2-microglobulin gene (M-17987) and the mitochondrial gene (NC-012920) (Table S1). Each sample was assessed in triplicate. The reaction mixture contained 11.5 µl of Luna™ Universal Probe qPCR Master Mix (Cat, # M3004X, New England BioLabs), 4 µl of DNAse-free water, 1 µl of each primer, 1 µl of each probe, and 2.5 µl of DNA in a total reaction volume of 20 µl. Thermal cycling was undertaken using CFX Connect™ Real-Time System (BIO-RAD) according to the following parameters (i) initial denaturation step at 95°C for 1 min, (ii) 60 cycles of denaturation at 95°C for 15 s, (iii) annealing at 55°C for 15 s, and (iv) elongation at 60°C for 15 s.

2.4 | Statistical analysis

Statistical analyses were carried out using IBM SPSS Statistics (Version 28, IBM Corporation) while the associated graphs were generated using GraphPad Prism (Version 9.4). The level of significance was set as $p < 0.05$. The chi-square test of independence was undertaken to assess the association between categorical variables. Shapiro–Wilk's test of normality was calculated to assess if continuous variables follow a normal distribution, p-values less than 0.05 indicate deviation from normality. Nonparametric tests (Mann–Whitney *U* test and Kruskal–Wallis H test) were performed to assess the associations between cfDNA and cf-mtDNA with other variables. Spearman's correlation assessed the associations between two continuous variables. A receiver operating characteristic (ROC) curve was undertaken to assess the diagnostic accuracies of the proposed diagnostic indices. Yoden's index was calculated to identify the optimal cut-off based on the coordinates of the ROC curve. Accuracy, sensitivity, specificity, positive predictive value, and negative predictive value were calculated based on the optimal cut-off points and according to their standard equation.

3 | RESULTS

3.1 | General characteristics of participant variables

A total of 270 participants were included in this study, and more than half were males (58.9%). The mean age of the participants was 48.1 years (SD ± 15.9) and ranged from 25 to 77 years. Males were significantly older than females, 51.5 (SD ± 14.2) and 43.2 (SD ± 17) years, respectively ($p < 0.0005$).

The participants were categorised according to their diagnosis into three diagnostic categories (1) 102 patients diagnosed with HNSCC (64 with laryngeal SCC and 38 with oral cavity SCC), 31 patients diagnosed with OLK, and 137 healthy controls (Table 1). The mean age of HNSCC patients was statistically significantly higher than OLK patients and controls, $p < 0.0005$ (Table 1).

The diagnostic categories were statistically significantly associated with the participant's gender, smoking status, and duration of smoking ($p < 0.0005$), post hoc analyses were performed to further explicate these relations (Table 1).

The microscopic examination of OLK surgical biopsies revealed the following grades of epithelial disorders: (1) 10 patients with epithelial keratosis with no OED, (2) four patients with mild OED, (3) five patients with moderate OED, and (4) 12 patients with severe OED. None of OLK cases underwent malignant transformation.

3.2 | The association between cfDNA and study variables

The age of the participants was statistically significantly associated with the mean of cfDNA copies/mL, $p = 0.003$. Shapiro–Wilk's normality tests showed that cfDNA copies/ml were not normally distributed based on gender, smoking status, diagnostic category, location of SCC, and stage of SCC, $p < 0.05$. Thus, nonparametric tests (Mann–Whitney U test and Kruskal–Wallis H test) were the tests of choice to assess the associations between these variables.

TABLE 1 General description of the participant variables and their association with the diagnostic categories

Variables	HNSCC	OLK	Control	All participants	p-Value
Gender				Total *N* (%)	< 0.0005
Male *N* (%)	82 (80.4)[a]	22 (71)	55 (40.1)	159 (58.9)	
Female *N* (%)	20 (19.6)[a]	9 (29)	82 (59.9)[a]	111 (41.1)	
Mean age				Mean (SD)	< 0.0005
All participants (SD)	58.8 (11.2)[b]	46.58 (9.9)	40.4 (15.5)	48.1 (15.9)	
Male (SD)	58.5 (9.8)	47.8 (9.4)	42.4 (15.8)	51.7 (14.2)	0.45
Female (SD)	60 (16)	43.6 (11.1)	39 (15.3)	43.1 (17)	
Smoking status				Total *N* (%)	< 0.0005
Yes *N* (%)	78 (76.5)[a]	22 (71)	13 (9.5)[a]	113	
No *N* (%)	24 (23.5)	9 (29)	124 (90.5)[a]	157	
Duration of smoking (years)					< 0.0005
All participants (SD)	33.8 (12)[b]	21.8 (10.5)	19.8 (9)	29.8 (12.8)	
Total *N* (%)	102 (37.8)	31 (11.5)	137 (50.7)	270 (100)	-

Abbreviations: HNSCC, Head and neck squamous cell carcinomata; OLK, oral leukoplakia.
[a]Post hoc analysis, Bonferroni-adjusted p-value <0.0083.
[b]Games–Howell post hoc test p-value <0.05.

TABLE 2 The associations between cf-mtDNA, cfDNA, and cf-mtDNA/cfDNA with other participant variables

	cfDNA			Cf-mtDNA		
Variables	Median (mega copies/ml)	Mean rank	p-Value	Median (mega copies/ml)	Mean rank	p-Value
Gender[a]	4.3	-	0.121	1.69	-	0.019
Male	5.33	127.6		1.91	130.1	
Female	2.78	113.4		1.55	108.7	
Smoking status[a]	4.3	-	0.076	1.69	-	<0.0005
Yes	5.64	131.1		3.12	161.1	
No	3.56	115		1.01	95.3	
Diagnosis[b]	4.3		<0.0005	1.69	-	<0.0005
Control	2.29	96.3		0.92	93.2	
OLK	2	98.6		1.44	103.5	
HNSCC	7.31	159.4		5.12	173.9	
HNSCC location[a]	7.32			5.12		
Oral cavity	6.78	42.7	0.085	4.49	34.7	0.1
Laryngeal	8.16	53.1		6.1	43.5	

Abbreviations: HNSCC, Head and neck squamous cell carcinomata; OLK, oral leukoplakia.
[a]Mann–Whitney U test.
[b]Kruskal-Wallis H test.

According to Mann–Whitney U tests, neither the participant's gender nor their smoking status was statistically significantly associated with the median score of cfDNA copies/ml ($U = 6226.5$, $z = -1.549$, $p = 0.121$) and ($U = 6296.0$, $z = -1.776$, $p = 0.076$), respectively (Table 2).

The median score of cfDNA was associated significantly with the diagnostic categories according to a Kruskal-Wallis H test, $\chi^2(2) = 46.59$, $p < 0.0005$ (Table 2). Further post hoc analysis revealed statistically significant differences in the median score of cfDNA between controls and HNSCCs ($p < 0.0005$) and between OLKs and HNSCCs ($p < 0.0005$) but not between controls and OLKs ($p = 0.877$) (Figure 1A).

There were no statistically significant associations with both the location and the stage of HNSCC ($U = 857.0$, $z = -1.724$, $p = 0.085$) and ($\chi^2(4) = 2.287$, $p = 0.683$), respectively (Table 2 and Figure S1). On the contrary, the grade of epithelial disorders for OLK patients was statistically significantly associated with the median score of cfDNA ($\chi^2(3) = 15.804$, $p = 0.001$). Pairwise comparisons showed that the median score of cfDNA was significantly higher for patients with severe OED than patients with epithelial keratosis and patients with mild OED, $p = 0.015$ and $p = 0.01$, respectively.

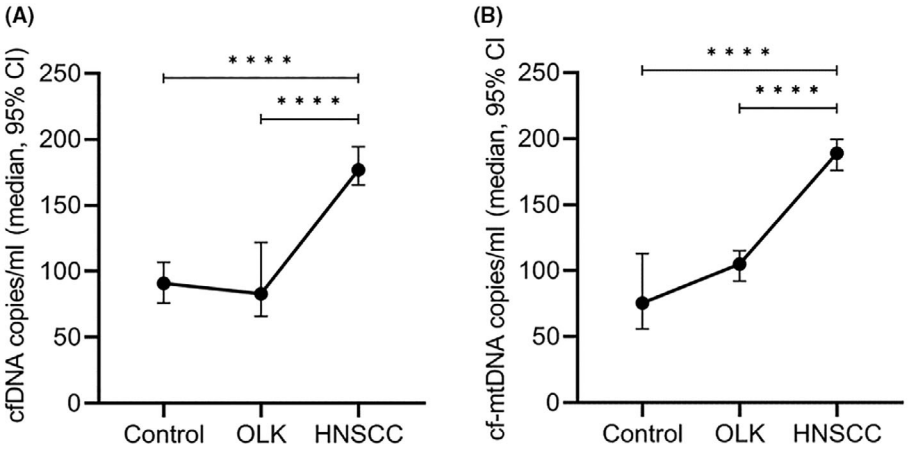

FIGURE 1 The associations between the three diagnostic categories with A: the median score of cfDNA, B: the median score of cf-mtDNA. **** $p < 0.0005$

A multistep multinominal regression model was run to understand the effect of six variables (age, gender, smoking status, duration of smoking, and HNSCC location and stage) on the values of cfDNA, accordingly, this model was statistically not significant, $p = 0.7$.

3.3 | The association between cf-mtDNA and study variables

There was a statistically significant association between the participant age and the mean of cf-mtDNA copies/ml, $p < 0.0005$. The absolute number of copies/ml for cf-mtDNA was not normally distributed among the participants based on their gender, smoking status, diagnostic category, location of SCC, and stage of SCC as assessed by Shapiro–Wilk's normality test, $p < 0.05$.

The median score of cf-mtDNA was statistically significantly higher for the male participants than the female ones, $U = 5846.5$, $z = -2.354$, $p = 0.019$ (Table 2). Likewise, the median score of cf-mtDNA was statistically significantly higher among smokers in comparison to nonsmokers, $U = 3137.5$, $z = -7.145$, $p < 0.0005$ (Table 2). Post hoc analysis showed that the median score of cf-mtDNA increased among smokers in the control and HNSCC groups, $p = 0.003$ and $p = 0.038$, respectively (Figure 2).

The median score of cf-mtDNA was statistically significantly associated with the diagnosis, $\chi^2(2) = 68.261$, $p < 0.0005$ (Table 2). Subsequently, post hoc analysis for pairwise comparisons revealed statistically significant differences in the median score cf-mtDNA copies/ml between controls and HNSCCs ($p < 0.0005$) and between OLKs and HNSCCs ($p < 0.0005$), but not between controls and OLKs ($p = 0.46$) (Figure 1B).

Neither the stage nor the location of HNSCC was statistically significantly associated with the median score of cf-mtDNA, ($\chi^2(4) = 3.437$, $p = 0.487$) and ($U = 587.5$, $z = -1.643$, $p = 0.1$), respectively (Table 2 and Figure S1).

There was no association between the grade of epithelial disorders for the OLK patients and the median score of cf-mtDNA, $\chi^2(3) = 8.148$, $p = 0.063$. A multinominal regression model to predict values of cf-mtDNA based on six variables (age, gender, smoking status, duration of smoking, location of HNSCC and stage of HNSCC) was statistically not significant, $p = 0.288$.

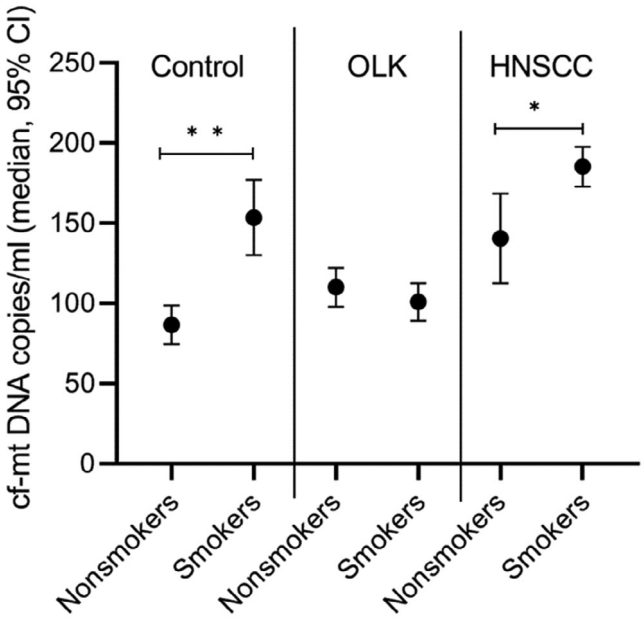

FIGURE 2 The median score of cf-mtDNA among the participants in the different diagnostic categories based on their smoking status. * $p < 0.05$, ** $p < 0.005$

3.4 | Diagnostic utility of cfDNA and cf-mtDNA

The absolute number of copies/ml for each of cfDNA and cf-mtDNA was used to propose a diagnostic index for each of them. ROC curves to discriminate HNSCC patients from OLK and healthy individuals showed AUC values of 0.758 (95% CI, 0.691–0.825) for cfDNA-based index and 0.826 (95% CI, 0.773–0.88) for cf-mtDNA-based index (Figure 3). A third index based on the ratio of cf-mtDNA and cfDNA was tested and did not show a useful utility in terms of the AUC value (AUC = 0.608, 95% CI, 0.527–0.689).

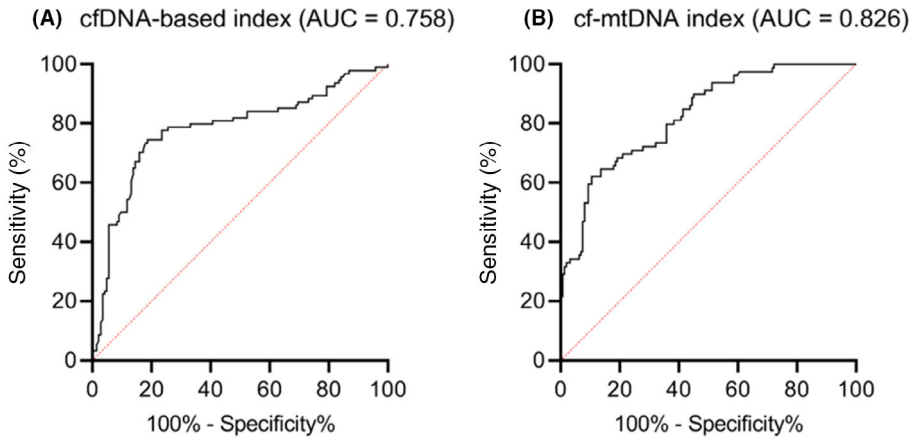

FIGURE 3 The ROC curves for the utility of the proposed diagnostic indices in the discrimination between HNSCC and other diagnostic categories, (A) cfDNA-based index, (B) cf-mtDNA-based index

TABLE 3 Optimal cut-off values based on the number of cfDNA and cf-mtDNA mega copies/ml and the related statistics

Indices optimal cut-off values	Sensitivity % (95% CI)	Specificity % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	Accuracy % (95% CI)
cfDNA	71.43	81.38	72.16	80.82	77.37
HNSCC ≥5.74	(61.4–80.1)	(74.1–87.4)	(64.3–78.8)	(75.3–85.3)	(71.6–82.5)
cf-mtDNA	74.24	82.9	62	89.51	80.5
HNSCC ≥3.85	(61.9–84.2)	(76.4–88.1)	(53.4–70)	(84.9–92.8)	(74.9–85.3)

Abbreviations: HNSCC, Head and neck squamous cell carcinomata; NPV, negative predictive value; PPV, positive predictive value.

Youden's index was calculated based on the coordinates of the ROC curve to provide optimal cut-off levels for both cfDNA copies/ml and cf-mtDNA copies/ml. Accordingly, the cut-off value for the cfDNA was 5.74 mega copies/ml and the associated accuracy of this approach in the discrimination between HNSCC and non-HNSCC conditions was 77.37%. While the cut-off value for cf-mtDNA was 3.85 mega copies/ml and showed an accuracy of 80.5% (Table 3).

4 | DISCUSSION

In modern medicine, precision medicine and early detection integrate into a significant paradigm: point-of-care diagnostics. Point-of-care diagnostics aims to achieve fast, minimally invasive, accurate, and cost-effective diagnostic results.[18] All efforts are currently directed to maximise standards of care; interestingly, this approach showed specificities exceeded 98% among patients with certain malignancies like colon and cervical cancers.[18]

Nonetheless, to date, HNSCC has no standard tool for screening or triaging patients,[6] contributing figures that show 50% of patients with head and neck cancer are diagnosed at late stages. Reported delays in HNSCC diagnosis ranged from 2 to 15 months once symptoms appear.[19,20] This study was designed with a primary aim of providing a non-invasive tool that can be employed for early detection and/or triaging patients with potential HNSCCs. To achieve this goal, saliva-based liquid biopsies were employed to quantify specific molecules among a cohort of patients with different diagnostic categories.

Saliva is known for its diagnostic utility because it includes various components like DNA, RNA, proteins, and metabolites.[3] A high-throughput genotyping study concluded that saliva is a valid alternative to blood samples regarding DNA genotype and quality.[21] Moreover, saliva-based liquid biopsy to detect HNSCC has several advantages over other body fluids because (i) it reflects genomic, proteomic and pathological changes in the oral cavity, larynx and pharynx, (ii) noninvasive, readily available and accessible in high volumes, and (iii) allows real-time monitoring of patients by repetitive self-sampling.[3]

The biomarkers of choice for this purpose were cfDNA and cf-mtDNA since their utility as diagnostic biomarkers was already established in several human malignancies. In this study, the quantity of saliva-based cfDNA and cf-mtDNA has increased significantly among HNSCC patients relative to others; these results are harmonious with previous plasma-based findings for these molecules.[13,22] On the contrary, two studies did find a significant difference in cfDNA between oral cancer patients and others.[23,24] For the first study, the authors quantified DNA by spectrophotometry, not qPCR,[23] however, this method alone is known for its inconsistency and inability to distinguish between double-stranded DNA, single-stranded DNA, oligonucleotides, and free nucleotides.[25] For the second paper, cfDNA increased among oral cancer patients but not significantly.[24] This is probably attributed to the small sample size of that study, mainly because their proposed cfDNA integrity index showed a significant association with oral cancer patients.[24]

Noteworthy, it has been proposed that cf-mtDNA has a distinct benefit over cfDNA due to its shorter length and more straightforward

organisation.[12] Due to the mitochondrial DNA's location near the mitochondrion's inner membrane, it is highly susceptible to damage by reactive oxygen species and mutagens, making cf-mtDNA easy to identify in liquid samples with low DNA yields.[12] This may also explain the significant increase in the quantity of cf-mtDNA, but not cfDNA, among smokers compared to nonsmokers. Tobacco-associated products are one of the exogenous sources that enhance the accumulation of mutations in the mitochondrial DNA as well as produce endogenous reactive oxygen species, which upon persistence, causes mitochondrial dysfunction starts due to exceeding its anti-oxidant response level.[26]

On the other hand, the quantities of cfDNA and cf-mtDNA were comparable between the OLK patients and the healthy controls. According to a previous systematic review in 2019, only one study assessed the quantity of cfDNA among oral potentially malignant disorders where no significant difference was reported.[23] Likewise, a proposed cfDNA integrity index did not show a statistically significant difference between oral cancer and OLK patients.[27] We found significant differences between patients with mild OED and severe OED and between patients with keratosis and severe OED, which probably highlights the potential of using cfDNA for estimating the grade of epithelial dysplasia among patients. However, due to the limited number of patients diagnosed with OLK in this study, further investigations including large cohorts of patients with oral potentially malignant disorders are required to provide conclusive outcomes.

The age of the participants in this study was statistically significantly associated with quantities of cfDMA and cf-mtDNA. This finding was not surprising as these molecules have been reported previously as biomarkers of ageing.[28] While this was attributed to an ageing-related phenomenon of increased cellular stress and death accompanied by low-grade inflammation,[29,30] it importantly highlights the utility of saliva to provide real-time snapshots of body dynamics.

In this study, there are three main limitations. Firstly, being a cross-sectional study without follow-up data limited the ability to assess the prognostic values of the investigated molecules. Second, there were no blood samples to assess the concordance between saliva and blood at the patient level. Considering blood as a gold standard to measure cfDNA and cf-mtDNA, such data are valuable to further assess the significance of this approach. Finally, the number of patients in the OLK was relatively small and without follow-up data, which hindered our ability to assess the utility of using cfDNA and cf-mtDNA in detecting patients with oral potentially malignant disorders and predicting their risk of malignancy.

In conclusion, quantifying cfDNA and cf-mtDNA in saliva-based liquid biopsy samples showed great potential utility as a precision medicine tool to detect patients with HNSCC. While our results are based on a single centre, further multi-centre longitudinal studies are warranted for further validation and optimization.

AUTHOR CONTRIBUTIONS

Lana Sayal: Conceptualization; data curation; investigation; writing-original draft. **Omar Hamadah**: Conceptualization; methodology; project administration; supervision; writing-review and editing. **Aroub Almasri**: Methodology; resources; supervision. **Majdy Idrees**: Formal analysis; writing-review and editing. **Peter Thomson**: writing-review and editing. **Omar Kujan**: Conceptualization; formal analysis; writing-review and editing.

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CONFLICTS OF INTEREST

Authors declare no conflict of interests.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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