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Stability of circulating miRNA in saliva: The influence of sample associated pre-analytical variables

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ABSTRACT

Background and aims: Increasing evidence supports the practicability of salivary cell-free (cf) miRNA as liquid biopsy markers in cancers. Its successful translation in the clinical setting requires reproducible approaches for saliva manipulation, in order to control for pre-analytical variables influencing miRNA stability. This study aims to define the optimal conditions to maintain the integrity of saliva during collection, transport and processing with respect to cf-miRNA quantification.

Materials and methods: Saliva was collected from 20 healthy subjects and 8 oral cancer patients. Two sampling methods were tested and different storage temperatures and times were evaluated. Salivary expression level of target miRNAs was quantified by qPCR. Comparison between group mean values at specific conditions were performed using paired t-tests. Agreement between measurements was evaluated using a Bland-Altman plot.

Results: Different collection methods revealed comparable levels of salivary miR-484 and miR-106b-5p in both subject cohorts. MiRNAs were stable for up to 48 h at 4 °C in saliva supernatant, showing significant alteration after 96 h. Mid-term storage of supernatant at −20 °C decreased miRNA stability significantly compared to standard −80 °C.

Conclusions: Cf-miRNA in saliva were slightly altered by collection methods and storage conditions, both in healthy and in pathological contexts, and remained stable for a period of time compatible with main clinical routine needs.

1. Introduction

MicroRNAs (miRNAs) are small non-coding RNA molecules with altered expression in a variety of cancers and remarkable stability in biological fluids. These are promising characteristics for defining the diagnosis and prognosis of tumors using liquid biopsy. In this regard, saliva is a suitable biofluid for monitoring oral disease by measuring circulating levels of miRNAs, also considering the permanent contact of saliva with oral cancer cells and the potential transfer of genetic material to it [1,2]. Numerous studies have demonstrated the clinical efficacy of extracellular circulating miRNA in the diagnosis and prognostic stratification of patients with oral cancer, demonstrating their potential as non-invasive biomarkers [3–5].

Although promising, translation of salivary miRNAs in clinical practice faces a number of obstacles, such as the need to establish optimal conditions for saliva processing and preservation of miRNAs

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following collection, particularly circulating extracellular miRNAs. This field of study is still in its infancy, and little is known about the susceptibility of this class of molecules to pre-analytical variables [6]. Common methods for collecting saliva rely on passive sampling by spittle, which ensures a more uniform saliva composition, followed by separation of cleared supernatant and long-term storage at −80 °C [7]. It is recommended that the time between collection, which is typically performed during routine clinical procedures, and processing in the laboratory be as brief as possible to ensure the preservation of saliva's inherent characteristics and the stability of its extracellular miRNA pool. Indeed, all of these steps may affect the quality and quantity of circulating miRNAs and introduce potential sources of error in subsequent analysis, especially for qPCR-based assays, where template quality is one of the most important factors determining the reproducibility of miRNA expression measurement [8].

In this study, we investigated the stability of cell-free (cf) miRNAs in the saliva of healthy donors under a variety of experimental conditions, taking into account the most important variables that could affect miRNA recovery and stability. These include alternative collection strategies and storage temperatures, in addition to the effect of delayed saliva processing on miRNA expression levels. In addition, a pilot study was conducted on a cohort of patients with squamous cell carcinoma of the head and neck (HNSCC) to test experimental reproducibility in a pathological context.

2. Materials and methods

2.1. Patient cohort

Unstimulated whole saliva was collected from 20 healthy volunteers over a 3-month period between December 2021 and February 2022, and from 8 HNSCC patients diagnosed and treated at the Unit of Otorhinolaryngology – Head and Neck Surgery of the Spedali Civili, University of Brescia, Italy, between September 2022 and February 2023. Written informed consent was obtained from all participants under the IDENTIFY protocol at Spedali Civili.

of Brescia (Ethical Committee approval nr NP 4551). The healthy control group was comprised of 13 females and 7 males (age range, 25–57 years, median 32). The HNSCC group was comprised of 2 females and 6 males (age range, 42–71 years, median 57).

2.2. Sample collection and processing

Saliva samples were collected in two different ways, either by spitting in a plastic container or by using the LolliSponge® device (Copan Italia S.p.A.). Patients were requested to refrain from smoking, eating, drinking and oral hygiene procedures for at least 1 h prior to saliva collection. After mouth rinsing with 10 ml of sterile PBS, they were asked to spit into a sterile 50 ml Falcon tube and saliva was immediately centrifuged at 2600 g for 15 min at 4 °C to remove cellular components,

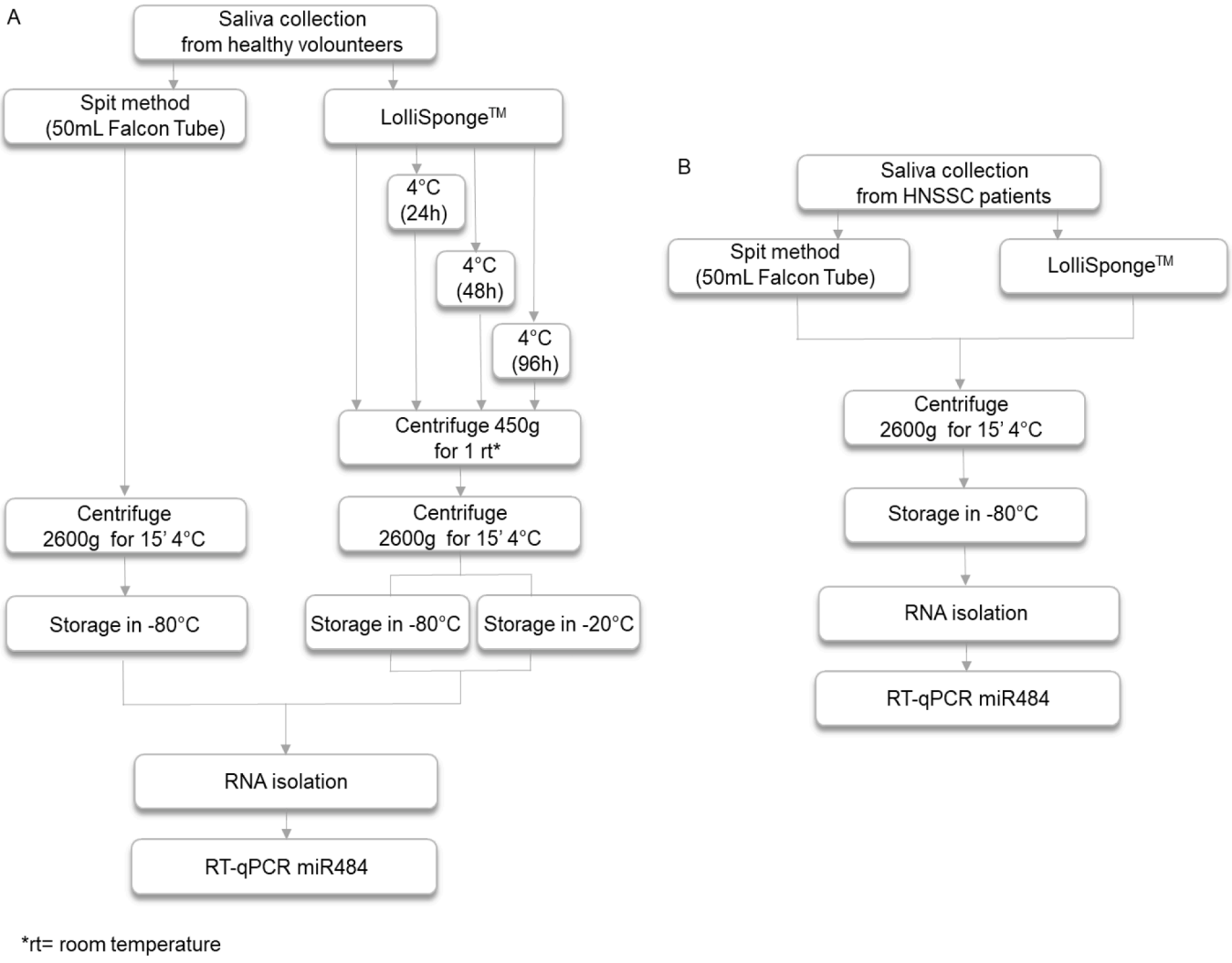


Fig. 1. Flowchart of whole saliva collection by passive spit or Lollisponge® in healthy (A) and HNSCC (B) subjects.

according to published protocols [3]. Patients were then asked to accumulate saliva again on their mouth floor for 10 s prior to collection with the LolliSponge® device by gently pressing the sponge with the tongue for 60 s, according to the Manufacturer's instructions. For healthy volunteers, sampling was repeated and LolliSponge® left at 4 °C for up to 96 h before processing.

The LolliSponge® was centrifuged at 450g for 60 s to release the saliva from the sponge, and processed as described above to separate the supernatant from cells. Cell-free saliva supernatants were stored in −80 °C or in −20 °C until RNA isolation. A flow-chart of the process is depicted in Fig. 1A-B.

2.3. RNA extraction and miRNA quantification by qPCR

Total RNA was extracted from 200 μl of cell-free saliva using miRNeasy Mini kit (Qiagen,

Hilden, Germany), with a modified protocol for co-purification of small RNAs from liquid samples as previously described [9]. The miRCURY locked-nucleic acid Universal real-time (RT) miRNA PCR system (Exiqon, Qiagen, Germany), based on universal reverse transcription followed by RT PCR amplification with miR-specific primers (miRCURY LNA miRNA PCR assay YP00205636 and YP00205884 Exiqon, Qiagen), was used for first-strand cDNA synthesis and SYBR Green-based amplification. All samples were run in duplicate using Bio-Rad CFX96 Real-Time system (Bio Rad).

2.4. Data analysis

The expression levels of miRNA were described using the mean and standard deviation. qPCR data were normalized by calculating the difference between the quantitation cycle (Cq) values of the target miRNA and the endogenous reference. To account for within-sample correlation, the longitudinal trend in miRNA expression was estimated using linear mixed models (LMM) with random intercept [10]. Comparison between group mean values at specific conditions were performed using paired t-tests. Results are reported as estimated means or differences, with associated 95 % confidence intervals. Agreement between measurements was evaluated using a Bland-Altman plot [11]. All tests were two-sided and assumed a 5 % significance level. All the analyses were performed using R (version 4.3.0) and GraphPad.

3. Results

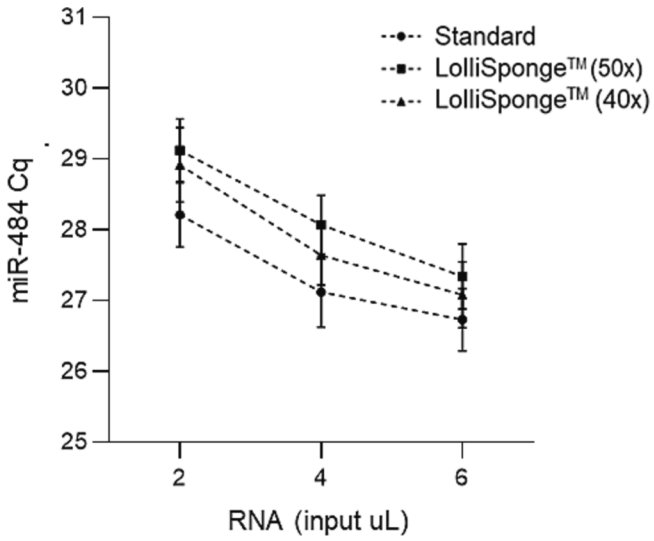
Collection of whole saliva by passive sampling followed by separation of supernatant and immediate storage at low temperature is currently recommended for quantification of circulating levels of miRNA by means of molecular biology techniques [5,12].

We therefore considered saliva spit as a standard reference method (from now on Standard) against which an alternative device, such as LolliSponge®, may be compared. The latter uses inert absorbent material as the collection media that effectively release biomolecules from the pad. Considering that the amount of RNA that can be recovered from saliva, as well as the amount of inhibitors left after purification, can vary from method to method, we first checked the quality of RNA purified either by Standard and LolliSponge® samples in order to: *i)* monitor the purification yield and identify possible signs of enzymatic reaction inhibition related to both the collection strategies, and *ii)* identify comparable RNA input amount for RT and qPCR reactions. We thus performed independent RNA isolation from saliva supernatants and diluted purified RNA in a ratio of 1:2 for subsequent cDNA synthesis and miRNA amplification, as recommended by guidelines for miRNA profiling in biofluids [13]. Expression level of endogenous miR-484, which is typically present and stably expressed in human fluids [14], including saliva [5], was measured.

Result of dilution series shows a linear relationship between RNA input and Cq value for both Standard and LolliSponge® samples, with no sign of PCR inhibition and indicative that accurate miR-484 quantification is obtained at each datapoint (Fig. 2). For each of the RNA input amounts, the level of miR-484 did not differ significantly between Standard and LolliSponge®samples, nor considering the cDNA dilution factor (LMM, all $p > 0.1$). For LolliSponge®, the 40x dilution factor yields miR-484 Cq value more similar to Standard and thus we set this experimental condition before expanding the comparison to a cohort of 20 healthy subjects and 8 HNSCC patients. In healthy controls, miR-484 was consistently amplified in all samples (Standard average^{Cq} = 27.10, SD = 1.38; LolliSponge® average^{Cq} = 27.74, SD = 1.61), with invariable expression in Standard compared to matched LolliSponge® (paired *t*-test: mean of differences = 0.642, $IC_{95\%}$ −0.1896 to 1.474, p = 0.122). Similar trend in miR-484 expression was observed in HNSCC (Standard average^{Cq} = 30.81, SD = 2.96; LolliSponge® average^{Cq} = 30.66, SD = 2.97; paired *t*-test: mean difference = -0.143, $IC_{95\%}$ −0.739; 0.452, p = 0.59). We used Bland-Altman plot to assess the agreement between miR-484 measurement using Standard and Lollisponge® collection methods. Data plotted in Fig. 3A-B, which show the mean difference between two independent measurements for each subject in both healthy and HNSCC, indicate that no collection-related systematic miR-484 under- or over-estimation occurred in either subject's cohorts. Concordance is greater in the HNSCC than in the healthy group (HNSCC Bias = 0.143, SD = 0.71; healthy Bias = -0.642, SD = 1.77), where Lollisponge® tends to underestimate the expression of miR-484. In this regard, we highlight that the average volume of saliva recovered in HNSCC was highly consistent among methods, as in these patients salivation is generally limited by the pathological condition, by the effect of the treatments themselves and by the other drugs frequently employed in these patients, in particular opioids [15]. Conversely, a greater heterogeneity exists among controls, and this discrepancy may reasonably account for the lower agreement between methods observed in this setting, although differences remain in a biologically irrelevant range of values.

We further measured the expression of miR-106b-5p, previously reported as a candidate diagnostic biomarker for oral cancer detection [5], to test experimental reproducibility in a clinically relevant context. Using miR-484 Cq value to normalize miR-106b-5p expression in the HNSCC cohort, the Bland Altaman analysis still evidenced the good agreement between methods, with Bias = -0.175 and LOA interval = 1.43 (Fig. 3C). Together these data suggest that both collection methods could be used as a substitute for one other.

Since for cf-miRNA analysis no preservative can be added to stabilize RNA, processing of saliva within 30 min after collection followed by storage in −80 °C has been recommended to minimize sample degradation [16]. To test whether and to what extent the stability of cf-miRNA is biased by delayed sample processing, saliva was either *i)* immediately centrifuged after collection and supernatant freezed at −80 °C (T0) or *ii)* placed at 4 °C for various times that mimic realistic short-term transport and storage conditions in the clinical lab. At 3 different time points, 24 h (T1), 48 h (T2), and 96 h (T3), the supernatant was recovered and stored at −80 °C before RNA extraction. MiR-484 maintained a stable profile across T0-T1-T2 experimental time points (average^{Cq} = 29.31, SD = 1.33; average^{Cq} = 29.77, SD = 1.24; average^{Cq} = 29.75, SD = 1.53, respectively), revealing a slight but significantly decreased expression, corresponding to a higher Cq value, at T3 (average^{Cq} = 30.33, SD = 1.27) (Fig. 4A), suggestive that keeping saliva at a refrigerated temperature of 4 °C up to 48 h before processing has no relevant effect on miR-484 amplification. To further explore the influence of storage temperature on miRNA stability, aliquote of saliva supernatant taken from donors at T1 were kept in parallel in −80 °C and in −20 °C for 1 year before analysis. The effect of storage in −20 °C as opposed to recommended −80 °C resulted in significant increase of more than 2 Cq values (average^{Cq} = 31.02, SD = 1.83; average^{Cq} = 28.26, SD = 1.27, respectively; paired *t* test: mean of differences = 2.759, IC95 1.986 to 3.532, $p < 0.0001$; Fig. 4B), evidence of miR-484 degradation after prolonged storage of saliva in −20°.



RNA (input uL)	Comparison	Difference [CI 95%]	p-value
2	Lollisponge (40x) vs Standard	0,28 [-0,68 ; 1,25]	0,558
	Lollisponge (50x) vs Standard	0,71 [-0,25 ; 1,68]	0,147
4	Lollisponge (40x) vs Standard	0,42 [-0,54 ; 1,40]	0,381
	Lollisponge (50x) vs Standard	0,71 [-0,25 ; 1,68]	0,147
6	Lollisponge (40x) vs Standard	0,14 [-1,11 ; 0,83]	0,769
	Lollisponge (50x) vs Standard	0,14 [-0,83 ; 1,11]	0,769

Fig. 2. MiR-484 is consistently amplified in saliva samples irrespective of collection strategy. RNA was purified by either Standard or LolliSponge® samples from healthy donors (n = 10) and different input amounts in the cDNA synthesis reaction were tested (2 uL, 4uL, 6uL in a 10 uL RT reaction). Four uL of a 50x dilution of cDNA were used for Standard qPCR, whereas for LolliSponge® samples an alternative 40x dilution factor of cDNA was tested. The linear relationship between input amount and miR-484 signal expression obtained in qPCR at different dilution factors is shown.

4. Discussion

The quantification of circulating cf-miRNAs has been used in various cancer types in order to provide useful biomarkers for early diagnosis, prognosis, or outcome prediction [17]. While there is general agreement on miRNA purification methods and measurement platforms [8,18], and much research is focused on defining proper normalization procedures [19], few studies have addressed the effect of pre-analytical variables on the stability of circulating miRNA in saliva specimens. However, one of the major impediments to routine acceptance and use of miRNA is the lack of standard sample preparation methods. Thus, research in this area is critical for determining the best way to handle, transport, and store saliva samples for downstream miRNA analysis, whether for biobanking, multicenter studies, or future diagnostic applications, and is relevant to their potential utility as biomarkers. These assumptions prompted us to compare various saliva manipulation strategies in order to monitor the effect of different equipment and procedures on cf-miRNA yield and quality under defined experimental conditions. The latter were designed to replicate specimen collection during typical routine procedures while accounting for potential logistical constraints posed by the clinical context, such as the fact that reliable refrigeration may not be guaranteed for an extended period of time and the availability of dedicated equipment, such as a −80 °C refrigerator, may be limited.

We herein showed that an intact RNA with unaltered miRNA expression can be isolated from saliva supernatant, regardless of collection method, and remain stable for a period of time compatible with main research and clinical routine needs. Our findings, based on qPCR quantification of endogenous miR-484 and cancer-related miR-106b-5p, show strong agreement between measurements in matched saliva samples collected by spit or LolliSponge®, both in healthy and pathological contexts. At the moment, in the study of circulating miRNAs, the need to limit cell lysis to avoid contamination from the cellular miRNA pool precludes the use of commercially available saliva collectors [20], in favor of the easier collection of whole saliva by spit. While preferred, due to the viscous nature of saliva, this method can result in difficult sample handling and inaccurate volume measurement [21]. Furthermore, saliva adsorption on various devices may have unpredictable effects on sample integrity [22]. The data presented here indicate that the use of absorbent sponge-based devices is appropriate for cf-miRNA analysis. In comparison to spit, saliva harvested with Lollisponge® results in higher sample uniformity, both in terms of quantity and quality, as the absorbent pad likely acts as a filter to remove mucinous material and cellular debris from whole saliva. This supports the potential use of Lollisponge® in difficult settings such as HNSCC irradiated patients, where the very high viscosity of saliva due to post-treatment xerostomia may severely limit its use [15]. Saliva can be stored at 4 °C for up to 48 h after collection before supernatant recovery and miRNA isolation without causing significant changes in overall cf-miRNA stability or the expression of the miRNA tested. The proper storage of supernatant at −80 °C, on the other hand, appears to be critical to maintain the integrity of cf-miRNA in the mid-term period, as we observed a significant drop in detection of miR-484 in matched samples stored at −20 °C for more than 12 months. Similar findings were reported for other biologic fluids, such as plasma and urine, where cf-miRNA remained stable after 24 h at 4 °C, whereas freezing at −80 °C was recommended to ensure long-term miRNA storage [23]. Among the practical implications of our findings are the ability to collect saliva even in locations not directly connected to a laboratory, as well as the ease of

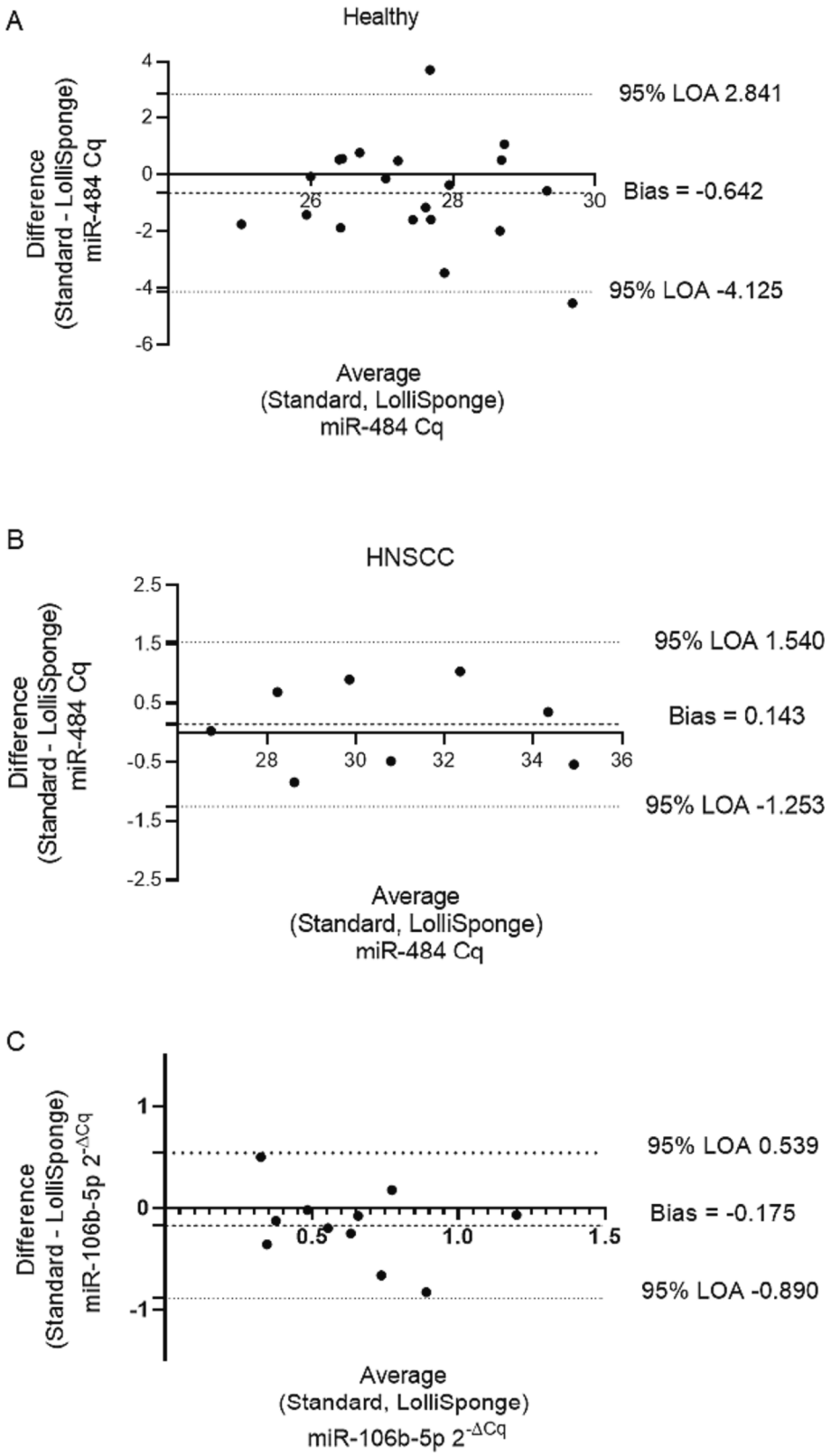
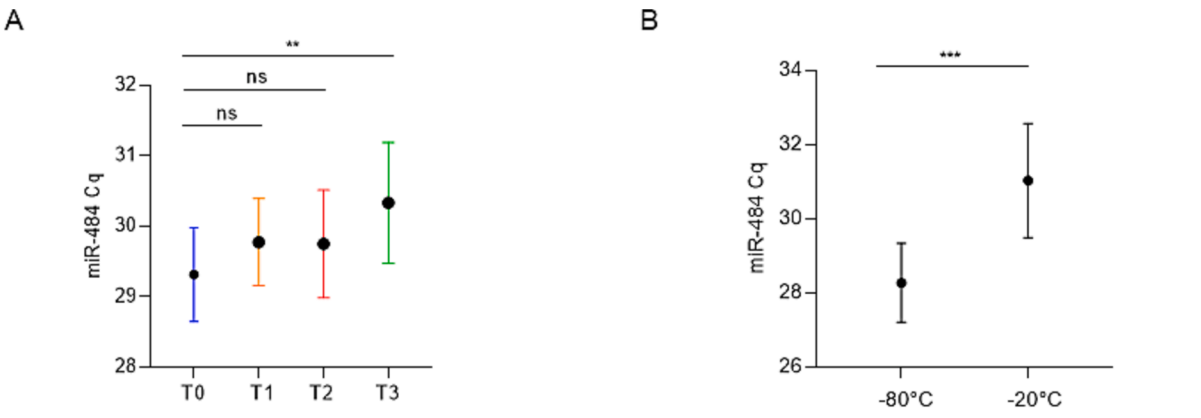


Fig. 3. Agreement between cf-miRNA measurement using Standard and Lollisponge® collection methods. Endogenous miR-484 and tumor-specific miR-106b-5p were measured by qPCR in Standard and Lollisponge® saliva samples: A) Bland-Altman plot for agreement analysis of Standard and Lollisponge® in healthy subjects and B) HNSCC patients. The mean difference between miR-484 Cq values (Bias, solid line) and 95% limits of agreement (LOA, dotted lines) are shown; C) Bland-Altman plot for agreement analysis of Standard and Lollisponge® in HNSCC patients. The mean difference between miR-106b-5p $2^{-\Delta Cq}$ values (Bias, solid line) and 95% limits of agreement (LOA, dotted lines) are shown.



Comparison	Difference [CI95%]	p-value
T0 *vs* T1	-0,46 [-1.19 ; 0.27]	0,2029
T0 *vs* T2	-0,43 [-1.22 ; 0.34]	0,2575
T0 *vs* T3	-1,02 [-1.60 ; -0.43]	0,0032**
T1 *vs* T2	0,02 [-0.41 ; 0.46]	0,9047
T1 *vs* T3	-0,55 [-1.47 ; 0.35]	0,2032
T2 *vs* T3	-0,58 [-1.46 ; 0.29]	0,1707

Comparison	Difference [CI95%]	p value
-80°C *vs* -20°C	2,75 [1.98 ; 3.53]	<0.0001***

Fig. 4. Influence of delayed processing and storage temperature of saliva on cf-miRNA expression level. (A) Saliva collected from healthy subjects (n = 20) was immediately centrifuged (T0) or left at 4 °C for up to 96 h (T3) before processing and storage in −80 °C. MiR-484 Cq values were compared (low Cq value denote high miRNA level) and significant difference are shown (LMM, $p < 0.01$). (B) The midterm storage (>= 12 months) of saliva in −20 °C compared to standard −80 °C results in significant drop in miR-484 expression level detected by qPCR (paired *t*-test, ***$p < 0.001$).

sample handling during transport or analytic procedures without concern for sample integrity. These statements are obviously limited to the miRNA tested, and we cannot rule out the possibility that other miRNAs will behave differently. Furthermore, the relatively small number of samples analyzed necessitates caution in data generalization and future validation in large prospective multicenter studies to strengthen statistical significance. Nonetheless, the results show that there are no significant differences between the methods and procedures used, and they experimentally support the stability of miRNAs, which has previously been predicted in other biological fluids [24], including saliva supernatant.

5. Conclusions

Increasing evidence supports the practicability of salivary cf-miRNA as liquid biopsy markers in cancers. Defining a common procedure for saliva management is mandatory to minimize preclinical variability affecting the quantity and quality of circulating miRNAs and downstream targets analysis, to reinforce the basis for robust comparison of results between studies and ultimately accelerate the translation of salivary miRNA in the clinical context.

CRediT authorship contribution statement

Chiara Romani: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. **Maria Baronchelli:** Investigation. **Claudia Assoni:** Investigation. **Davide Mattavelli:** Writing – review & editing. **Stefano Calza:** Conceptualization, Data curation, Methodology, Funding acquisition, Writing – original draft. **Cesare Piazza:** Funding acquisition, Supervision, Writing – review & editing. **Paolo Bossi:** Conceptualization, Funding acquisition, Writing – original draft.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: PB reports advisory board participation and/or research activities outside the object of the current research with Merck, Sanofi-Regeneron, Merck Sharp & Dohme, Sun Pharma, Angelini, Nestlè, Elevar.

Data availability

Data will be made available on request.

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References

[1] Mahmodul Hasan Sohel, Extracellular/Circulating MicroRNAs: Release Mechanisms, Functions and Challenges, Achiev. Life Sci. 10 (2) (2016) 175–186, https://doi.org/10.1016/j.als.2016.11.007.
[2] A. Roi, et al., Circulating miRNA as a Biomarker in Oral Cancer Liquid Biopsy, Biomedicines 11 (3) (2023) 965, https://doi.org/10.3390/biomedicines11030965.
[3] F. Momen-Heravi, A.J. Trachtenberg, W.P. Kuo, Y.S. Cheng, Genomewide study of salivary microRNAs for detection of oral cancer, J Dent Res 93 (7 Suppl) (2014) 86s–93s.
[4] O. Rapado-González, C. Martínez-Reglerod, A. Salgado-Barreira, R. López-López, M.M. Suárez-Cunqueiro, L. Muinelo-Romay, miRNAs in liquid biopsy for oral

squamous cell carcinoma diagnosis: Systematic review and meta-analysis, Oral Oncol 104465 (2019).
[5] C. Romani, E. Salviato, A. Paderno, et al., Genome-wide study of salivary miRNAs identifies miR-423-5p as promising diagnostic and prognostic biomarker in oral squamous cell carcinoma, Theranostics 11 (6) (2021) 2987–2999, https://doi.org/10.7150/thno.45157.
[6] N. Kosaka, H. Iguchi, T. Ochiya, Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis, CancerSci 101 (2010) 2087–2092, https://doi.org/10.1111/j.1349-7006.2010.01650.x.
[7] R. Sullivan, S. Heavey, D.G. Graham, R. Wellman, S. Khan, S. Thrumurthy, B. S. Simpson, T. Baker, S. Jevons, J. Ariza, V. Eneh, H. Pye, H. Luxton, R. Hamoudi, H. Whitaker, L.B. Lovat, An optimised saliva collection method to produce high-yield, high-quality RNA for translational research, PLoS One. 15 (3) (2020 Mar 9) e0229791.
[8] L. Moldovan, K.E. Batte, J. Trgovcich, J. Wisler, C.B. Marsh, M. Piper, Methodological challenges in utilizing miRNAs as circulating biomarkers, J Cell Mol Med. 18 (3) (2014 Mar) 371–390, https://doi.org/10.1111/jcmm.12236.
[9] P. Todeschini, E. Salviato, L. Paracchini, M. Ferracin, M. Petrillo, L. Zanotti, G. Tognon, A. Gambino, E. Calura, G. Caratti, P. Martini, L. Beltrame, L. Maragoni, D. Gallo, F.E. Odicino, E. Sartori, G. Scambia, M. Negrini, A. Ravaggi, M. D'Incalci, S. Marchini, E. Bignotti, C. Romualdi, Circulating miRNA landscape identifies miR-1246 as promising diagnostic biomarker in high-grade serous ovarian carcinoma: A validation across two independent cohorts, Cancer Lett. 1 (388) (2017 Mar) 320–327, https://doi.org/10.1016/j.canlet.2016.12.017.
[10] A. Gelman, J. Hill, Data Analysis Using Regression and Multilevel/Hierarchical Models, Cambridge University Press, Cambridge, England, 2006.
[11] J.M. Land, D. Altman, Statistical methods for assessing agreement between two methods of clinical measurement, Lancet 327 (8476) (1986) 307–310, https://doi.org/10.1016/S0140-6736(86)90837-8.
[12] Y. Li, M.A. St John, X. Zhou, Y. Kim, U. Sinha, R.C. Jordan, D. Eisele, E. Abemayor, D. Elashoff, N.H. Park, D.T. Wong, Salivary transcriptome diagnostics for oral cancer detection, Clin Cancer Res. 10 (24) (2004 Dec 15) 8442–8450, https://doi.org/10.1158/1078-0432.CCR-04-1167.
[13] T. Blondal, S. Jensby Nielsen, A. Baker, D. Andreasen, P. Mouritzen, M. Wrang Teilum, I.K. Dahlsveen, Assessing sample and miRNA profile quality in serum and plasma or other biofluids, Methods 59 (1) (2013 Jan) S1–S6, https://doi.org/10.1016/j.ymeth.2012.09.015.
[14] S. Wei, S. Hu, N. Han, G. Wang, H. Chen, Q. Yao, Y. Zhao, J. Ye, A. Ji, Q. Sun, Screening and evaluation of endogenous reference genes for miRNA expression analysis in forensic body fluid samples, Forensic Sci Int Genet. 63 (2023 Mar), 102827, https://doi.org/10.1016/j.fsigen.2023.102827.
[15] U. Almhöjd, H. Cevik-Aras, N. Karlsson, et al., Stimulated saliva composition in patients with cancer of the head and neck region, BMC Oral Health 21 (2021) 509, https://doi.org/10.1186/s12903-021-01872-x.
[16] B.S. Henson, D.T.C. Wong, Storage, and Processing of Saliva Samples for Downstream Molecular Applications, in: G. Seymour, M. Cullinan, N. Heng (Eds.), Oral Biology. Methods in Molecular Biology, vol 666, Humana Press, Totowa, NJ, 2010, https://doi.org/10.1007/978-1-60761-820-1.
[17] Y. He, J. Lin, D. Kong, M. Huang, C. Xu, T.K. Kim, A. Etheridge, Y. Luo, Y. Ding, K. Wang, Current State of Circulating MicroRNAs as Cancer Biomarkers, Clin Chem. 61 (9) (2015 Sep) 1138–1155, https://doi.org/10.1373/clinchem.2015.241190. Epub 2015 Aug 3 PMID: 26319452.
[18] P. Mestdagh, N. Hartmann, L. Baeriswyl, et al., Evaluation of quantitative miRNA expression platforms in the microRNA quality control (miRQC) study, Nat Methods 11 (2014) 809–815, https://doi.org/10.1038/nmeth.3014.
[19] R. Sullivan, A. Montgomery, A. Scipioni, P. Jhaveri, A.T. Schmidt, S.D. Hicks, Confounding Factors Impacting microRNA Expression in Human Saliva: Methodological and Biological Considerations, Genes 2022 (1874) 13, https://doi.org/10.3390/genes13101874.
[20] A. Urbizu, L. Arnaldo, K. Beyer, Obtaining miRNA from Saliva—Comparison of Sampling and Purification Methods, Int. J. Mol. Sci. 24 (2023) 2386, https://doi.org/10.3390/ijms2403238.
[21] E. Presser, M. Simuyandi, J. Brown, The effects of storage time and temperature on recovery of salivary secretory immunoglobulin A, Am. J. Hum. Biol. 26 (2014) 417–420, https://doi.org/10.1002/ajhb.22525.
[22] C. Golatowski, M.G. Salazar, V.M. Dhople, E. Hammer, T. Kocher, N. Jehmlich, U. Völker, Comparative evaluation of saliva collection methods for proteome analysis, Clin Chim Acta. 18 (419) (2013 Apr) 42–46, https://doi.org/10.1016/j.cca.2013.01.013.
[23] B.N. Chorley, E. Atabakhsh, G. Doran, J.C. Gautier, H. Ellinger-Ziegelbauer, D. Jackson, T. Sharapova, P.S.T. Yuen, R.J. Church, P. Couttet, R. Froetschl, J. McDuffie, V. Martinez, P. Pande, L. Peel, C. Rafferty, F.J. Simutis, A.H. Harrill, Methodological considerations for measuring biofluid-based microRNA biomarkers, Crit Rev Toxicol. 51 (3) (2021 Mar) 264–282, https://doi.org/10.1080/10408444.2021.1907530.
[24] A. Turchinovich, L. Weiz, A. Langheinz, B. Burwinkel, Characterization of extracellular circulating microRNA, Nucleic Acids Res. 39 (16) (2011 Sep 1) 7223–7233, https://doi.org/10.1093/nar/gkr254. Epub 2011 May 24.