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Lipoproteins as overlooked RNA carriers in saliva: implications for extracellular vesicle biomarker studies

Tiago Degani Veit[1,*], Fernanda Visioli[2,3] and José Artur Bogo Chies[4]

[1] Department of Microbiology, Immunology and Parasitology, Institute of Health's Basic Science, Federal University of Rio Grande do Sul, Porto Alegre, Brazil
[2] Dentistry School, Federal University of Rio Grande do Sul, Porto Alegre, Brazil
[3] Experimental Center Research, Porto Alegre Clinical Hospital, Porto Alegre, Brazil
[4] Postgraduate Program in Genetics and Molecular Biology, Laboratory of Immunobiology, Department of Genetics, Federal University of Rio Grande do Sul, Porto Alegre, Brazil

* Correspondence author; E-mail: tiago.veit@ufrgs.br.

Abstract: Extracellular vesicles (EVs) are nanoscopic structures released by all cell types, playing a crucial role in cellular communication. EVs contain various types of RNAs, and a significant number of studies in the field of biomarker research have focused on the RNA content of these vesicles, particularly microRNAs. Saliva is an easily and non-invasive obtainable body fluid that is being increasingly studied for the identification of biomarkers associated with oral and systemic disorders. Early studies investigating salivary RNA distribution reported that the majority of its microRNA content seemed to be associated with EVs. Recently, an RNAseq analysis of host and microbial salivary RNA content in different salivary fractions reported that a majority of the most abundant microRNAs (miRNAs) were detected in both EV-enriched and unenriched saliva fractions. In this letter we raise the hypothesis that this high correlation regarding the miRNA content among saliva fractions might be partially explained by the presence of alternative, overlooked sources of miRNAs in saliva such as lipoproteins. The focus of this report is to raise awareness regarding potential contaminants in EV saliva preparations and to emphasize the need of further research aimed at directly assessing the contribution of these alternative miRNA carriers.

Keywords: extracellular vesicles; saliva; lipoproteins; miRNAs; biomarker research; cancer; exosomes

1. Introduction

Extracellular vesicles (EVs) are nanoscopic vesicles released by all cell types, which encompass exosomes, microvesicles and apoptotic bodies, among others. Importantly, EVs are majorly involved in cellular communication [1,2]. Because they are ubiquitous and carry different types of biomolecules, EVs have been the subject of intense investigation in the search for biomarkers in different types of pathological, as well as physiological conditions. EV-associated biomarkers may provide valuable information about the patient's pathophysiological status, thus guiding therapeutic management. In particular, the content of EVs has been studied in different types of neoplastic conditions and a growing body of literature has accumulated regarding EV-based cancer biomarkers in the last few years [3]. Besides their protein and lipid content, EVs carry different types of nucleic acids, such as genomic and mitochondrial DNA, as well as several RNA species, including microRNAs (miRNAs), messenger RNAs (mRNAs), ribosomal RNAs (rRNAs) and long non-coding RNAs (lncRNAs), and a significant number of studies at the biomarker research field has focused on the miRNA content of such vesicles. A quick search on Scopus using the keywords “extracellular AND vesicle AND cancer AND (microRNA OR miRNA) AND biomarker” by October 30th, 2024 returned 1401 entries, most of which (1039 entries) from 2020 to date.

Saliva is an easily obtainable body fluid that is being increasingly studied for the identification of biomarkers associated with oral and systemic disorders. One of the main advantages of using saliva as a sample for diagnostic purposes is the non-invasive nature of its collection, unlike blood. Like other body fluids, saliva is rich in EVs, which can have diverse cellular origins, including the cells from the oral mucosa epithelium, salivary glands, secondary lymphoid tissues (e.g., tonsils) and the microbiota that colonizes different niches of the oral cavity. Moreover, EVs from saliva may be informative of diseases at distant sites, as was demonstrated in mice with melanoma. In this animal model, RNAs could make their way from the tumor site into the circulation and then be detected in the EV fraction from saliva [4]. However, the isolation of EVs from saliva presents some challenges, such as saliva’s high viscosity due to the presence of high molecular weight mucins. Additionally, saliva composition is highly complex, with the presence of a wide range of biomolecules and extracellular particles in varying concentrations, although generally lower when compared to other fluids such as plasma [5].

Early studies investigating salivary RNA profiles reported that the majority of its RNA content seemed to be associated with EVs such as exosomes and microvesicles [6,7], with little RNA observed outside these vesicular bodies. Although several works have already addressed either the RNA content of whole saliva or salivary EV preparations, a more thorough investigation of salivary RNA content has been lacking. In a recently published study, Tong *et al.* reported a detailed analysis of host and microbial salivary RNA content by RNAseq [8]. They analyzed the RNA from whole saliva (after depletion of cellular debris) and salivary fractions obtained by differential centrifugation. Remarkably, they found that both cell-free saliva (CFS) and its three derived fractions—named EV-D (for EV-depleted

saliva), MV (for microvesicles) and EXO (for exosomes)—were indeed abundant in several different species of human and microbial RNA. In particular, all salivary fractions shared considerable amounts of host derived RNAs species such as rRNAs, mRNAs, miRNAs, tRNAs, lncRNAs. Microbial RNA contributed to approximately 50% of the total RNA encountered in saliva, which could be traced back to several bacterial phyla, suggesting that the microbiome significantly contributes to the RNA content of saliva. While the presence of microbiome RNA in saliva samples is established, its ubiquitous presence in different salivary fractions may reflect the diversity of secretion mechanisms by different bacterial populations. The biologic significance and biomarker potential of these foreign RNAs remain largely unexplored and certainly warrant further investigation.

2. miRNA profiles in saliva preparations

A somewhat puzzling finding of the study from Tong *et al.* regarding host RNA expression was that the miRNA profiles from all four salivary preparations were highly correlated. Remarkably, most (84%) of the 200 most abundantly expressed miRNAs were detected in all saliva fractions. Specifically, regarding the differences between the "exosome" fraction (EXO) and the "EV-depleted" fraction (EV-D), the authors observed that, among the 28 differentially expressed miRNAs, only 3 EXO-associated miRNAs were enriched more than tenfold in relation to EV-D, while no EV-D-associated miRNA was enriched above four-fold in relation to EXO. A plausible explanation for these findings would be an incomplete separation of the pelletable EVs from soluble RNA-carrier proteins. It is possible that the differential ultracentrifugation (UC) method used to obtain the saliva fractions (10,000 × g for 1 hour plus a single 100,000 × g step for 1 hour with no further UC washing steps) might not have been long enough to pellet all EVs, thus accounting for the relatively poor enrichment of EV-associated miRNAs in EXO compared to other salivary fractions. Noteworthily, the Nanoparticle Tracking Analysis graph from the EV-D fraction showed peaks that were compatible with the size range of small EVs (Figure S1 in [8]). The issues associated with the isolation of EVs from viscous fluids such as plasma and saliva have long been addressed by seminal UC protocols that employed longer centrifugation times as well as multiple centrifugation steps in order to augment EV recovery and purity [9].

The high miRNA correlation observed among salivary fractions could also be explained by the presence of additional non-vesicular sources of extracellular RNA (exRNA) in saliva. While the exRNA landscapes of most body fluids have remained uncharted until recently, the complexity of the extracellular RNA (exRNA) landscape of blood has already been explored for more than a decade. In blood, ribonucleoproteins (RNPs, e.g., AGO2) and circulatory lipoproteins (LPP) such as HDL (high-density lipoprotein) and LDL (low-density lipoprotein) are established alternative sources of extracellular miRNA [10,11] and other short RNA (sRNA) species. Specifically, it was also shown that HDL from mice can transport tRNA-derived sRNAs (tDRs) and rRNA-derived sRNAs, while both HDL and LDL were shown to transport nonhost microbial sRNAs [12,13]. Recently, it was shown that VLDL (very low-density lipoprotein) also carried miRNAs and presented a miRNA profile highly similar to that

of HDL [14]. HDLs have similar density ranges as exosomes/small EVs (1.10–1.21 g/mL) and thus can be pelleted down as well through UC, albeit at a much slower rate. LDLs are larger (22–29 nm) and also denser than saliva (1.019–1.063 *vs.* 1.002–1.012 g/mL) but pellet even slower than HDLs due to the smaller difference in density. However, even the minor fraction of those LPP particles that are expected to copellet with EVs through UC would still largely surpass EVs as the main component of a UC pellet due to being much more abundant than plasma EVs. This is a major concern for researchers aiming at the discovery of EV-associated biomarkers in this blood, either being lipid-, protein- or nucleic acid-based [15–17]. Due to the potential contamination issues regarding the copurification of lipoproteins in plasma and serum EV preparations, several methods have been used in order to eliminate such contaminants. Such methods included several combinations of classically employed EV isolation steps (e.g., UC followed by size exclusion chromatography; multiple UC steps), the selective degradation of LPP or their selective removal by using magnetic beads coated with adsorptive substances [9,17–21].

3. Current evidence of the presence of lipoproteins in saliva

Regarding saliva, there has been accumulating evidence of the presence of lipoproteins in recent years. Two recent bioinformatics studies conducted by the Extracellular RNA Communication Consortium aimed at elucidating the endogenous sources of exRNA in biological fluids suggested that saliva samples not only exhibited a high diversity of RNPs but also a robust molecular signature associated with the presence of lipoproteins [22,23]. Further evidence of the presence of lipoproteins in saliva was provided by the proteomic data from Yamamoto *et al.*, who aimed at determining the buoyant densities of salivary EV subclasses by applying UC followed by prolonged density gradient ultracentrifugation (UC-DG). Remarkably, all salivary samples presented many of the hallmark apolipoproteins (APOs) found in HDL, LDL and VLDL, including ApoA-1, ApoB-100 and ApoE at significant amounts. Moreover, most APOs were detected at the UC-DG fractions corresponding to expected buoyant densities of their respective lipoprotein classes [24].

4. Lipoproteins as potential contaminating miRNA sources in salivary EV preparations

We hypothesized that, even considering a 10–100-fold reduction in HDL and LDL levels compared to plasma, as suggested by previous studies analyzing salivary cholesterol [25,26], lipoproteins would still constitute a significant amount of contaminating particles after single-step EV isolation methods which could potentially bias downstream analyses of EV RNA biomarkers. It is also conceivable that, while some amount of lipoproteins would be pelleted down by UC, a substantial fraction would remain unpelleted in the supernatant, thus contributing to the high similarity in miRNAs profiles observed among the four saliva fractions evaluated by Tong *et al.*

We thus aimed to investigate if, among the differentially expressed miRNAs reported by the authors in the results and supplementary data, we could identify microRNAs known to be carried by circulating lipoproteins. We opted to use the data from Rossi-Herring *et al.* [14], who

conducted miRNA microarray analyses in isolates of VLDL, LDL and HDL obtained by a combined protocol for the isolation of serum lipoproteins with high purity (differential flotation followed by size-exclusion chromatography). As shown in Table 1, from the 15 miRNAs enriched in the EXO fraction and assessed in serum lipoproteins, six had been stably detected in circulating HDL samples. Interestingly, from those six stably expressed miRNAs, five were also stably expressed in VLDL and two also stably expressed in LDL, *i.e.*, by all lipoprotein classes. Moreover, among the nine miRNAs enriched in non-EXO fractions and previously assessed in serum, three were stably detected in HDL, of which one was also stably detected in VLDL. Overall, this data suggests that some of the miRNAs enriched in EXO (thus regarded as EV-derived miRNAs) could be, in fact, derived from co-pelleted lipoproteins. These findings also make it plausible that the high miRNA similarity among the four saliva fractions could be explained, at least in part, by a) the partial pelleting of lipoproteins and/or b) the relatively high degree of identity between the miRNAs carried by lipoprotein classes presenting variable degrees of pelletability. Very few other studies in the literature applied a one-step UC protocol to the analysis of miRNA content from salivary EVs. Besides, in those studies, a full analysis of datasets is difficult due to the non-availability of raw data (see, for example the study from Langevin *et al.* 2017) [27]. However, in a later work from the same group [28], the salivary EVs of healthy subjects were isolated by a protocol encompassing three consecutive UC steps, specially designed for isolating EVs while removing lipoproteins and other contaminants from viscous fluids [9]. Therefore, it was possible to compare the RNAseq data from this study with the data from the Tong *et al.* study. Notably, among the six miRNAs enriched in EXO and stably detected in HDL, three (let-7b-5p, miR-125a-5p and miR-335-3p) could not be detected by the RNAseq analysis of Langevin *et al.* (2020) (see Table 1). Altogether, this data corroborates our hypothesis that LPPs might be contributing to the miRNA content of salivary EV preparations obtained by insufficiently stringent isolation protocols.

Table 1. Differentially expressed miRNAs in saliva fractions and their presence in circulating lipoproteins.

miRNAs Enriched in EXO[a]	**Presence in cHDL[b]**	**Presence in cVLDL[b]**	**Presence in cLDL[b]**	**Presence in EVs[c]**
let-7a-5p (vs. EV-D)	stable	stable	low/undetected	stable
let-7b-5p (vs. EV-D)	stable	stable	detected	undetected
let-7c-5p (vs. EV-D)	stable	stable	low/undetected	stable
let-7e-5p (vs. EV-D, CFS)	detected	low/undetected	low/undetected	undetected
let-7f-5p (vs. EV-D)	detected	low/undetected	low/undetected	stable
miR-125a-5p (vs. EV-D, CFS)	stable	stable	stable	undetected
miR-141-3p (vs. CFS)	low/undetected	low/undetected	low/undetected	stable
miR-187-3p (vs. CFS)	-	-	-	stable

Table 1. *Cont.*

miRNAs Enriched in EXO[a]	Presence in cHDL[b]	Presence in cVLDL[b]	Presence in cLDL[b]	Presence in EVs[c]
miR-200a-3p (vs. EV-D, CFS)	low/undetected	low/undetected	low/undetected	stable
miR-200b-3p (vs. CFS)	-	-	-	stable*
miR-200b-5p (vs. CFS)	-	-	-	stable*
miR-200c-3p (vs. CFS)	low/undetected	low/undetected	low/undetected	stable
miR-30a-3p	-	-	-	stable*
miR-30a-5p (vs. EV-D, CFS)	stable	low/undetected	low/undetected	stable*
miR-30c-2-3p (vs. EV-D)	-	-	-	stable
miR-335-3p (vs. EV-D, CFS)	stable	stable	stable	undetected
miR-574-5p (vs. EV-D, CFS)	detected	low/undetected	low/undetected	stable
miR-885-3p (vs. CFS)	low/undetected	low/undetected	low/undetected	stable
miR-99b-5p (vs. EV-D)	detected	low/undetected	low/undetected	stable
miRNAs Enriched in non-EXO fractions				
miR-10399-3p (EV-D)	-	-	-	undetected
miR-106b-3p (CFS)	-	-	-	stable
miR-12136 (MV)	-	-	-	undetected
miR-132-3p (EV-D)	low/undetected	low/undetected	low/undetected	detected
miR-140-3p (EV-D, CFS)	detected	detected	low/undetected	stable
miR-145-5p (EV-D)	detected	detected	low/undetected	undetected
miR-15b-5p (EV-D)	stable	detected	low/undetected	detected
miR-193b-5p (EV-D)	-	-	-	stable
miR-23a-3p (EV-D)	stable	stable	detected	stable
miR-27a-5p (EV-D)	-	-	-	stable
miR-29a-3p (EV-D)	stable	low/undetected	low/undetected	stable
miR-345-5p (EV-D)	-	-	-	stable
miR-425-3p (EV-D)	low/undetected	detected	low/undetected	stable*
miR-425-5p (EV-D)	detected	detected	low/undetected	stable*
miR-576-5p (EV-D)	-	-	-	stable
miR-652-3p (EV-D)	detected	detected	low/undetected	stable
miR-6842-3p (CFS)	-	-	-	stable
miR-769-5p (EV-D, CFS)	-	-	-	stable
miR-941 (CFS)	-	-	-	undetected

[a] data from Tong *et al.*2023, n=10; [b] data from Rossi-Herring *et al.* 2023, n=6; [c] data from Langevin *et al.* 2020; n=4; * no distinction was made between 5' and 3' transcripts for the given miRNA.
EXO: exosome; CFS: cell-free saliva; EV-D: EV-depleted; MV: microvesicle; stable: detected all samples; detected: detected in ≥ 50% of samples; low/undetected: detected in ＜50% of samples; dashes (-): not assessed.

5. Conclusion and future perspectives

The potential contamination of EVs isolated from serum/plasma with lipoproteins is a longtime known problem. Despite this, most publications in the field of RNA EV biomarker research still employ one-step purification protocols for the isolation of serum/plasma EVs,

mostly by 1) precipitation with commercial kits or in-house polyethylene glycol (PEG) preparations or 2) by UC. Notably, it is quite rare to find research articles on sRNA EV markers from blood that include markers for lipoprotein contaminants in their methods, such as ApoA-1, ApoB-100 or ApoE. With regards to salivary EVs, the possibility of lipoprotein contamination seems to be quite neglected notwithstanding the accumulating evidence from proteomic data [24,29] and from electron microscopy micrographs from salivary EV studies, which frequently show electron-lucent particles with spherical or quasi-spherical morphology and sizes compatible with that of lipoproteins together with the larger, electron-dense, cup-shaped EVs [24,30,31].

Possibly, one of the primary reasons for the lack of adherence to higher purity protocols for EV isolation of saliva is the relatively limited knowledge among researchers regarding saliva composition. This includes unawareness about: 1) the potential presence of lipoproteins in saliva and/or 2) their rich and diverse sRNA cargo. The main focus of our report is to raise awareness regarding potential contaminants in saliva preparations and also to emphasize the need of further research aimed at directly assessing the contribution of these alternative non-EV RNA carriers. This will enable the emergence of more meaningful sRNA biomarker discoveries in saliva. For research groups aiming at EV-associated biomarkers in this and other bodily fluids, the safest way to avoid contamination pitfalls is to follow the guidelines for the study of extracellular vesicles published by the International Society for Extracellular Vesicles, which recommend the use of both positive and negative markers of EVs in the biofluid of choice [32]. In the case of saliva, particularly when focusing on sRNA biomarkers, the use of lipoprotein marker(s) should, in our opinion, be mandatory.

While lipoproteins may constitute a real contamination problem for the study of EV-associated sRNA biomarkers in blood and saliva, the study of the lipoprotein sRNA content is itself a recent and promising topic of research. The recent advances in the knowledge of the highly diverse sRNA cargo of those extracellular particles have added another layer of complexity to the landscape of cellular communication. Progress in this field will certainly assist EV researchers in unraveling several inconsistencies observed in EV cargo analyses. Moreover, lipoproteins themselves might emerge in salivomics research, alongside EVs, as potential sources of RNA biomarkers for local and systemic diseases.

Finally, the recent discovery that EVs themselves may carry several plasma proteins and lipoproteins attached to their surface introduces yet another level of complexity for the study of EV biomarkers and their role in cellular communication. While the presence of this EV *corona* awaits confirmation in salivary EVs, it may prove to be a biologically relevant phenomenon as previously described *in vitro* [33]. Although EVs certainly constitute a relevant signaling entity in saliva, the understanding of their biologic and biomedical potential is just in its infancy. The recent discovery regarding the high diversity of host RNA carriers in saliva might foster new avenues of research aimed at comprehending the biological relevance of these carriers in health and disease.

Conflicts of interests

The authors declare no conflicts of interest.

Authors' contribution

Conceptualization, T.D.V.; methodology, T.D.V and J.A.B.C.; formal analysis, T.D.V, F.V. and J.A.B.C; writing—original draft preparation, T.D.V.; writing—review and editing, F.V. and J.A.B.C. All authors have read and agreed to the published version of the manuscript.

References

[1] Théry C, Zitvogel L, Amigorena S. Exosomes: composition, biogenesis and function. *Nat. Rev. Immunol.* 2002, 2(8):569–579.

[2] Mittelbrunn M, Sánchez-Madrid F. Intercellular communication: diverse structures for exchange of genetic information. *Nat. Rev. Mol. Cell Biol.* 2012, 13(5):328–335.

[3] Möller A, Lobb RJ. The evolving translational potential of small extracellular vesicles in cancer. *Nat. Rev. Cancer* 2020, 20(12):697–709.

[4] Kim J, Shin H, Park J. RNA in Salivary Extracellular Vesicles as a Possible Tool for Systemic Disease Diagnosis. Journal of Dental Research. *J. Dent. Res.* 2017, 96(8):938–944.

[5] Cecchettini A, Finamore F, Puxeddu I, Ferro F, Baldini C. Salivary extracellular vesicles *versus* whole saliva: new perspectives for the identification of proteomic biomarkers in Sjögren's syndrome. *Clin. Exp. Rheumatol.* 2019, 37(Suppl 118):240–248.

[6] Gallo A, Tandon M, Alevizos I, Illei GG. The Majority of MicroRNAs Detectable in Serum and Saliva Is Concentrated in Exosomes. *PLoS One* 2012, 7(3):e30679.

[7] Bahn JH, Zhang Q, Li F, Chan TM, Lin X, *et al.* The Landscape of MicroRNA, Piwi-Interacting RNA, and Circular RNA in Human Saliva. *Clin. Chem.* 2015, 61(1):221–230.

[8] Tong F, Tang G, Wang X. Characteristics of Human and Microbiome RNA Profiles in Saliva. *RNA Biol.* 2023, 20(1):398–408.

[9] Théry C, Amigorena S, Raposo G, Clayton A. Isolation and Characterization of Exosomes from Cell Culture Supernatants and Biological Fluids. *Curr. Protoc. Cell Biol.* 2006, 30(1):3–22.

[10] Turchinovich A, Weiz L, Langheinz A, Burwinkel B. Characterization of extracellular circulating microRNA. *Nucleic Acids Res.* 2011, 39(16):7223–7233.

[11] Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat. Cell Biol.* 2011, 13(4):423–433.

[12] Michell DL, Allen RM, Cavnar AB, Contreras DM, Yu M, *et al.* Elucidation of physico-chemical principles of high-density lipoprotein–small RNA binding interactions. *J. Biol. Chem.* 2022, 298(6):101952.

[13] Allen RM, Michell DL, Cavnar AB, Zhu W, Makhijani N, *et al.* LDL delivery of microbial small RNAs drives atherosclerosis through macrophage TLR8. *Nat. Cell Biol.* 2022, 24(12):1701–1713.

[14] Rossi-Herring G, Belmonte T, Rivas-Urbina A, Ben ítez S, Rotllan N, *et al.* Circulating lipoprotein-carried miRNome analysis reveals novel VLDL-enriched microRNAs that strongly correlate with the HDL-microRNA profile. *Biomed. Pharmacother.* 2023, 162:114623.

[15] Simonsen JB. What Are We Looking At? Extracellular Vesicles, Lipoproteins, or Both? *Circ. Res.* 2017, 121(8):920–922.

[16] Johnsen KB, Gudbergsson JM, Andresen TL, Simonsen JB. What is the blood concentration of extracellular vesicles? Implications for the use of extracellular vesicles as blood-borne biomarkers of cancer. *Biochim. Biophys. Acta Rev. Cancer* 2019, 1871(1):109–116.

[17] Brennan K, Martin K, FitzGerald SP, O'Sullivan J, Wu Y, *et al.* A comparison of methods for the isolation and separation of extracellular vesicles from protein and lipid particles in human serum. *Sci. Rep.* 2020, 10(1):1039.

[18] Iannotta D, Lai A, Nair S, Koifman NA, Lappas M, *et al.* Chemically‐Induced Lipoprotein Breakdown for Improved Extracellular Vesicle Purification. *Small* 2024, 20(18):2307240.

[19] Yang R, Lou D, Xia K, Sun L, Zhu Q. A pH-Mediated Highly Selective System Enabling Simultaneous Analysis of Circulating RNAs Carried by Extracellular Vesicles and Lipoproteins. *Anal. Chem.* 2023, 95(51):18803–18813.

[20] Zhang Y, Deng Z, Lou D, Wang Y, Wang R, *et al.* High-Efficiency Separation of Extracellular Vesicles from Lipoproteins in Plasma by Agarose Gel Electrophoresis. *Anal. Chem.* 2020, 92(11):7493–7499.

[21] Chou CY, Chiang PC, Li CC, Chang JW, Lu PH, *et al.* Improving the Purity of Extracellular Vesicles by Removal of Lipoproteins from Size Exclusion Chromatography- and Ultracentrifugation-Processed Samples Using Glycosaminoglycan-Functionalized Magnetic Beads. *ACS Appl. Mater. Interfaces* 2024, 16(34): 44386–44398.

[22] Murillo OD, Thistlethwaite W, Rozowsky J, Subramanian SL, Lucero R, *et al.* exRNA Atlas Analysis Reveals Distinct Extracellular RNA Cargo Types and Their Carriers Present across Human Biofluids. *Cell* 2019, 177(2):463–477.

[23] LaPlante EL, Stürchler A, Fullem R, Chen D, Starner AC, *et al.* exRNA-eCLIP intersection analysis reveals a map of extracellular RNA binding proteins and associated RNAs across major human biofluids and carriers. *Cell Genom.* 2023, 3(5):100303.

[24] Yamamoto S, Okamura K, Fujii R, Kawano T, Ueda K, *et al.* Specimen-specific drift of densities defines distinct subclasses of extracellular vesicles from human whole saliva. *PLoS One* 2021, 16(4):e0249526.

[25] Al-Rawi NH. Oxidative stress, antioxidant status and lipid profile in the saliva of type 2 diabetics. *Diab. Vasc. Dis. Res.* 2011, 8(1):22–28.

[26] Choudhry AA, Sharma P, Mohapatra T, Prasad M, Kumar P. Assessment of Salivary Lipid Profile Parameters in Healthy and Type 2 Diabetes Mellitus Patients. *J. Clin. Diagn. Res.* 2021, 15(6).

[27] Langevin S, Kuhnell D, Parry T, Biesiada J, Huang S, *et al.* Comprehensive microRNA-sequencing of exosomes derived from head and neck carcinoma cells *in vitro* reveals common secretion profiles and potential utility as salivary biomarkers. *Oncotarget* 2017, 8(47):82459.

[28] Langevin SM, Kuhnell D, Biesiada J, Zhang X, Medvedovic M, *et al.* Comparability of the small RNA secretome across human biofluids concomitantly collected from healthy adults. *PLoS One* 2020, 15(4):e0229976.

[29] Loo JA, Yan W, Ramachandran P, Wong DT. Comparative Human Salivary and Plasma Proteomes. *J. Dent. Res.* 2010, 89(10):1016–1023.

[30] Reseco L, Molina-Crespo A, Atienza M, Gonzalez E, Falcon-Perez JM, *et al.* Characterization of Extracellular Vesicles from Human Saliva: Effects of Age and Isolation Techniques. *Cells* 2024, 13(1):95.

[31] Jangholi A, Bark JM, De Lima LTF, Lima LG, Möller A, *et al.* Method optimisation to enrich small extracellular vesicles from saliva samples. *Clin. Transl. Med.* 2023, 13(8):e1341.

[32] Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* 2018, 7(1):1535750.

[33] Tóth EÁ, Turiák L, Visnovitz T, Cserép C, Mázló A, *et al.* Formation of a protein corona on the surface of extracellular vesicles in blood plasma. *J. Extracell. Vesicles* 2021, 10(11):e12140.