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1 **Salivary extracellular vesicles isolation methods impact the**  
2 **robustness of downstream biomarkers detection**

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16

17 **Abstract**

18 Extracellular vesicles (EVs), crucial mediators in cell-to-cell communication, are implicated in both  
19 homeostatic and pathological processes. Their detectability in easily accessible peripheral fluids like  
20 saliva positions them as promising candidates for non-invasive biomarker discovery. However, the lack  
21 of standardized methods for salivary EVs isolation greatly limits our ability to study them. Therefore,  
22 we rigorously compared salivary EVs isolated using two scalable techniques—co-precipitation and  
23 immuno-affinity—against the long-established but labor-intensive ultracentrifugation method.  
24 Employing Cryo-Electron Microscopy, Nanoparticle Tracking Analysis, Western blots (WB), and  
25 proteomics, we identified significant method-dependent variances in the size, concentration, and  
26 protein content of EVs. Importantly, our study uniquely demonstrates the ability of EV isolation to  
27 detect specific biomarkers that remain undetected in whole saliva by WB. RT-qPCR analysis targeting  
28 six miRNAs confirmed a consistent enrichment of these miRNAs in EV-derived cargo across all three  
29 isolation methods. We also found that pre-filtering saliva samples with 0.22 or 0.45  $\mu\text{m}$  pores adversely  
30 affects subsequent analyses. Our findings highlight the untapped potential of salivary EVs in  
31 diagnostics and advocate for the co-precipitation method as an efficient, cost-effective, and clinically  
32 relevant approach for small-volume saliva samples. This work not only sheds light on a neglected  
33 source of EVs but also paves the way for their application in routine clinical diagnostics.

34

35 **Keywords:** Saliva, Extracellular vesicles, Exosomes, miRNA, Biomarkers, Diagnostic, Filtration.

36

## 37 Introduction

38 In recent decades, the exploration of extracellular vesicles (EVs) has ignited a surge of interest among  
39 researchers, unveiling a complex network of intercellular communication mechanisms. These small,  
40 membrane-bound entities have emerged as pivotal players in various physiological processes (both  
41 healthy and pathological), orchestrating a symphony of molecular interactions between cells[1].

42 EVs are nanometric particles released by all cell types across diverse tissues and can be found  
43 abundantly in various body fluids, including blood, urine, cerebrospinal fluid (CSF), milk and saliva [2-  
44 6]. Their nanometric size range and lipid bilayer membrane structure are distinctive features that  
45 discriminate them from other soluble extracellular factors, rendering them particularly adept at  
46 traversing biological barriers and ensuring efficient intercellular communication [1]. A key aspect of  
47 EVs lies in their heterogeneity, which encompasses a wide array of subtypes, each characterized by  
48 specific biogenesis pathways and molecular cargo and are classified in three main groups: exosomes,  
49 microvesicles and apoptotic bodies [1, 7, 8]. In fact, these three categories forms a continuum and  
50 being able to separate EVs by size alone is very challenging.

51 EVs are also heterogeneous regarding their membrane composition and molecular cargo. The  
52 repertoire of molecules harbored by EVs includes proteins, nucleic acids (such as miRNAs, mRNAs, and  
53 non-coding RNAs), lipids and even metabolites, enabling EVs to transfer functional biomolecules  
54 between cells [9]. These distinct origins confer unique biomolecular compositions, making EVs versatile  
55 mediators of diverse biological signals. Moreover, the features of this molecular cargo reflects the  
56 physiological and pathological state of the parent cell, underscoring the diagnostic and prognostic  
57 potential of EVs in various diseases [10].

58 Most of the EVs studies in biofluids focus primarily on blood, urine, milk and CSF. However, on a  
59 diagnostic perspective, these biofluids suffer from a number of limitations, like invasiveness,  
60 requirement for trained personnel, risk of infection, dilution effect (especially for urine), or small  
61 volumes (CSF) which may hinder conducting multiple tests or repeated sampling. In this regard, saliva  
62 can be seen as a valuable biofluid alternative due to its non-invasiveness and stress-free collection  
63 procedure. Its easy access enhance compliance, facilitate repetitive sampling and require minimal  
64 expertise and equipment for sample collection. Saliva is a very dynamic biofluid, and its composition  
65 varies according to the physiological or pathological processes taking place within the body, reflecting  
66 the local and systemic health and making it usable as a proxy to assess diverse oral and systemic  
67 pathologies[11]. Moreover, several biomarkers usually measured in blood can also be found in saliva,  
68 with a good correlation between both biofluids [12]. Therefore, it could easily become the matrix of  
69 choice for the development and use of Point Of Care (POC) and auto-tests, eliminating the necessity

70 of long and expensive lab tests. This was well illustrated during the COVID-19 pandemic, as salivary  
71 auto-tests like EasyCOV® allowed numerous airports, train stations, schools, nursing houses, and low  
72 income countries to increase their testing capacities at low cost [13]. Thus, saliva has the potential to  
73 facilitate and improve the tests rapidity and sensitivity relying on biomarkers provided they are present  
74 in this biofluid. However, the presence of contaminants, bacteria and inhibitors can be seen as severe  
75 inconveniences, which may explain in part why this biofluid is not as extensively studied as the others  
76 [14].

77 EVs were described in saliva [6, 15] and could represent a good alternative to bypass all the limitations  
78 cited above and reveal all the diagnostic potential of this biofluid. Therefore, there is an urgent need  
79 to develop and standardize EVs isolation methods adapted to salivary samples for diagnostic use.

80 Currently, ultracentrifugation (UC) is the gold standard method employed to isolate EVs [16]. UC can  
81 isolate EVs from large volume of samples at low cost with relatively high purity. However, it requires  
82 high speed centrifuge, an expensive lab equipment, is quite time consuming and does not allow a large  
83 number of samples to be processed in parallel which hinders its usage on a clinical perspective.  
84 Recently, several kits relying on polymer-based precipitation (PEG) to isolate EVs in a short time, at low  
85 speed centrifugation have been tested in several studies in different human biofluids [17-19]. Immuno-  
86 affinity-based EV isolation is also an interesting alternative to UC because it requires small volumes of  
87 samples, it is fast and, due to its specificity, enable to isolate highly pure EVs [20]. Moreover, both  
88 methods could be used at larger scales than UC, which is a huge advantage regarding clinical  
89 compliance required for diagnostic.

90 To date, no direct comparizon in saliva has been done to extensively show how these three different  
91 isolation methods influence the isolation results of salivary EVs regarding both their proteic and nucleic  
92 acid content.

93 Published protocols from researchers and/or manufacturers instructions often mention the use of  
94 filtration in order to improve EVs recovery [21, 22]. However, the assumed efficiency of this additional  
95 step has never been properly assessed and it remains unclear if this step is useful or, on the contrary,  
96 detrimental in terms of the molecular content of the EV.

97 In this study, we compared three methods relying on very different mechanisms to isolate EVs from  
98 human saliva, namely UC, co-precipitation and immuno-affinity. We propose a comprehensive analysis  
99 regarding the characterization of EVs, their proteic cargo and miRNA content. We also assessed the  
100 usefulness of adding a filtration step on protein and miRNA recovery for each method.

## 101 **Materials and methods:**

### 102 **Saliva collection and sample processing**

103 Unstimulated saliva samples were obtained from nine healthy males volunteers aged between 18 and  
104 40 years old. Subjects were asked to refrain from eating, drinking or smoking for at least 1 hour prior  
105 to saliva collection. Whole saliva was collected into polystyrene tubes and centrifuged at  $300 \times g$  for  
106 10 min at  $4^{\circ}\text{C}$  to remove cells and large debris. The supernatant was separated from the pellet and  
107 centrifuged again at  $3000 \times g$  for 30 min at  $4^{\circ}\text{C}$  to remove residual organelles, cell fragments and small  
108 debris. The whole saliva supernatant (WS) was separated from the pellet and kept on ice for EV  
109 isolation. For the purpose of some experiments, an optional filtration step was performed  
110 subsequently. Briefly, the whole saliva supernatant was filtered through  $0.45 \mu\text{m}$  or  $0.22 \mu\text{m}$  PVDF  
111 syringe filters (Merck) before proceeding to the next step. All methods were performed in accordance  
112 with relevant guidelines and regulations. All participants signed informed consent prior to participating  
113 as part of an ethically approved study by a French national ethic committee (CPP-NORD OUEST III) on  
114 April 21, 2023 (N° ID RCB : 2023-A00188-37). The study was registered at [www.clinicaltrials.gov](http://www.clinicaltrials.gov) (2023-  
115 A00188-37).

### 116 **Isolation and concentration of EVs**

117 **Ultracentrifugation (UC).** 1 mL of the WS was diluted with 24 mL of phosphate-buffered saline (PBS)  
118 and transferred to 50 mL, Open-Top Thickwall Polypropylene Tube (Beckman Coulter) for  
119 ultracentrifugation at  $100,000 \times g$  for 1 hour at  $4^{\circ}\text{C}$  (JXN-30 centrifuge, JA-30.50 Ti Rotor, Beckman  
120 Coulter). The pellet was washed with PBS and centrifuged again at  $100,000 \times g$  for 1 hour at  $4^{\circ}\text{C}$  to  
121 remove soluble contaminant. The final pellet was resuspended in  $100 \mu\text{L}$  PBS and then kept on ice or  
122 at  $-80^{\circ}\text{C}$  for further analysis.

123 **Co-precipitation (Q).** The extracellular vesicles from WS was isolated using miRCURY® Exosome Kit  
124 (Qiagen) according to the manufacturer's recommendations. Briefly,  $400 \mu\text{L}$  of Precipitation Buffer B  
125 were added in 1 mL of whole saliva supernatant and incubated for 60 min at  $4^{\circ}\text{C}$ . At the end of the  
126 incubation time, the samples were centrifuged at  $10,000 \times g$  for 30 min at room temperature, the  
127 supernatant was removed, and the pellet was resuspended in  $100 \mu\text{L}$  of Resuspension Buffer and then  
128 kept on ice or at  $-80^{\circ}\text{C}$  for further analysis.

129 **Immunomagnetic separation of EVs (M).** The extracellular vesicles from WS was isolated using  
130 Exosome Isolation Kit Pan, Human (Miltenyi Biotec) according to the manufacturer's  
131 recommendations. Briefly,  $50 \mu\text{L}$  of Exosome Isolation MicroBeads were added in 1 mL of WS and  
132 incubated for 1 hour at room temperature. The mixture was applied to an equilibrated  $\mu$  column ( $100$   
133  $\mu\text{L}$  of equilibration buffer, then washed 3 times with  $100 \mu\text{L}$  of isolation buffer) and placed in a  $\mu\text{MACS}$

134 Separator attached to the MACS MultiStand (Miltenyi Biotec). Afterwards,  $\mu$  column was washed 4  
135 times with 200  $\mu$ L of isolation buffer. EVs and beads were co-eluted outside the magnetic field with  
136 100  $\mu$ L of isolation buffer using a dedicated plunger. EVs and beads were then kept on ice or at  $-80^{\circ}\text{C}$   
137 for further analysis.

### 138 **Characterization of EVs**

139 EVs were characterized as recommended by the International Society of Extracellular Vesicles (ISEV)  
140 [23, 24]. EV size and particles number were measured by Nanoparticles Tracking Analysis using the  
141 NanoSight NS300 (Malvern Panalytical). EVs integrity and the absence of large aggregates were  
142 analysed by cryo-EM using the JEOL 2200 FS transmission electron microscope (Jeol). Small RNA  
143 content was isolated using the miRNeasy Serum/Plasma Kit (Qiagen). Protein content was measured  
144 using the Micro BCA Protein Assay Kit (ThermoFisher Scientific) and characterized by western blot, as  
145 described [25]. EVs were used freshly prepared.

### 146 **Proteomics**

147 LC-MS/MS analysis were done at Plateforme de Proteomique Fonctionnelle de Montpellier (FPP).  
148 Triplicates of WS, EV UC, EV Q and EV M were analysed with 14.5  $\mu$ g of proteins per sample. Briefly,  
149 protein digestion was performed using S-Trap micro columns (ProtiFi) following the manufacturer's  
150 instructions. Peptide samples were injected for analysis using a nano flow HPLC (RSLC U3000, Thermo  
151 Fisher Scientific) coupled to a mass spectrometer equipped with a nanoelectrospray source (Q Exactive  
152 HF, Thermo Fisher Scientific). Peptides were separated on a capillary column (0.075 mm  $\times$  500 mm,  
153 Acclaim Pepmap 100, reverse phase C18, NanoViper, Thermo Fisher Scientific) following a gradient of  
154 2-40% buffer B in 128 min (A = 0.1 % formic acid B = 0.1% formic acid, 80% acetonitrile) at a flow rate  
155 of 300 nl/min. Spectra were recorded via Xcalibur 4.2 software (Thermo Fisher Scientific) with the  
156 128.meth method. The spectral data were analyzed using MaxQuant v2030 and Perseus v16150  
157 software, using the leading FPP v3.5 script. RefProteome\_HUMAN-  
158 cano\_2023\_03\_UP000005640\_559292.fasta databases and a base of common contaminants, with the  
159 following fixed modification: Carbamidomethylation (C) and the following variable modifications:  
160 Oxidation (M); Acetyl (Protein N-term) were used. Data validation was performed with the following  
161 filters: FDR peptides and proteins at 1%.

### 162 **SDS-PAGE and Western blot (WB) analysis**

163 Samples were lysed in RIPA buffer containing protease inhibitor (Roche) and Laemmli buffer containing  
164 2.5% of  $\beta$ -mercaptoethanol. 10  $\mu$ g of proteins were loaded on homemade 12.5% SDS polyacrylamide  
165 gel and run at 200 V for 50 min. Proteins were transferred to nitrocellulose membrane for 1 hour using  
166 Trans-Blot cell (Bio-Rad). After transfer, the membranes were blocked with 5% skim milk in PBS-Tween

167 and incubated over night at 4 °C with primary antibodies: anti-CD9 (1/1000), anti-CD63 (1/1000), anti-  
168 CD81 (1/1000), anti-TSG101 (1/1000), anti-albumin (1/20,000), anti-mucine-16 (1/1000), anti-SAA1  
169 (1/1000) from Abcam and anti-CD59 (1/400) from Sigma. The membranes probed with the appropriate  
170 primary antibodies were incubated with secondary antibodies linked to horseradish peroxidase (HRP)  
171 (Sigma). Blots were revealed using Clarity Max Western ECL Substrate (Bio-Rad) and quantified with  
172 ChemiDoc MP Imaging System (Bio-Rad) and ImageJ software.

### 173 **Bioinformatics tools**

174 The enrichment in EV proteins was checked by comparizon with ExoCarta Top 100 list  
175 (<http://www.exocarta.org>) [26]. The visualization of Venn diagram was performed using online Draw  
176 Venn Diagram tool (<https://bioinformatics.psb.ugent.be/webtools/Venn/>).

### 177 **RNA Extraction and RT-qPCR**

178 Total small RNA was extracted from EVs or WS samples using the miRNeasy Serum/Plasma Kit (Qiagen).  
179 miRNAs were quantified using Qubit™ microRNA Assay Kits (ThermoFisher Scientific) according to the  
180 manufacturer's recommendations. Labchip analysis was performed to assess the size of small RNAs  
181 according manufacturer's instructions (PerkinElmer).The Reverse transcription was performed on 4 ng  
182 RNA using miRCURY® LNA® RT Kit (Qiagen). Real-time quantitative PCR was performed on 40 pg cDNA  
183 using specific primers (Supplementary Table 1) and miRCURY® LNA® miRNA SYBR® Green PCR (Qiagen).  
184 Values were normalized to miRNA quantity and expressed as relative expression to control WS using  
185 formulae  $2^{-\Delta CT}$ .

### 186 **Statistical Analysis**

187 Statistical analyses were performed using the GraphPad 9 Prism Software. Data distribution was  
188 assessed using the normality test. A Wilcoxon signed-rank test was done to compare one group to the  
189 control normalized to 1. If controls were not normalized, the statistical analyses between three groups  
190 were compared using the the Friedman's test when values were paired and non-parametric, or Kruskal-  
191 Wallis test when values were unpaired and non-parametric, followed by Dunn's multiple comparizon.  
192 Data are presented as mean  $\pm$  SEM. \* p <0.05; \*\* p <0.01; \*\*\* p <0.001, \*\*\*\*: p < 0.0001.

193

## 194 **Results**

### 195 **Workflow for EV isolation by ultracentrifugation (UC), co-precipitation (Q) and immuno-** 196 **affinity (M)**

197 EVs were isolated from the volunteers' saliva by three different isolation methods. We compared a co-  
198 precipitation based method (Qiagen) and an immuno-affinity based method (Miltenyi) with the  
199 differential ultracentrifugation technique (UC), the latter being currently considered as the gold  
200 standard for EV isolation. An overview of the experimental workflow for saliva collection, processing  
201 and EVs isolation is depicted in figure 1 (see material and methods for detailed procedure). Once  
202 isolated, we assessed the characteristics of saliva derived EVs for each isolation method in parallel,  
203 namely EV UC, EV Q and EV M (for ultracentrifugation, co-precipitation and immuno-affinity isolated  
204 EVs respectively) following the MISEV guidelines [23, 24].

### 205 **Particle size distribution and concentrations of EVs isolated from human saliva by** 206 **Nanoparticle Tracking Analysis**

207 After isolation, particle size distribution and concentrations of EVs were measured using NTA (NanoSight  
208 NS300) (fig. 2). For this experiment, EV samples from four volunteers were analyzed in triplicates. All  
209 samples were submitted to  $\mu$ BCA assay in order to adjust them at the same proteic concentration  
210 before proceeding to NTA.

211 EV UC shows the wider size distribution, with a mean size of 264 nm (+/- 13 nm) followed by EV Q: 227  
212 nm (+/- 7 nm) and EV M: 84 nm (+/- 4 nm) (fig. 2A and D). The observed smaller size distribution and  
213 mean size of EV M is partly attributed to the immuno-affinity kit's use of antibodies coupled to  
214 magnetic beads for EV capture. These beads, in excess in the sample, have a size centered around 50  
215 nm according to the manufacturer and their measurement cannot be uncoupled from the EVs. This  
216 phenomenon was also observed in another study [20]. When looking at the mode, and the D10 (fig. 2  
217 E, F), the size measurement is clearly biased towards small size. However, the D50 and D90 (fig. 2F)  
218 clearly show that even the larger vesicles of EV M samples are way smaller than EV UC and EV Q (D90  
219 mean : EV UC 422 (+/- 21) ; EV Q 339 (+/- 9) ; EV M 131 (+/- 13)). Moreover, when EVs below 100 nm  
220 were removed from analysis to account for the beads bias, the EV M size distribution was still  
221 significantly narrower than those obtained by the two other methods (fig. S1), confirming that  
222 immuno-affinity method tend to isolate small vesicles.

223 Regarding the total number of vesicles, the immuno-affinity based technique allows to isolate more  
224 particles than the other two: EV UC  $2.34 \times 10^{10}$ ; EV Q  $1.96 \times 10^{10}$ ; EV M  $2.25 \times 10^{11}$  particles (fig. 2C).  
225 Again, this is partly due to the presence of magnetic beads in the sample. In fact, there are fewer

226 measured particles in EV M than in EV UC, although the difference is not statistically significant (fig.  
227 S1B). When normalized by the quantity of proteins measured in the samples, EV M has the highest  
228 concentration, with  $1.36 \times 10^9$  particles/ml/ $\mu\text{g}$ . In contrast, EV UC has a concentration of  $4.51 \times 10^8$   
229 particles/ml/ $\mu\text{g}$ , and EV Q has  $1.77 \times 10^8$  particles/ml/ $\mu\text{g}$  of protein. This represents a 2.5-fold lower  
230 concentration of EVs in the EV Q condition compared to EV M (fig. 2A and supplementary table 2). This  
231 ratio of particle number to protein concentration is generally used to estimate the EV purity [27]. It  
232 clearly shows that EVs isolated by immuno-affinity and ultracentrifugation are more pure than those  
233 isolated by co-precipitation. This result is in contradiction with others studies, where PEG isolation was  
234 shown to have a higher recovery efficiency than UC in saliva and blood [19, 28].

235 This is likely due to the fact that co-precipitation kits relies on polycationic polymers (PEG) to help the  
236 EV precipitation at low centrifugation speed. It may also co-precipitate a significant quantity of non-  
237 vesicular proteins that bias the final protein concentration within these samples. This observation was  
238 previously made in several studies using PEG-based precipitation methods like ExoQuick-TC (System  
239 Biosciences Inc), ExoQuick-CG (System Biosciences Inc) or miRCURY Exosomes isolation kits (Exiqon)  
240 for instance, that relies on the same principle to isolate EVs [19, 20, 29-31].

241 Based on NTA results, the immuno-affinity method seems to isolate rather small EVs than UC and co-  
242 precipitation methods. The latter one isolates less particles than ultracentrifugation and immuno-  
243 affinity when normalized by their proteic content. This raise the concern that EVs isolated by co-  
244 precipitation may have a higher proteic contamination than the two other isolation methods.

### 245 **Morphological characterization of EVs from saliva**

246 We next seek to visualize by cryo-EM the morphology and the structural integrity of the EVs. The main  
247 advantage of the cryo-EM technique lies in its ability to maintain membranes in a state that closely  
248 resembles their natural condition. This preservation enables clear observation of lipid bilayers and  
249 internal structures of vesicles. We were able to visualize EVs in all conditions (fig. 2B). EV UC acquired  
250 pictures show nice round shaped vesicles with a bilayered membrane (fig. 2B, upper left panel). They  
251 were well dispersed with only a few couples of vesicles stucked together. EV Q were less easy to detect  
252 and appear more blurry (fig. 2B, upper right panel). This is likely due to remaining traces of PEG within  
253 the samples interfering during the acquisition. These vesicles are round shaped though and tend to  
254 agglomerate more than the UC ones. For UC M, we could also visualize the EVs (fig. 2B, lower panel)  
255 linked to the beads with clear round shapes, similar to the UC condition. However, many magnetic  
256 beads aggregates were observed within the microscope field. Despite differences, the EVs obtained  
257 using the three described isolation methods can be assessed by cryo-EM.

## 258 **Protein content-based characterization of EVs isolated from saliva**

259 Based on the MISEV guidelines [23, 24], we did a systematic proteic quantification for all EV isolation  
260 methods and the starting WS. 27 biological samples from nine healthy volunteers were assessed. We  
261 used the  $\mu$ BCA kit to estimate the protein quantity in each sample (fig. 3A). As expected, whole saliva  
262 contains a huge amount of proteins, with a mean concentration of 4.7 mg/ml. Regarding EV isolation,  
263 EV UC has the lowest recovery rate, followed by EV M and EV Q (both being significantly higher than  
264 EV UC ( $p < 0.01$  and  $p < 0.001$  respectively) with a mean protein quantity of 43.2  $\mu$ g ; 195.8  $\mu$ g and 123.5  
265  $\mu$ g per mL of saliva respectively.

266 A SDS-PAGE gel was first Coomassie stained in order to look at the global profile of WS, EV UC, EV Q  
267 and EV M. The same volume of sample was loaded for each lane. As shown in figure 3B, the global  
268 intensities observed for each condition correlated well with the relative abundance measured using  
269 the  $\mu$ BCA assay. However, despite sharing many similarities, there are several noticeable differences  
270 between the WS condition and the EV conditions. Some large bands present in WS tend to become  
271 very faint or disappear in the EV UC, EV M samples and, to a lesser extent, EV Q samples. This is  
272 expected because some proteins like mucins or albumin are very prominent in saliva but not in EVs.  
273 Unexpectedly, the three EV isolated profiles also show differences from one to each other. EV Q closely  
274 resemble to the WS profile. EV UC also shares similarities, but several large bands tend to become  
275 faint and several small bands become visible all along the lane. EV M is the one showing the most  
276 striking differences, without any strong bands but many small discrete bands all along the lane. This  
277 result may indicate that the three isolation methods leads to different proteic contents.

278 We next wanted to assess by WB the presence of specific markers indicative of EV enrichment: the  
279 transmembrane tetraspanins CD9, CD81, CD63 and a cytosolic protein belonging to the ESCRT  
280 complex: TSG101. We also selected albumin as a negative marker of EVs, abundantly present in whole  
281 saliva, as an indicator of EV purity (fig. 3C). 20 $\mu$ g of protein were loaded for each lane. In WS, we  
282 observe a strong signal for albumin and TSG101 but we could not detect the three tetraspanins CD9,  
283 CD81 and CD63. For EV UC, all tetraspanins and TSG101 were detectable and only a faint signal was  
284 visible for albumin, indicating that EVs isolated by UC are relatively free of protein contamination (fig.  
285 3C, 3D). For EV Q, TSG101, CD9 and CD81 were detectable. The CD63 signal was not detected in all  
286 samples, and in instances where it was present, the band was consistently weaker compared to those  
287 obtained through UC and M isolation methods (fig. S2). A significant fraction of albumin was also  
288 present in these samples, confirming that using co-precipitation methods leads to some salivary  
289 proteic contamination of the EV fraction (fig. 3C, 3D). For EV M, TSG101, CD9, CD63 were present and  
290 the samples were almost free of albumin contamination, but unexpectedly, we failed to detect CD81  
291 signal. This surprising result may be due to distinct EV subtypes preferentially isolated by this method

292 in saliva or to some steric hindrance impeding the binding of the CD81 antibody to its target, resulting  
293 in an important loss of these EVs or the impossibility to detect this marker in our WB experiments.  
294 Only EV UC fraction allows the systematic detection of all three tetraspanins. EV M show a comparable  
295 profile except for CD81 (fig. 3C, 3D). EV Q protein bands are systematically weaker and show a shift in  
296 mobility compared to the other methods which is likely due to remaining traces of PEG containing  
297 buffer (fig. 3C, 3D). Given the fact that there is probably protein contamination from WS to some extent  
298 and that we loaded equal protein amount in all lanes, this result is expected. These western blots  
299 clearly show a differential enrichment in specific markers of EVs for the three methods (fig. 3C, 3D),  
300 which may again suggest that we isolate different population of EVs. These results are representative  
301 of four independent experiments, using saliva from four volunteers (fig. S2A) revealing a greater  
302 variability inter-methods than inter-individuals.

303 In order to confirm these data at a larger scale and to study the whole proteomic landscape resulting  
304 from the different isolation methods, we performed a quantitative label free LC-MS/MS proteomic  
305 analysis on WS and the EVs isolated by the three methods. We used three biological replicates for each  
306 condition. However, one EV M sample failed to be analyzed and was removed in the subsequent  
307 analysis.

308 We identified 648 proteins in WS, 800 in EV UC, 711 in EV Q and 394 in EV M samples (Supplementary  
309 Table 3). For each EV isolation method, we compared their proteic content to the top 100 EV markers.  
310 EV UC had the highest overlap with the top 100 (85), closely followed by EV Q (78), but EV M showed  
311 only a moderate overlap (66) (fig. 3E).

312 To be the more stringent and robust possible, we looked at the proteins identified in all three replicates  
313 for EV UC and EV Q and the two EV M replicates (fig. 3F). Importantly, these proteins were unique to  
314 EVs and absent from the WS samples. 140 proteins were uniquely found in EV UC, 45 in EV Q and 12  
315 in EV M. Only 29 were identified in EV isolated by the three methods (8.5% of total proteins identified).  
316 This clearly demonstrates that the choice of EV isolation method is crucial, as it directly affects the  
317 harvested protein content and, consequently, has a significant impact on the success or failure of  
318 detecting biomarkers. To verify this last assertion, we selected three proteins known to be upregulated  
319 in several diseases and well described as strong biomarkers in their corresponding pathologies. We  
320 chose mucin-16, a cancer biomarker [32], the Serum Amyloid A1 protein (SAA1), a traumatic brain  
321 injury marker [33] and CD59, an early biomarker for gestational diabetes mellitus [34]. These three  
322 proteins were identified in the LC-MSMS analysis with higher intensities in EVs compared to the WS.

323 We could confirm by WB the presence of these three proteins only in the EVs and not in WS samples  
324 (fig. 3G). The stronger signal was always observed in EV UC, followed by EV Q. We failed to detect

325 MUC16 and SAA1 in the EV M condition (fig. 3G and fig. 3H). The experiment was repeated seven times  
326 (fig. S2B,C and D). This result emphasize the relevance and the high potential of salivary EVs for  
327 diagnostic. From a clinical perspective, this kind of differential analysis can enhance the discovery of  
328 new biomarkers, improve the sensitivity of detection of existing ones and facilitate their transposition  
329 to the clinic.

### 330 **MiRNA content-based characterization of EVs isolated from saliva**

331 As we observed strong differences in the proteic cargo of EVs depending on the isolation method  
332 chosen, we wondered if the nucleic acid cargo could be also affected by it.

333 MiRNAs, small non-coding RNAs crucial in regulatory roles [35], impact about 60% of protein-encoding  
334 genes, orchestrate cellular signaling, and can translocate to the nucleus [36-38], making them  
335 increasingly recognized as potential biomarkers in various biofluids [39-41] including saliva [42] for  
336 many diseases [41-43].

337 It is now well established that EVs contains small non-coding RNA (sncRNA) and miRNA in particular  
338 with more than 10 000 entries in Vesiclepedia (<http://microvesicles.org/>). Therefore, we chose to  
339 focus only on miRNA to assess the nucleic acid cargo of the method-dependent isolated vesicles.

340 Small RNAs from WS and isolated EVs were purified using miRNeasy Serum/Plasma Kit (Qiagen) that  
341 has a cut-off of 200 bases. The total small RNAs contained in WS, EV UC, EV Q and EV M was then  
342 quantified by Qubit™ microRNA Assay Kit (ThermoFisher Scientific) (fig. 4A). To compare the total  
343 quantity of small RNA recovered among the samples, we normalized the total RNA quantity measured  
344 by the initial amount of saliva used for the extraction. Figure 4A shows the total amount of small RNA  
345 (ng/ml of saliva) for all samples. Ten biological replicates were used in this experiment. The WS  
346 condition contained a significantly larger fraction of small RNA compared to the EV UC and EV M  
347 conditions, with 179 ng/ml of saliva versus 30.9 ng/ml and 8.3 ng/ml in EV UC and EV M respectively.  
348 Although the small RNA content in EV Q is lower, it's not statistically significant when compared to WS  
349 (62.8 ng/ml), yet it is significantly higher than that in EV M (fig. 4A). This observation was confirmed by  
350 the lab chip analysis using small RNA kit (PerkinElmer) (fig. 4B).

351 To assess whether EV isolation techniques influence the outcome of miRNA analysis and to study  
352 whether EVs are enriched in miRNAs compare to the WS content, we selected a small set of six miRNAs  
353 known to be present in saliva [44] including hsa-Let-7a-5p, hsa-Let-7f-5p, hsa-miR-148a-3p, hsa-miR-  
354 26b-5p, hsa-miR-103a-3p and hsa-miR-107 for our analysis. Based on the Qubit results, we used 4 ng  
355 of total small RNA to proceed the reverse transcription. 1/10<sup>th</sup> of the RT reaction was subsequently  
356 used in the qPCR reaction. Although the same RNA quantity was used in all conditions, some strong  
357 and reproducible differences were observed between the different conditions tested. Figure 4C shows

358 the relative levels of the miRNA targets in EVs expressed as a fold change to WS. All tested miRNAs  
359 were significantly more enriched in EV conditions than in WS, with enrichment levels ranging from 4-  
360 to 50-fold, depending on the specific miRNA. This result confirms the observation made in several  
361 articles [44, 45] that the main miRNA source in saliva arise from the EVs rather than from circulating  
362 free miRNA. However, slight differences in the expression of the six tested miRNAs were observed  
363 among the three techniques. In the EV Q condition, all targets have p-values less than 0.01. For EV M,  
364 all p-values are below 0.01, except for hsa-miR-103a-3p, which has a p-value less than 0.05. In the EV  
365 UC condition, three targets have p-values below 0.05, and three others have p-values below 0.01. EV  
366 Q also shows the more reproducible results, with the smallest error bars compared to the other  
367 technics. Based on this result, the three methods are suitable regarding miRNA measurements with EV  
368 Q being the most consistent one. Another interesting point is that the quantity of the six miRNAs  
369 measured is not identical in the EV isolated using the same methods, nor is the fold change to WS,  
370 highlighting the variability of miRNAs contained in EVs (fig. 4C).

#### 371 **Effect of filtration on EVs isolation, recovery and subsequent analysis**

372 Filtration is an optionnal step often recommended by manufacturer or by the scientific community in  
373 order to improve EVs purity. However, the importance of this step and its assumed improvement in  
374 recovery has never been strictly assessed. We used a similar workflow to the one depicted in figure 1,  
375 with the addition of a filtration step through a 0.45  $\mu\text{m}$  or a 0.22  $\mu\text{m}$  filter on whole saliva right before  
376 proceeding to EV's isolation (fig. 5A). In the EV UC condition, adding filtration to the procedure made  
377 it impossible to quantify protein concentration anymore, as shown in Figure 5B. Additionally, the  
378 quantity of small RNA in these EVs dramatically decreased after filtration, dropping from 5.31 ng in the  
379 unfiltered condition compared to 2.26 ng and 2.47 ng in the 0.45  $\mu\text{m}$  and 0.22  $\mu\text{m}$  filtration conditions,  
380 respectively (fig. 5C). RT-qPCR results indicated a substantial reduction in both miRNA recovery and  
381 miRNA target detection for both filtration conditions, with the extent of loss varying depending on the  
382 target (fig. 5D). Notably, the miRNA recovery appeared more adversely affected in the 0.22  $\mu\text{m}$   
383 condition than in the 0.45  $\mu\text{m}$  condition, although this difference was not statistically significant.

384 Similar patterns were observed in the EV Q samples with regard to protein and small RNA contents, as  
385 detailed in Figure S3. The miRNA targets assessed by RT-qPCR in these samples also demonstrated a  
386 decrease in recovery, except for hsa-Let-7f-5p, which showed an increased fold change compared to  
387 the non-filtered condition (fig. S3C).

388 In contrast, the protein quantities in EV M samples remained unchanged between non-filtered and  
389 filtered conditions (fig. S4A, left panel). This consistency may be partially attributed to the presence of  
390 mouse immunoglobulin chains on the beads, which represent a significant portion of the measured

391 proteins. Alternatively, it might reflect a preference of this technique for isolating smaller vesicles, as  
392 observed in our experiments (Fig. 2A, D, E, F, and S1). These smaller EVs might be less impacted by  
393 filtration, given their size relative to the filter pores.

394 However, for the EV M samples, both the total small RNA quantity (fig. S4B) and the fold changes in  
395 miRNA targets relative to the non-filtered condition (fig. S4C) experienced a drastic reduction, similar  
396 to what was observed in the EV UC conditions (fig. 5). This is likely due to significant loss or degradation  
397 of EVs during filtration.

398 Taken together, these data show that in a global manner, filtration either on 0.45 or 0.22  $\mu\text{m}$  pores  
399 dramatically affect EVs recovery in all the isolation conditions and strongly impede subsequent  
400 analysis. Filtration of saliva before EV isolation should thus be avoided.

401

## 402 Discussion

403 Over the last 15 years, several studies have underlined the great potential of saliva as a very interesting  
404 and promising biofluid for biomarker discovery. However, limitations such as variability, the presence  
405 of inhibitors and bacteria makes this biofluid still understudied compared to blood, urine or even CSF.  
406 The identification of EVs in saliva[6] has raised a profound interest, since this could overcome the  
407 limitations cited above. Nevertheless, only a few EV biomarkers isolated from human biofluids have  
408 been implemented in clinical practice so far due to the fact that EV isolation and characterization  
409 remain challenging and complex [46]. Several researchers have looked at ways to improve and  
410 standardize isolation methods for the analysis of human samples, but these studies concern mainly  
411 blood, urine and CSF [30, 47-49], with only a few assessing saliva [19, 31, 50]. Ultracentrifugation is by  
412 far the most used technique even despite being time-consuming, weakly parallelizable and requires  
413 big lab instruments. Alternative methods employing co-precipitation which are faster, scalable,  
414 cheaper and more suitable for clinical implementation have been tested. Immuno-affinity-based EV  
415 isolation also offers advantages, including the need for smaller sample amounts, faster processing, and  
416 the potential to yield highly pure EV fractions.

417 For the first time, in this study, we conducted in parallel an extensive comparison between these three  
418 types of EV isolation methods in human saliva. We characterized the integrity of EVs isolated by these  
419 three methods and compared their protein and miRNA content.

420 First, we observed that co-precipitation and immunoaffinity kits allow isolating efficiently EVs from  
421 saliva even if they were primarily designed for other biofluids. However, we found that despite the use  
422 of identical starting volume of saliva to isolate EVs, the relative enrichment level is very different  
423 depending on the chosen method. As observed by the NTA experiments, the size distribution and  
424 concentration of particles vary drastically depending on the isolation method, which strongly suggest  
425 that each method likely isolate different EV subpopulations associated with various degrees of purity  
426 and recovery rates. Compared to EV UC, EV Q shows the same size distribution but with more than  
427 twice as less particles. Cryo-EM data shows nice although blurry vesicles. This suggest that remaining  
428 traces of buffer are still present in the preparations and that debris or protein aggregates forms when  
429 EVs are coprecipitated. When we looked at specific proteins by western blot, by loading the same  
430 amount of total proteins for each condition, the band intensities were less pronounced for EV Q  
431 compared to EV UC regarding CD9 and CD81. We also observed a systematic shift in protein migration  
432 for all targets tested in the EV Q samples. Interestingly, TSG101 profiles (double band) in EV Q were  
433 comparable to a merge between the profile of WS and EV UC or EV M. In saliva, the upper band was  
434 predominant whereas in EV UC and EV M, only the lower band was visible. For EV Q, both bands

435 appears in equal proportions as if the total protein contained in EV Q was a mix between an EV isolated  
436 fraction and the whole saliva. The fact that albumin was detectable only in whole saliva and to a lower  
437 extend in EV Q but almost never in the two other methods confirms that the EV Q method coprecipitate  
438 a large portion of soluble proteins present in the whole saliva. Based on these observations, we  
439 propose that additionally to albumin, TSG101 migration profile could constitute another purity  
440 indicator of EV preparations in saliva. To further investigate these apparent discrepancies, we  
441 proceeded to an extensive proteomic analysis. EV Q appeared to contain a set of proteins similar to EV  
442 UC, with almost as much Top 100 EV markers as EV UC (85 vs 78) and a small subset of uniquely  
443 identified proteins. These data are in line with a recent study comparing UC and ExoQuick-CG™ and  
444 showing 190 and 171 proteins differentially expressed in UC and PEG conditions respectively.  
445 Moreover, these enriched proteins seems to be related to very different biological processes and  
446 functions, advocating for a selective enrichment in different EV populations [19].

447 Importantly, EV Q allowed the detection by WB of known biomarkers (MUC16, CD59 and SAA1) albeit  
448 less intense than EV UC, confirming the high diagnostic potential of this method to improve the  
449 detection of proteins that fail to be detected in the whole saliva.

450 EV M seems to isolate significantly smaller particles with an efficiency close to the EV UC condition.  
451 This was also confirmed by WB, where band intensities were comparable in EV UC and EV M for CD9  
452 and CD63. Surprisingly, we failed to detect CD81 by WB. This was unexpected, as the beads are  
453 designed to recognize the three tetraspanins CD9, CD63 and CD81. Two out of three samples were  
454 devoided of CD81 in the proteomic analysis, confirming the lack of CD81 protein in several samples. A  
455 plausible explanation would be that we preferentially isolate EVs having CD63 and CD9 on their  
456 surface. A subset of EVs harbouring the CD81 tetraspanin would be captured in a smaller proportion,  
457 resulting in a too faint signal that failed to be detected by WB. Alternatively, steric hindrance could  
458 impede the antibody binding to its target. Moreover, the global proteic content as well as the Top 100  
459 EV markers of these isolated EVs weakly overlap the EV UC and EV Q ones. Cryo-EM data show very  
460 nice vesicles though, with one or several beads at their surface.

461 Apart from the EV characterization experiments, the immuno-affinity-based technique seems to be  
462 suitable for EVs isolation. They preferentially isolate small vesicles, require small volumes of samples  
463 and can be processed easily and quickly. However, caution must be taken as this method tend to isolate  
464 a different and less exhaustive proteic content and sometimes fail to identify markers (MUC16 and  
465 SAA1) that are present in the EVs isolated by UC or co-precipitation.

466 Taken together, these results point to the fact that different EV isolation methods lead to preferential  
467 enrichment of different EVs subpopulations harbouring various size, concentration and protein cargo.

468 Despite the presence of contaminants, the co-precipitation method yields results similar to the UC  
469 method in terms of EV size distribution, isolated protein cargos, and the detection of EV-enriched  
470 proteins by WB compared to WS. This makes it a preferred method for rapid, cost-effective, and large-  
471 scale diagnostic purposes.

472 In the literature, contradictory results exist regarding whether or not miRNAs are preferentially  
473 contained in a free or protein-bound form within the saliva or if they are mainly enclosed within the  
474 EV's cargo [44, 45, 51, 52]. We systematically assessed our miRNAs derived from EVs and those  
475 contained in the whole saliva. After extracting small RNA, the quantified miRNAs in human salivary  
476 samples revealed a relatively high concentration in whole saliva compared to the three EV isolation  
477 methods. Notably, the levels in EV Q and EV UC were significantly higher than in the EV M condition,  
478 with EV M exhibiting the lowest recovery rate. We next assessed a specific set of six miRNAs known to  
479 be present in saliva [44] and for which dysregulations are associated to diverse pathologies [53-58].  
480 For all the targets, we clearly observed that miRNAs were enriched within EVs, confirming several  
481 previous observations [44, 45]. This was true for all samples tested in all individuals. In this regard, the  
482 three tested methods are suitable for RT-qPCR miRNA analysis as they give similar results (EV Q being  
483 the more consistent one though). A more systematic investigation using small RNA sequencing would  
484 be necessary to strengthen this statement.

485 Finally, we wanted to assess the usefulness of the often recommended filtration step assumed to  
486 improve EV's purity. We tried two different pore sizes; 0.22 and 0.45 $\mu$ m, as they are often used in  
487 several studies involving EV isolation. Surprisingly, filtration not only failed to improve EV purity, but it  
488 also dramatically decreased the overall recovery of EVs. This was clearly shown by the protein  
489 quantification, the Qubit measurements of small RNAs and the RT-qPCR experiments. In a global  
490 manner, all the Ct increased significantly (data not shown) suggesting a general loss of nucleic acid  
491 material directly due to the filtration process. Compared to the non-filtered conditions, all EVs samples  
492 recovered after filtration show a drastic drop of their measured level. This can be interpreted by a  
493 loss/destruction of an important fraction of EVs when applied to the filter. The pressure exerted on  
494 the salivary sample and the increasing pressure of drop across the filters result probably in pores  
495 obstruction. And the pores filled with extracellular vesicles may damage the harvested population and  
496 impede greatly the EV recovery as suspected in [59]. This result also emphasizes the fact that most of  
497 the miRNAs (at least for all the targets we tested) are preferentially contained in EVs and the free  
498 fraction circulating in saliva is clearly not the main source of miRNA.

499 Altogether, these data underline the importance of the EV isolation method to be chosen depending  
500 on the focus of the research studies, the necessity of developing standard protocols and the great

501 potential of salivary EVs for reliable biomarker identification and non-invasive diagnostic. The ease of  
502 collection, the possibility of repetitive sampling in a stress-free and non-invasive way are the strongest  
503 assets of saliva. Coupled to a cheap, rapid, scalable and reliable EVs isolation technique as the co-  
504 precipitation method, it opens the way to a new diagnostic era. We believe that with a few  
505 improvements, co-precipitation can become this method of choice.

506 To our knowledge, it is the first time that a direct comparizon of three EV isolation methods relying on  
507 very different principles is conducted in human saliva. We have shown that the different methods  
508 employed likely result in the isolation of different EV subpopulations. Based on the advantages and  
509 inconveniences observed in this study, we highly recommend that researchers take the time to  
510 precisely evaluate several isolation methods and choose the one that suits bests their aim and their  
511 downstream analysis. From a diagnostic perspective, the co-precipitation method is the one that fits  
512 the best the purpose of diagnostic and biomarker discovery in saliva. Filtration should also be avoided.  
513 In this regard, this article participate to increase the knowledge on salivary EVs and pave the way for a  
514 future use of EVs isolated from saliva towards potent and non-invasive tool for biomarker research.  
515 More experiments at a larger scale are required to assess the whole proteome and sncRNAome in  
516 order to grasp the whole landscape of isolated EVs by these three methods.

517

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## 525 **Author contributions**

526 Study concept and design: J.B., F.M. and M.K. Acquisition, analysis, interpretation of data: All authors.  
527 Drafting of the manuscript and critical revision of the manuscript for important intellectual content:  
528 J.B., F.M. and M.K. Statistical analysis: J.B. Funding acquisition: F.M. All authors critically reviewed and  
529 edited the manuscript.

## 530 **Data availability statement**

531 The authors declare that all relevant data have been provided within the manuscript and its supporting  
532 information files.

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## 535 **Disclosure of interest**

536 The authors declare that there are no conflicts of interest.

537

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## 669 **Figure legends**

670 **Fig. 1: Workflow of human salivary extracellular vesicles isolation.** Representative scheme of the  
671 workflow. Briefly, 4 mL of saliva were collected from donors and centrifuged successively at 300 x g  
672 and 3000 x g to remove cells and large debris and then small debris respectively. Then, whole saliva  
673 supernatant was split in four fractions: 0.5 mL whole saliva supernatant (WS) used as a control and  
674 1 mL dedicated to each EV isolation methods.

675 **Fig. 2: Characterization of human salivary extracellular vesicles.** (A) Mean size distribution of  
676 extracellular vesicles (EV UC, Q and M) assessed by NTA (n=4). (B) Representative pictures of EVs  
677 isolated by ultracentrifugation (EV UC), co-precipitation (EV Q) and by immuno-affinity (EV M) by cryo-  
678 transmission electron microscopy. Image bars represent 100 nm. (C) Quantification by NTA of EV  
679 particles isolated from 1mL of human saliva (n=4). (D) Mean size of EVs derived from saliva (n=4). (E)  
680 Mode size of EVs derived from saliva (n=4). (F) Particle size distribution D10, D50, and D90  
681 corresponding to the 10% smallest particles, 50% (median), and 10% largest particles within a sample  
682 respectively (n=4). \*: p < 0.05

683 **Fig. 3: Characterization of human salivary extracellular vesicles by protein markers.** (A) Total proteins  
684 contained in human saliva and salivary EVs (n>26). (B) Commassie blue staining of total proteins from  
685 whole saliva or EV UC, EV Q and EV M. (C) Western blot analysis of salivary markers (albumin,  
686 endosomal (TSG101) and tetraspanins (CD9, CD63, CD81)) in EV UC, EV Q and EV M protein extracts.  
687 (D) Relative quantification to WS of proteins shown in (C). Results are given as fold-change vs. control  
688 whole saliva normalized at 1. (n=4). (E) Venn diagram showing overlap between the ExoCarta Top 100  
689 proteins list (yellow) and the salivary EV proteins in EV UC (blue), EV Q (red) and EV M (green)  
690 determined by proteomics. (F) Venn diagram showing the number of EV proteins identified in EV UC  
691 (blue), EV Q (red) and EV M (green) not identified in WS determined by proteomics. (G) Western blot  
692 analysis of mucin-16, CD59 and serum amyloid A1 biomarkers in EV UC, EV Q and EV M protein extracts.  
693 (H) Relative quantification to WS of proteins shown in (G). Results are given as fold-change vs. control  
694 whole saliva normalized at 1. (n>5). \*: p < 0.05 ; \*\*: p < 0.01 ; \*\*\*: p < 0.001 ; \*\*\*\*: p < 0.0001.

695 **Fig. 4: MiRNA cargo assesement in human salivary extracellular vesicles.** (A) Quantity of total small  
696 RNA contained in human saliva and extracellular vesicles (UC, Q and M) (n=10). (B) Gel-like image of  
697 small RNA isolated from whole saliva and EVs, generated from the LabChip® GX system. (C) miRNA  
698 levels in EVs (UC, Q and M) relative to whole saliva (WS). Results are given as fold-change of whole  
699 saliva normalized at 1. (n>9). \*: p < 0.05 ; \*\*: p < 0.01 ; \*\*\*: p < 0.001.

700 **Fig. 5: Impact of filtration on saliva-derived extracellular vesicles isolated by ultracentrifugation.** (A)  
701 Representative scheme of the workflow. Briefly, saliva collected from donors was centrifuged  
702 successively at 300 x g and 3000 x g to remove cells and large debris and then small debris respectively.  
703 Then, whole saliva supernatant was filtered on 0.45µm or 0.22µm filter. The filtrate was subsequently  
704 used for EVs isolation as described previously. (B) Concentration of total proteins contained in EVs  
705 isolated by ultracentrifugation from 1mL of human non-filtered saliva (NF) and 0.45µm or 0.22µm  
706 filtered saliva (n=3). (C) Quantity of total small RNA contained in EVs isolated by ultracentrifugation  
707 from 1mL of human non-filtered saliva (NF) and 0.45µm or 0.22µm filtered saliva (n=4). (D) miRNA  
708 levels in EVs isolated by ultracentrifugation from filtered 0.45µm or 0.22µm saliva relative to EVs  
709 isolated from non-filtered saliva (NF). Results are given as fold-change vs. control non-filtered saliva  
710 normalized at 1. (n=4). \*: p < 0.05 ; \*\*: p < 0.01.

711

Fig. 1.

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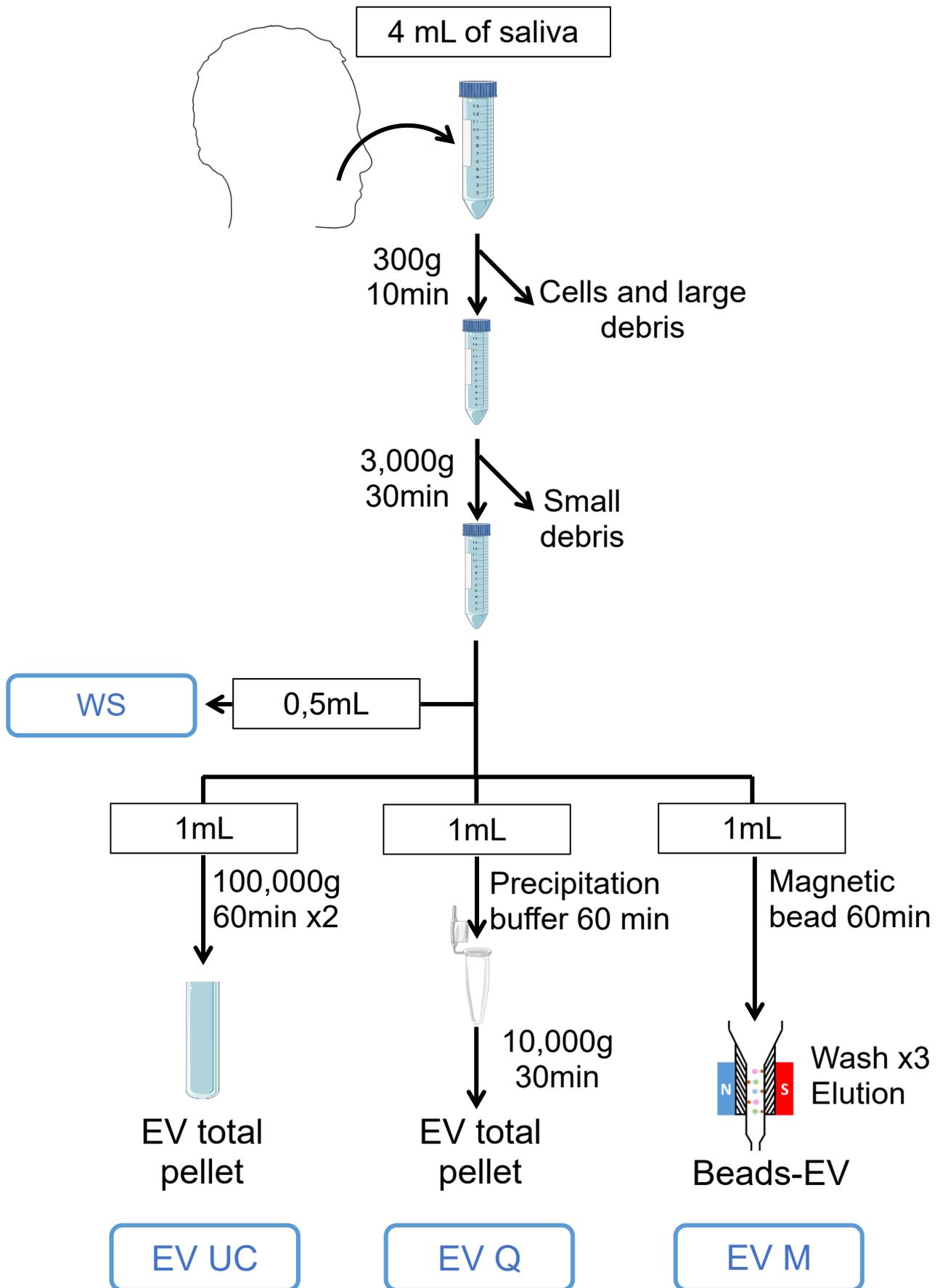
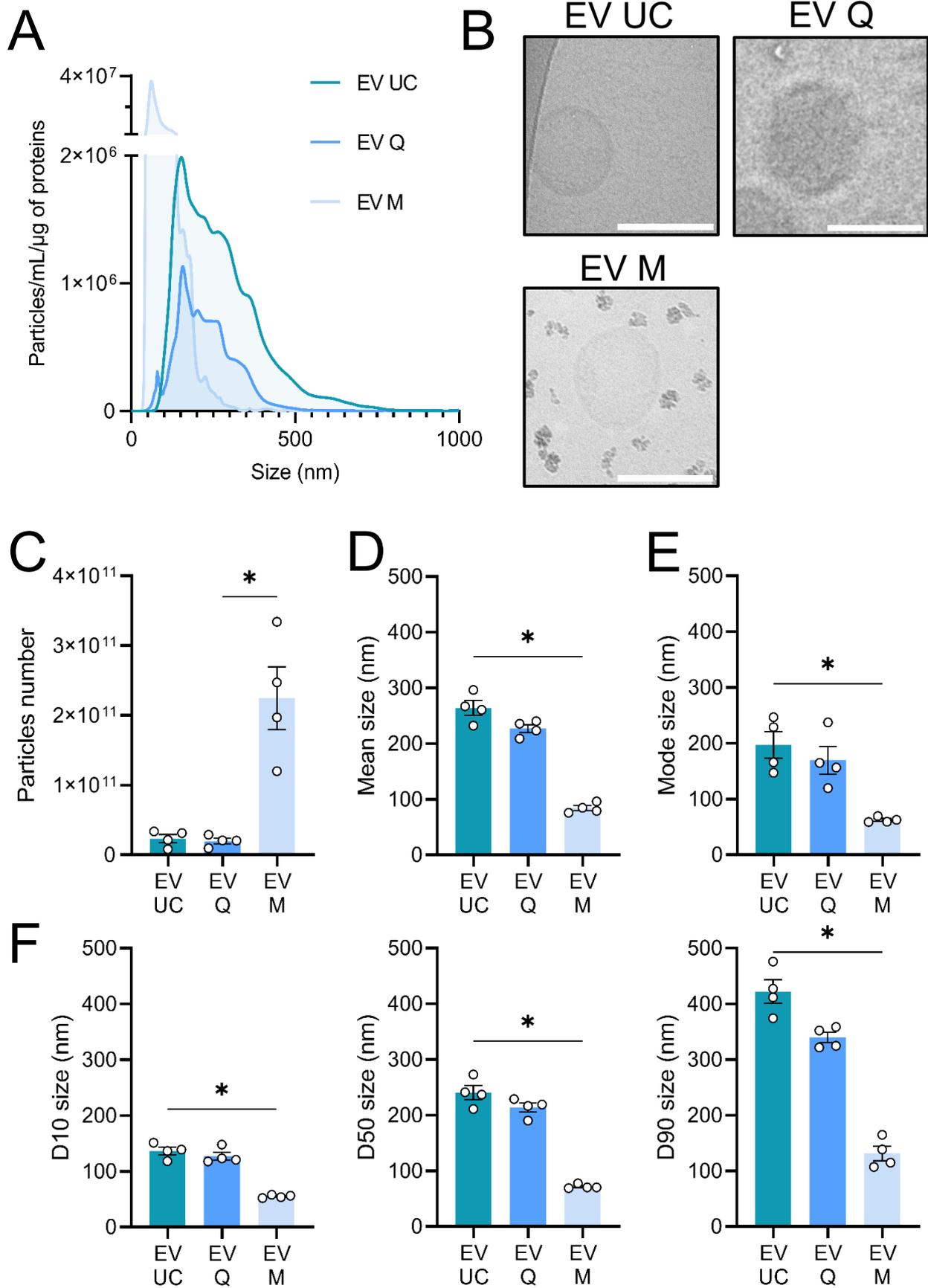


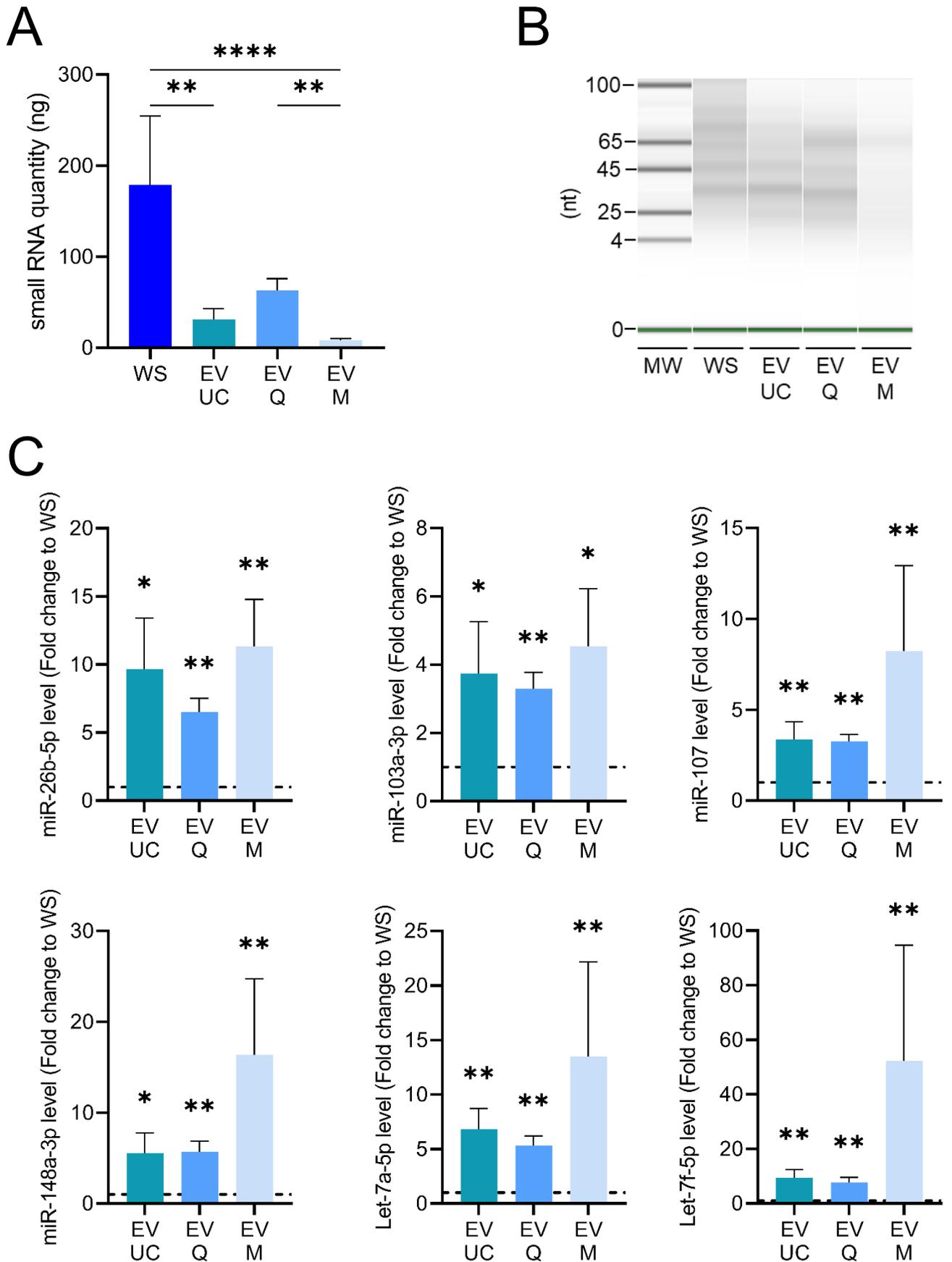
Fig. 2.

713





715 Fig. 4.



716 Fig. 5.

