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The salivary proteome reflects some traits of dietary habits in diabetic and non-diabetic older adults

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Abstract

1 **Purpose** : Objective markers of usual diet are of interest as alternative or validating tools in
2 nutritional epidemiology research. The main purpose of the work was to assess whether saliva
3 protein composition can reflect dietary habits in older adults, and how type 2 diabetes impacted on
4 the saliva-diet correlates.

5 **Methods** : 214 participants were selected from two European cohorts of community-dwelling older
6 adults (3C-Bordeaux and Seniors-ENRICA-2), using a case-control design nested in each cohort. Cases
7 were individuals with type 2 diabetes. Dietary information was obtained using the Mediterranean
8 Diet Adherence Screener (MEDAS). Saliva was successfully obtained from 211 subjects, and its
9 proteome analyzed by liquid chromatography-tandem mass spectrometry.

10 **Results** : The relative abundance of 246 saliva proteins was obtained across all participants. The
11 salivary proteome differed depending on the intake level of some food groups (especially vegetables,
12 fruits, sweet snacks and red meat), in a diabetic status- and cohort-specific manner. Gene Set
13 Enrichment Analysis suggested that some biological processes were consistently affected by diet
14 across cohorts, for example enhanced platelet degranulation in high consumers of sweet snacks.
15 Minimal models were then fitted to predict dietary variables by sociodemographic, clinical and
16 salivary proteome variables. For the food group « sweet snacks », selected salivary proteins
17 contributed to the predictive model and improved its performance in the Seniors-ENRICA-2 cohort
18 and when both cohorts were combined.

19 **Conclusion** : Saliva proteome composition of elderly individuals can reflect some aspects of dietary
20 patterns.

21

22 **Keywords** : salivary biomarkers, proteomics, usual diet, ageing, diabetes, Gene Set Enrichment
23 Analysis

24 **Declarations**

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29 **Conflicts of interests** : nothing to disclose

30 **Ethics approval** : Study protocols of the 3C and Seniors-ENRICA-2 cohorts were approved
31 respectively by the Committee for Protection of Persons (CPP) participating in Biomedical Research of
32 the Kremlin-Bicêtre University Hospital (Paris) and the Ethics Research Committee of the « La Paz »
33 University Hospital (Madrid).

34 **Consent to participate / Consent to publish** : Participants provided written informed consent to
35 participate and for the results of the study to be published.

36 **Availability of data and material** : Data described in the manuscript will be made available upon
37 request addressed to the corresponding author.

38 **Code Availability** : The R scripts will be made available upon request addressed to the corresponding
39 author.

40 **Author's contributions** : EN, CF, FRA, ELG, GP & MM conceived and designed the study. CC, TS & PB
41 performed saliva analysis. RD, FH & DGCL performed statistical analyses. PA, EGGE, CF, FRA & ELG
42 provided epidemiological data and sampled saliva. CC & MM drafted the manuscript. All authors
43 revised the manuscript and approved its final version.

44 Introduction

45 There is vast evidence linking intakes of nutrients, specific foods, food groups or even dietary
46 patterns with health and well-being outcomes. Some studies have focused on the older population,
47 targeting health issues more prevalent in this age group. For example, a link has been established
48 between usual intake of protein and frailty [1] or between coffee consumption and risk of falling [2].
49 Higher adherence to the so-called Mediterranean diet (MeDi) has also been associated with
50 decreased cognitive decline [3] or decreased risk of frailty [4,5]. Concerning another pathology with
51 higher prevalence among the elderly population, higher adherence to MeDi diet has been associated
52 with lower risk of type 2 diabetes mellitus (T2DM) [6,7] and not only through its impact on body
53 weight [8]. Recording one's diet can therefore be a useful tool for the clinician as a starting point to
54 implement dietary guidelines and/or to follow adherence to dietary advice.

55 One challenge in nutritional epidemiology research is to capture real dietary intakes. Assessment
56 usually relies on questionnaires (such as 24-hour recalls or Food Frequency Questionnaire) which
57 may be prone to memory or social desirability bias. Therefore, objective markers of dietary intake
58 are required as alternative or validating tools to increase the reliability and accuracy of diet
59 information [9]. With this objective in mind, markers of dietary intake have been sought for mainly in
60 serum and in urine. For example, metabolome markers of usual consumption of citrus fruit or fish
61 were identified in serum of adult participants [10]. Urinary metabolome markers of usual nut intake
62 were also identified and correlated to cognitive decline in older adults [11]. Recently, plasma
63 metabolome markers of adherence to the Mediterranean diet have been associated with
64 cardiovascular disease risk [12].

65 Saliva is a biological fluid which presents some advantages over blood or urine, particularly regarding
66 its sampling which minimizes pain, privacy or safety issues. Saliva sampling also presents some
67 limitations, for example in case of minimal saliva flow. Young children and the oldest or frail elderly
68 subjects are thus two groups for which it may be difficult to obtain saliva samples [13]. However,
69 saliva was successfully sampled and its protein or peptide composition analyzed on 3- and 6-month-

70 old infants [14], on premature babies [15] or on old persons with a mean age of 82 years [16]. Saliva
71 composition was successfully associated with usual intake of carbohydrates in adults [17], with diet
72 transition in infants [14,18] or with dietary patterns in children with or without eating difficulties
73 [19]. In addition, saliva (together with blood) proved more resilient than urine to recent dietary
74 intake when focusing on metabolome composition [20]. For all these reasons, the primary objective
75 of this work was to evaluate whether saliva is a suitable source of objective protein markers of usual
76 dietary intake in older adults. We evaluated this with participants from two different cohorts, in
77 France and Spain, which provided information on the cross-cultural and/or geographical sensitivity of
78 the results. Finally, participants were part of a wider project on salivary biomarkers of Mediterranean
79 diet and type 2 diabetes mellitus [21]. The design of participants' selection therefore also enabled to
80 address a secondary objective, namely to assess the impact of this pathology on the association
81 between saliva composition and dietary habits.

82 **Methods**

83 Population-based cohorts

84 Participants originated from two population-based cohorts on ageing, the Bordeaux sample of the
85 Three-City Study (3C) in France [22] and the Seniors-ENRICA-2 cohort in Spain [23]. Study protocols of
86 the 3C and Seniors-ENRICA-2 cohorts were approved respectively by the Committee for Protection of
87 Persons (CPP) participating in Biomedical Research of the Kremlin-Bicêtre University Hospital (Paris)
88 and the Ethics Research Committee of the « La Paz » University Hospital (Madrid).

89 Participants

90 The 214 Individuals included in the study were selected using a case-control design nested in each
91 cohort. Cases were individuals affected by T2DM based on self-reported physician's diagnosis and/or
92 being on antidiabetic treatment (oral medication or insulin) at the time of data collection. Controls
93 were selected concurrently and were free of diabetes at the time of data collection. For the purpose
94 of the present study, we used the following information from each participant: cohort, sex, age

95 (years), body mass index (BMI; kg/m²), type 2 diabetic status, smoker status (never, former, current),
96 saliva flow (g/min) and food consumption.

97 Dietary surveys and assessment of adherence to the Mediterranean diet

98 Food consumption data were collected using a FFQ in the 3C cohort [24] and a validated electronic
99 diet history in the Seniors-ENRICA-2 cohort [25]. Adherence to the Mediterranean dietary pattern
100 was assessed by calculating a MEDAS score [26] modified by omitting the question on *sofrito*, since
101 this cooking technique is specific of the Spanish population. The MEDAS score could therefore range
102 from 0 to 13, with higher values indicating higher adherence to the Mediterranean diet. Differences
103 between cohorts or diabetic status was tested by a Wilcoxon test. In addition, to study the link
104 between saliva proteome and diet, we restricted the analysis to the 11 MEDAS items describing the
105 consumption frequency of food groups, namely olive oil, vegetables, fruits, red meat,
106 butter/margarine/cream, sweet drinks, wine, legumes, fish/shellfish, sweet snacks (confectionary,
107 biscuits and commercial pastries) and nuts. For each food group, the participants were classified as
108 high or low consumers based on the cut-off points defined for MEDAS score calculation (Online
109 Resource 1).

110 Saliva sampling

111 Sampling of unstimulated saliva was conducted at the participants' home. Sampling was proposed
112 early in the morning after overnight fasting. Drinking water was permitted up to 5 minutes before
113 saliva collection. Participants were instructed to sit comfortably and to tilt their head slightly
114 downwards. At their own rythmn, they spat the saliva pooling on the floor of the mouth into 40mL
115 polypropylene tubes. Sampling was performed for 10 minutes. In case a participant wished to stop
116 before the end of the 10 minutes, the time was recorded in order to be able to calculate the saliva
117 flow (expressed in g/min). Saliva samples were immediately placed on ice, transported to the
118 laboratory and placed at -80°C as soon as possible (never after 4 hours on ice). At the end of the
119 collection wave, samples were shipped to the analytical facilities in dry-ice.

120 Saliva proteome analyses

121 *Sample preparation*

122 Saliva was thawed at 4°C and vortexed. One mL of saliva (or the total volume of saliva when it was
123 lower than 1 mL) was centrifuged at 14000 g for 20 minutes. The supernatant was used for proteome
124 analyses. Protein concentration was measured by an infrared spectroscopy-based method using a
125 Direct Detect® spectrometer (Merck). Samples were diluted in water in order to adjust all samples to
126 the same protein concentration, then mixed with 1 volume of Laemmli denaturing buffer and heated
127 at 90°C for 5 minutes. Sample volumes corresponding to 3.5 µg of protein were loaded onto SDS-
128 PAGE gels containing 12% and 5% acrylamide in the resolving and stacking gels, respectively.
129 Electrophoresis was performed using a Mini-Protean II unit (BioRad, Marnes-La-Coquette, France) at
130 100 V until the dye front entered the resolving gel. Gels were stained for one hour in R-250
131 Coomassie. Bands were manually excised, reduced in 10 mM dithiotreitol in 50 mM ammonium
132 bicarbonate, and alkylated in 55 mM iodoacetamide in 50 mM ammonium bicarbonate. Destaining of
133 the excised bands was obtained by successive rinses in 25 mM ammonium bicarbonate / acetonitrile
134 (1 :1 v/v). Gel pieces were then dried by incubation in 100% acetonitrile for 10 min followed by
135 vacuum-drying in a SpeedVac. Finally, gel pieces were incubated overnight at 37°C with 30 µL of a
136 trypsin solution (V5111, Promega) at 10 ng/µL in 25 mM ammonium bicarbonate. Peptide extraction
137 was performed by addition of 40 µL of acetonitrile 100%, 0.5% formic acid and sonication for 15 min.
138 The trypsin digests were vacuum-dried in a SpeedVac and stored at 20 °C in a solution of 0.05%
139 trifluoroacetic acid before Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) analysis

140 *Mass Spectrometry analyses*

141 Five µL of the protein digests were injected into a nanoHPLC (Ultimate 3000, ThermoFisher
142 Scientific). The peptide mixture was first concentrated and desalted on a microcolumn (Acclaim, 300
143 µm, 5mm) equilibrated with trifluoroacetic acid (TFA) 0.05% in water. After 6 min, the microcolumn
144 was switched on-line to an analytical C18 nanocolumn (Acclaim, 75 µm, 25 cm, Pepmap) equilibrated
145 with 94.9 % H₂O, 5% dimethyl sulfoxide (DMSO), 0.1% formic acid (FA) and peptides were separated

146 at 35°C according to their hydrophobicity with a 4 to 25% linear gradient of acetonitrile (94.9 % ACN,
147 5 % DMSO, 0.1% FA) at a flow rate of 300 nL/min for 50 min. Peptides were electro-eluted with an
148 ESI nanosource (1.6 kV) in the mass spectrometer (Orbitrap Velos, ThermoFisher scientific). The
149 Orbitrap was used in top 15 data dependent mode, with gas phase fractionation (GPF1 400-480 m/z;
150 GPF2 480-560 m/z; GPF3 560-660 m/z; GPF4 660-850 m/z, GPF5 850-1401 m/z).

151 Samples were analyzed in 6 different series (different days). To monitor and normalize LC-MS
152 performance over time, a Quality Control (QC) was injected every 10 samples. This QC was prepared
153 by diluting 20 μL of a commercial mixture of 15 synthetic peptides (Thermo Scientific™ Pierce™
154 Peptide Retention Time Calibration Mixture) into 180 μL of a trypsin digest of a pool of 20 salivas. Five
155 μL of the QC was injected and analyzed similarly to samples, except that gas phase fractionation was
156 not performed.

157 Raw files were imported into ProgenesisQI Proteomics (Nonlinear dynamics) and the label-free
158 quantification workflow was followed (see www.nonlinear.com). This includes peak alignment,
159 establishment of a single ion map, normalization of intensities using data from 2 to 5x charged ions
160 and XIC type quantification of all ions detected. Peptide identification were performed using Mascot
161 interrogating the database homosapiens UniProt 201804 (71,600 sequences). Peptide mass tolerance
162 was set to 10 ppm and fragment mass tolerance was set to 0.5 Da. Two miscleavages were
163 authorized, and methionine oxidation, carbamidomethylation of cysteine and deamidated
164 asparagine or acid aspartic were set as variable modifications. Protein identification was then
165 validated when at least two unique peptides from one protein showed significant identification
166 Mascot scores with False Discovery Rate (FDR < 1%). For protein quantification, the sum of all unique
167 normalized peptide ion abundances for a specific protein was calculated for each sample. The final
168 dataset consisted in 246 proteins quantified in 211 samples.

169 Proteome data handling

170 Protein abundance as obtained above was corrected for any series effect using QC data. For each
171 synthetic peptide, the average abundance was calculated per series and for all runs combined. This

172 enabled calculating a correcting coefficient applicable to each series for normalization. The mean
173 correcting coefficient for the 15 synthetic peptides was calculated per series, and it was applied to
174 the protein abundance within that series. Missing values (0.3% of overall data) were handled
175 following two distinct imputation methods as described by Wei et al. 2018 [27]. Eighty-two percent
176 of the missing values corresponded to proteins which always showed low abundance values. These
177 missing values were regarded as Missing Not At Random and thus handled using the quantile
178 regression imputation of left-censored method. Remaining missing values were regarded as Missing
179 At Random thus handled using the Random Forest imputation method.

180 Statistics

181 First, for each food group, we tested differences in the salivary proteome between high and low
182 consumers Student t-tests corrected by the Benjamini-Hocberg method to control the False
183 Discovery rate (FDR set at 5%). This was performed for the entire sample or when participants were
184 stratified by diabetic status or by cohort.

185 Second, the Gene Set Enrichment Analysis (GSEA) method [28] was used to highlight biological
186 processes enriched depending on the level of consumption of a food group. Given the case-control
187 design of the study, we performed the analyses separately for diabetic and non-diabetic subjects. For
188 each of the 246 proteins of the dataset, ontology terms and related annotations were retrieved from
189 quickGO and the GO database. GO terms corresponding to at least two proteins (1850 in total) were
190 selected. For each condition (one condition = one diabetic status, one food group), proteins were
191 ranked in descending order of their p-values after attributing a negative sign to p-values when
192 proteins were under-expressed in “high consumers”. This way, a GO term is regarded as of interest if
193 its protein members are mostly in the top (over-expressed) or bottom (under-expressed) region of
194 the list. GO terms were tested by unweighted GSEA estimation algorithm with at least a million
195 permutations. With multifactorial dependencies occurring in the GO terms classification, (correlated
196 and/or co-occurring annotations, co-occurring proteins ...), controlling false positive occurrence is
197 challenging and usual methods may be over-restrictive. Post-hoc correction was therefore conducted

198 as follows: the community structure of the GO network (terms as nodes, relationships as edges) was
199 determined using the Girvan-Newman algorithm [29]. This revealed 223 separated communities with
200 a modularity of 0.85. GO terms within a community were considered as strongly dependent from
201 each other whereas communities were considered as independent from each other. P-values of the
202 223 communities were computed as the mean of p-values of its GO terms members, and the
203 Benjamini-Hochberg correction was performed (calculation of a q-value). This generated a correcting
204 factor for each community, which was then applied to p-values of all GO terms within this
205 community to calculate individual q-values. GO terms were considered significant for q-values <0.01
206 (FDR 1%).

207 Finally, we fitted minimal models predicting dietary variables by sociodemographic, clinical and
208 salivary proteome variables. For that purpose, the workflow was as follows for each food group. First
209 we reduced the number of protein variables by three successive selection steps : 1- a Kernel Partial
210 Least Square Regression model was computed and proteins with high Variable Importance in
211 Projection (VIP) values were selected (cutoff=1). 2- For these proteins, the difference in abundance
212 between high and low consumers of the food group was tested by a Student t-test corrected by the
213 Benjamini-Hochberg method to control the False Discovery rate (FDR). Variables with a low FDR were
214 selected (cutoff=0.05). 3- Correlation among these proteins were calculated. Candidates sharing at
215 least one high correlation (cutoff=0.7) were ordered by Ascendant Hierarchical Clustering, with
216 adequate number of clusters determined by bootstrapping. The final set of proteins was made of all
217 uncorrelated variables plus one variable per cluster (the one with the highest contribution). Second,
218 we fitted the minimal model by logistic model learning using selected proteins and sociodemographic
219 and clinical confounding factors (diabetic status, cohort, sex, age, BMI, smoker status and saliva
220 flow). Subjects were split into a learning set (75%) and a validation set (25%). From the learning set, a
221 logistic model was adjusted. Two stepwise procedures (Akaike Information Criterion, Bayesian
222 Information Criterion) were performed separately : the AUC (Area under the Curve) of the ROC
223 (receiver operating characteristic) curves were computed from the validation set and the model with

224 the highest AUC was selected. This process was repeated through 1000 simulations of
225 learning/validation sets, and the final model retained the variables selected in at least half of the
226 simulations. For the food groups where proteome variables were retained, we evaluated the
227 performance of the models by calculating the AUC of the ROC curve. More specifically, three models
228 were tested : Model 1 was the optimal model obtained as described above, Model 2 was Model 1
229 into which we forced some basic descriptive variables (cohort, age, sex, diabetic status), and Model 3
230 was Model 1 from which protein variables were removed. The comparison of Model 1 and Model 3
231 allows documenting how much value the saliva proteome variables add to the model.
232 Data management and statistical analyses were performed using the Rgui open-source software
233 (<https://cran.r-project.org>).

234 **Results**

235 Participants' characteristics

236 Among 570 participants from wave 8 (2017-2018, 18y after baseline) of the 3C-Bordeaux cohort, 65
237 were diabetics. Among them, 37 participants agreed to participate and a random sample of 71
238 participants were selected as controls. Among 3273 participants from baseline in the Seniors-
239 ENRICA-2 cohort, 669 were diabetics. A random **sample** of 53 diabetics and 53 matched controls
240 were selected. Out of those 214 participants, 3 were excluded because of insufficient saliva
241 production (Online Resource 2). The 211 remaining participant's characteristics are presented in
242 Table 1. The French sample was older on average (87.3 vs 71.0 y) and comprised more women (61.3
243 vs 36.2%) than the Spanish one. As expected, the diabetic participants exhibited higher BMI on
244 average than controls in both cohorts.

245 Saliva amounts collected and salivary flows

246 The amounts of saliva collected varied from 0.09 to 8.68 g. As reported in Table 1, the average at-rest
247 saliva flow varied from 0.18 to 0.26 g/min depending on the cohort and diabetic status. The
248 difference between the two cohorts tested by a Wilcoxon test was significant ($p < 0.001$), with higher

249 flow among Seniors-ENRICA-2 participants. The difference between diabetic and non-diabetic
250 subjects was not significant either when combining the two cohorts or within each cohort.

251 MEDAS scores and dietary intakes

252 The mean MEDAS scores and the proportions of high vs low consumers of 11 food groups among all
253 participants or stratified by diabetic status or cohort are presented in Table 2. Adherence to the
254 Mediterranean dietary pattern was slightly (but not significantly) lower in diabetic participants
255 compared to controls, while it was significantly ($p < 0.05$) lower in 3C-Bordeaux participants compared
256 to Seniors-ENRICA-2 participants. The dietary patterns were overall comparable between diabetic
257 and non-diabetic individuals : the only two food groups which were clearly different according to the
258 diabetic status were « nuts » and « sweets snacks » (confectionary, biscuits and commercial
259 pastries), with a higher proportion of low consumers among diabetic subjects. In contrast, there
260 were more differences between the two cohorts, with five food groups clearly different between
261 Seniors-ENRICA-2 and 3C-Bordeaux. In the Spanish cohort there were more high consumers of olive
262 oil, while in the French cohort there were more high consumers of vegetables, nuts, wine and butter.

263 Proteins differentially expressed between high and low consumers of 11 food groups

264 Table 3 provides the number of proteins significantly different (FDR 5%) between high and low
265 consumer of the 11 food groups recorded. When considering all participants, there were
266 differentially expressed proteins for three food groups : vegetables, butter and sweet snacks. The list
267 of proteins significantly different, details of mass spectrometry identifications and abundance in high
268 vs low consumers are available in Online resource 3. The intake level of vegetables, red meat and
269 butter altered more the saliva of non-diabetic subjects (20, 58 and 5 differential proteins,
270 respectively) than that of diabetic subjects (4, 0 and 0 differential proteins, respectively). In contrast,
271 the intake level of sweet snacks had a major impact on saliva proteome (65 differential proteins) but
272 only for diabetic subjects. More generally, the link between saliva proteome and diet was more
273 pronounced among non-diabetic controls, with 85 proteins in total linked to 4 food groups
274 (vegetables, legumes, red meat, butter), than among diabetic participants with 69 proteins linked to

275 2 food groups (vegetables, sweet snacks). The link between saliva proteome and diet was also more
276 expressed among Seniors-ENRICA-2 participants with 77 proteins linked to two food groups (fruits,
277 sweet snacks), than among 3C-Bordeaux participants with 2 proteins linked to the intake level of
278 olive oil.

279 Gene Set Enrichment Analysis (GSEA)

280 Based on protein expression levels in the entire proteomic dataset, GSEA identified the biological
281 processes that were significantly enriched or depleted depending on the participants' intake level of
282 a given food group. This was performed for diabetic and control participants separately and for the
283 food group « sweet snacks » because of its major and robust impact on the salivary proteome (see
284 Table 3). Results for all participants are presented in Figure 1 and results of the analyses performed
285 for each cohort separately are available in Online resource 4.

286 For the two cohorts combined, some biological processes were in common and similarly affected for
287 all participants. Thus, *regulation of cellular macromolecule biosynthetic process* was repressed while
288 *platelet degranulation, leukocyte migration involved in inflammatory response and regulation of*
289 *immune system* were enhanced in high consumers of sweet snacks, regardless of their diabetic
290 status.

291 In addition, the impact of sweet snacks intake level was particularly evident among diabetic
292 participants (Fig. 1). In those participants, three GO categories related to apoptosis were repressed in
293 high consumers of sweet snacks, as well as the category regulation of peptidase activity. In contrast,
294 all the other GO terms were enriched in diabetic high consumers of sweet snacks. These terms
295 covered a very large panel of biological functions such as hemostasis, protein and peptide
296 expression, modification or secretion, immunity, cell-cell communication or metabolism. Of special
297 interest is a group of GO terms related to carbohydrate metabolism, (black box in Fig. 1). In controls,
298 high consumption of sweet snacks also modified specifically some biological processes but to a lesser
299 extent.

300 Analyses performed for each cohort separately confirmed that the impact of sweet snacks intake
301 level was higher for diabetic participants than for controls in both cohorts. A vast majority of the
302 biological processes significant for the two cohorts combined were also identified and significant in
303 at least one of the two cohorts. Results were mostly consistent between the two cohorts combined
304 and one of the cohorts (lines highlighted in blue in Online resource 4, e.g. *platelet degranulation*).
305 Five biological processes (highlighted in yellow in Online resource 4, e.g. *regulation of*
306 *phosphorylation*) were significant for diabetics and controls in the Seniors-ENRICA-2 cohort, while
307 they were significant only for diabetics in the 3C-Bordeaux cohort and in the joint analysis. Finally, 14
308 biological processes were consistent across cohorts for diabetics subjects: *regulation of apoptotic*
309 *process, platelet degranulation, regulation of immune response, regulation of gene expression,*
310 *regulation of transport, regulation of cellular component organization, regulation of protein/peptide*
311 *secretion, regulation of cell morphogenesis involved in differentiation, interleukin-12-mediated*
312 *signaling pathway, regulation of substrate adhesion-dependent cell spreading, Arp2/3 complex-*
313 *mediated actin nucleation, actin polymerization-dependent cell motility and microtubule-based*
314 *movement*. The latter four processes, related to cell spreading and motility, are likely linked to the
315 oral epithelium repair potential.

316 Salivary markers of specific food groups' intake levels

317 Minimal models were sought for all food groups. Tables 4 and 5 present an overview of the variables
318 retained in such models for all participants combined or cohort by cohort, respectively.

319 Considering both cohorts combined, apart from fruit for which no minimal model was retained, the
320 descriptive characteristics of the subjects could predict the level of consumption (defined as meeting
321 the MeDi requirements or not) for all food groups. The descriptor « cohort » was most frequently
322 retained (4 occurrences for vegetables, olive oil, wine and butter) followed by sex (3 occurrences for
323 wine, red meat and sweet snacks), diabetic status (nuts and sweet snacks) and smoker status
324 (legumes and sweet drinks). Of special interest are the three food groups for which abundance of
325 salivary proteins are predictors of intake : vegetables (5 proteins : P22079 lactoperoxidase, O00391

326 sulfhydryl oxidase 1, P10909 clusterin, P37802 transgelin-2 and Q8TAX7 mucin-7), butter (4 proteins :
327 Q96DR5 BPI fold-containing family A member 2, P22079 lactoperoxidase, P07476 involucrin and
328 P23280 carbonic anhydrase 6) and sweet snacks (2 proteins : E7EQB2 lactotransferrin, P02749 beta-
329 2-glycoprotein 1). For the three food groups vegetables, butter and sweet snacks, the distribution of
330 AUCs for 10000 simulations and the corresponding means and 95% confidence intervals are
331 presented in Fig. 2. These show that compared to the minimal models (top panels), forcing into the
332 model additional subjects' basic descriptors (middle panels) did not improve the model performance.
333 Moreover, removing the protein descriptors from the minimal models (bottom panel) clearly
334 reduced the model performance with an AUC mean shift of approximately -0.05 for vegetables and
335 butter and -0.1 for sweet snacks. In other words, we confirmed that the salivary proteome added
336 value to the prediction models.

337 Results obtained cohort by cohort (Table 5) did not directly confirm the protein markers identified
338 when the two cohorts were combined. In agreement with the very low number of proteins
339 significantly different between high and low consumers of the different food groups among 3C-
340 Bordeaux participants (Table 3), no minimal models retained salivary proteins for French
341 participants. Among Seniors-ENRICA-2 participants, salivary proteins were predictors of intake of
342 fruits, red meat and sweet snacks.

343 Discussion

344 In this study, the salivary proteome differed depending on the intake level of some food groups
345 (especially vegetables, fruits, sweet snacks and red meat), in a diabetic status- and cohort-specific
346 manner. In addition, some saliva proteins were predictive of the intake level of sweet snacks after
347 adjusting for several sociodemographic and clinical confounding factors.

348 The first challenge of the study was the collection of saliva. Only 3 participants out of 214 were
349 excluded due to insufficient saliva production, and only one of the 211 donors requested to stop
350 before the end of the 10 min collection time. The average saliva flows were below the 0.3 g-0.4/min
351 generally reported [30,31], in accordance with the well-known reducing impact of ageing on both at-

352 rest and stimulated saliva flow [32]. Age is likely the main factor explaining the statistical difference
353 between the cohorts, with lower average flow in French participants who are on average 16 years
354 older than Spanish participants. The sex-ratio and the cases-controls proportions also differ between
355 the two cohorts. However, several studies reported that gender had no significant impact on saliva
356 flows of either healthy [33] or diabetic elderly subjects [34]. Furthermore, the diabetic status had no
357 significant impact on saliva flow in our study, in each cohort or combining the two. To conclude on
358 saliva collection, our study highlighted that it was feasible, well-tolerated and although the volumes
359 were sometimes limited especially in the oldest elderlies, they remained compatible with the needs
360 of proteome analytical methods.

361 The assessment of dietary intakes is also a challenge for any population. Diet history and Food
362 Frequency Questionnaire are often used to capture usual nutrient intake or dietary habits with a high
363 level of details. Here, we used two different tools which are standard and validated instruments for
364 collecting food consumption data. In a previous study where these same cohorts and respective diet
365 recording tools were used, consistent results for the association between fruit and vegetable intake
366 and functional outcomes in older adults were obtained [35]. Based on the data obtained, we used
367 the MEDAS score to represent adherence to the MeDi diet [26]. We confirmed previous
368 observational studies reporting no difference in adherence to MeDi between persons with diagnosed
369 diabetes and controls [36]. The MEDAS questionnaire also enabled to classify participants into high
370 and low consumers for 11 food groups. There was a limited impact of the diabetic status on the
371 intake levels, at the exception of nuts and sweet snacks. This latter finding is in accordance with
372 previous results observed within the Seniors-ENRICA cohort, showing that the diet of diabetics differs
373 mainly by a greater avoidance of sweet products while other food groups are little affected [36].
374 Other studies are also in general agreement with this idea. For example, a cross-cultural study
375 investigated the diet of diabetic and non-diabetic elderly men in Finland, the Netherlands and Italy
376 and highlighted that the only food group that was significant different in all three countries was the
377 consumption of added sugar [37]. In another study [38], the food group *sweets* differed largely
378 between individuals with or without diabetes both in a pan-European cohort (the EPIC study) and a

379 USA-based cohort (the MEC study). The two latter articles also evidenced that differences between
380 countries exceeded those between diabetics and non-diabetics, in line with our results on the
381 MEDAS score or the individual food groups. Some dietary differences that we observed between
382 cohorts corresponded to expected cultural specificities (more high consumers of wine in the
383 Bordeaux area in France, more high consumers of olive oil in Spain), while others were more
384 surprising. In particular, the proportion of high consumers of vegetables was extremely high in the 3-
385 C Bordeaux cohort, well above other estimations for France [39]. In the 3-C Bordeaux cohort, fruits
386 and vegetables consumption has already been associated with a lower risk of death [40] which may
387 explain that high consumers of vegetables are over-represented in our sample of very old
388 participants. In contrast, the proportion of high consumers of vegetables in the Seniors-ENRICA-2
389 cohort was extremely low (3.8%) compared to the 12.3% or 17.6% in the general Spanish population
390 [41] or in diabetic Spanish patients [36], respectively.

391 Keeping these issues in mind, and with the objective of describing broadly the impact of dietary
392 intake on salivary proteome, we used the GSEA approach. Many salivary proteins are multifunctional
393 [42] and thus GSEA provided an overwhelming wealth of information, but a first obvious result was
394 that the biological processes enriched according to the intake level were mostly different between
395 diabetic subjects and controls. The salivary proteome is therefore shaped differently by diet
396 depending on the physiopathological status of the subject.

397 The second finding is that intake of sweet snacks had a major effect on biological processes in
398 diabetics, **regardless of the cohort considered**. This is not surprising since dietary sugars have a
399 profound physiological effect in diabetic individuals, who by definition have difficulty in controlling
400 glycemia. For example 8 enzymes of the *glucose catabolic process to pyruvate* (out of the 10 of the
401 core glycolysis pathway) were over-represented in high consumers of sweet snacks among diabetic
402 participants. This is consistent with the long-known increase in glycolytic enzymes activity or
403 expression induced by glucose in various tissues or cells such as pancreatic cells [43] or the jejunum
404 [44]. Inter-individual variability in salivary glycolytic enzymes has been hypothesized to be at least
405 partly related to dietary habits of the healthy adult saliva donors [45]. Finally, several glycolytic

406 enzymes were found in saliva of diabetic subjects and these were overexpressed in subjects with the
407 most severe forms of retinopathy [46].

408 More unexpectedly, **when analyzing the two cohorts jointly**, the *endothelial cell apoptotic process*
409 was repressed in diabetic high consumers of sweets, while high glucose is known to trigger apoptosis
410 in endothelial cells [47]. However, the three proteins attached to this GO term in our dataset are the
411 fibrinogen α , β and γ -chains. They are linked to negative regulation of the apoptotic process in GO,
412 but their primary function is rather blood clotting: their over-expression could thus reflect the more
413 frequent gum bleeding in diabetic individuals with inadequate intake of sweet snacks. This is then in
414 accordance with a consensus report indicating that poor glycaemic control in diabetes is associated
415 with poorer periodontal status and outcomes [48]. This example illustrates that GSEA should be
416 regarded as indicative of biological processes to be investigated further, and findings should be
417 examined considering representativity of the proteins attached to each GO category. In that respect,
418 the example of *platelet degranulation* deserves special attention since it is associated with 30
419 proteins in our dataset (out of 129 in the human genome). Increased platelet degranulation is
420 observed in T2DM patients compared to healthy subjects [49,50] and a mechanistic study concluded
421 that high glucose *per se* increased platelet reactivity in blood of both diabetics and controls [51]. In
422 our study, **combining both cohorts**, the saliva proteome composition also suggested that high intake
423 of sweet snacks was linked with enhanced platelet reactivity both in diabetics or controls. **This was**
424 **confirmed in the Spanish cohort and among diabetic subjects in the French cohort.**

425 We also looked for markers of diet and adjusted the models for confounding factors (diabetic status,
426 cohort, age, sex etc.). **Combining both cohorts**, salivary proteins were retained in the model for three
427 food groups. **For « vegetables » and « butter », one should note that the proportions of high**
428 **consumers are much higher in the 3C-Bordeaux cohort. Results should therefore be taken cautiously**
429 **because there is a risk that results are partly confounded with a cohort effect. In addition, the**
430 **analyses performed for each cohort separately highlighted the differences between the two cohorts**
431 **and illustrated the difficulty of identifying universal markers.** Nevertheless, whatever the subjects'
432 characteristics in terms of cohort, age, sex, diabetic status, smoker status, BMI or saliva flow, higher

433 abundance of lactotransferrin (E7EQB2) and beta-2-glycoprotein 1 (P02749) in saliva was associated
434 with a higher chance of being a high consumer of sweet snacks. Interestingly, we had previously
435 observed in healthy children that another protein of the transferrin family (serotransferrin) was
436 positively associated with a number of food groups including biscuits & sweets [19]. There is also
437 some biological coherence among some of the identified markers. For example, the two positive
438 markers of vegetables intake (lactoperoxidase, sulhydryl oxidase 1) catalyze reactions involving H₂O₂
439 and contribute to cellular redox homeostasis. However, the main general lesson from those results is
440 that the abundance of some salivary proteins was linked to the consumption level of some food
441 groups in elderly subjects after adjusting for several sociodemographic and clinical confounding
442 factors. In addition, since including salivary proteins data improved the prediction models, this is the
443 proof-of-concept that saliva might be a source of objective markers of usual diet.

444 To conclude, it should be reminded that the studied population originated from two very contrasted
445 cohorts, especially in terms of age and dietary habits. This represented a challenge, particularly for
446 statistical methods which can not fully account for confounding factors. Dietary intake was also here
447 simply evaluated as compliance or not to the requirements of MeDi diet. However, the salivary
448 proteome data suggested biological functions affected by the dietary intake level of **sweet** snacks,
449 **across cohorts and diabetic status (e.g. platelet degranulation) or across cohorts and specific to one**
450 **diabetic status (e.g. functions linked to cell spreading and cell motility).** Proteome data also added
451 value to minimal models predicting the intake level of some food groups. It is now necessary to
452 confirm these results on a validation population, but also to assess more finely the link between the
453 potential markers' expression and the actual quantitative intakes.

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Table 1. Main characteristics of the 211 participants whose saliva was analyzed in the study

	Seniors-ENRICA-2		3C-Bordeaux	
	diabetics (n=52)	controls (n=53)	diabetics (n=37)	controls (n=69)
Age (years, mean \pm SD)	70.6 \pm 4.3	71.4 \pm 3.7	87.4 \pm 4.1	87.3 \pm 2.9
Sex (% men)	69	58	48	33
BMI (mean \pm SD)	30.0 \pm 5.0	26.6 \pm 3.9	29.0 \pm 3.9	24.4 \pm 4.0
Smoker (never/former/current) ^a	9/37/6	15/33/5	17/7/5	33/20/5
Saliva flow (g/min, mean \pm SD)	0.26 \pm 0.17	0.23 \pm 0.16	0.18 \pm 0.13	0.20 \pm 0.18

^a 19 missing values (3C-Bordeaux)

Table 2. Modified MEDAS score (0 to 13 points) and percentage of low and high consumers of 11 food groups among the 211 older adults, altogether or when individuals are stratified by diabetic status or cohort. The cut-off points to define low and high intake are provided in Online Resource 1. Cells shaded in gray highlight the food groups for which there is a large (>10%) difference between the two sub-samples.

		All subjects (n=211)	Diabetic status		Cohort	
			Diabetics (n= 89)	Controls (n=122)	Seniors-ENRICA-2 (n=105)	3C-Bordeaux (n=106)
13-point MEDAS score		6.24 ± 1.57	6.13 ± 1.52	6.32 ± 1.61	6.47 ± 1.39	6.02 ± 1.70
Vegetables						
	Low	56.4	60.7	53.3	96.2	17.0
	High	43.6	39.3	46.7	3.8	83.0
Fruits						
	Low	54.0	58.4	50.8	53.3	54.7
	High	46.0	41.6	49.2	46.7	45.3
Olive oil						
	Low	88.2	85.4	90.2	80.0	96.2
	High	11.8	14.6	9.8	20.0	3.8
Legumes						
	Low	91.0	88.8	92.6	88.6	93.4
	High	9.0	11.2	7.4	11.4	6.6
Nuts						
	Low	82.9	91.0	77.0	89.5	76.4
	High	17.1	9.0	23.0	10.5	23.6
Wine						
	Low	65.4	69.7	62.3	82.9	48.1
	High	34.6	30.3	37.7	17.1	51.9
Fish						
	Low	68.2	70.8	66.4	64.8	71.7
	High	31.8	29.2	33.6	35.2	28.3
Red meat						
	Low	92.4	91.0	93.4	90.5	94.3
	High	7.6	9.0	6.6	9.5	5.7
Butter						
	Low	61.6	66.3	58.2	89.5	34.0
	High	38.4	33.7	41.8	10.5	66.0
Sweet snacks						
	Low	63.5	70.8	58.2	63.8	63.2
	High	36.5	29.2	41.8	36.2	36.8
Sweet drinks						
	Low	91.5	86.5	95.1	88.6	94.3
	High	8.5	13.5	4.9	11.4	5.7

Table 3. Number of proteins significantly differently expressed (FDR<5%) in saliva of low vs high consumers of 11 food groups, altogether or when individuals are stratified by diabetic status or by cohort. The cut-off points to define low and high intake are provided in Online Resource 1.

	Vegetables	Fruits	Olive oil	Legumes	Nuts	Wine	Fish	Red meat	Butter	Sweet snacks	Sweet drinks
All participants (n=211)	22	0	0	0	0	0	0	0	3	8	0
Diabetics (n=89)	4	0	0	0	0	0	0	0	0	65	0
Controls (n=122)	20	0	0	2	0	0	0	58	5	0	0
Seniors-ENRICA-2 (n=105)	0	63	0	0	0	0	0	0	0	14	0
3C Bordeaux (n=106)	0	0	2	0	0	0	0	0	0	0	0

Table 4. Variables retained in minimal models of prediction of intake of 11 food groups (two cohorts combined). Quantitative variables are separated into positive (chance of being a high consumer increases with value) or negative (chance of being a high consumer decreases with value) predictors. For categorial variables, the category associated with high intake is reported. Proteins are identified by their UniProt entry reference.

Food groups	Quantitative variables		Categorial variables
	positive	negative	
Vegetables	P22079, O00391	P10909, P37802, Q8TAX7	French cohort
Fruits			
Olive oil	BMI		Spanish cohort
Legumes			Former or current smokers
Nuts			Non-diabetics
Wine			French cohort, Men
Fish		Saliva flow	
Red meat			Men
Butter	Q96DR5, P22079	P23280, P07476	French cohort
Sweet snacks	E7EQB2, P02749		Non-diabetics, Men
Sweet drinks		Age	Former or current smokers

Table 5. Variables retained in minimal models of prediction of intake of 11 food groups (for each cohort separately). Quantitative variables are separated into positive (chance of being a high consumer increases with value) or negative (chance of being a high consumer decreases with value) predictors. For categorical variables, the category associated with high intake is reported. Proteins are identified by their UniProt entry reference.

Food groups	Quantitative variables		Categorical variables
	positive	negative	
Seniors-ENRICA-2			
Vegetables	BMI	Age	Women
Fruits	Q86YZ3, P0DMV8	P06753, P01024	
Olive oil	BMI		
Legumes			Women
Nuts			Non-diabetics
Wine			Men
Fish			
Red meat		Q96DR5, Q8TDL5, P01024, Age	Men
Butter			
Sweet snacks	P61158, P07195, P02763, P61769, saliva flow	P06744	Non-diabetics
Sweet drinks		Age	
3C-Bordeaux			
Vegetables	Age		
Fruits		Age, saliva flow	
Olive oil			Non-diabetics
Legumes			Men
Nuts		Saliva flow	
Wine			Men
Fish		Saliva flow	
Red meat			Diabetics
Butter			
Sweet snacks	BMI		Non-diabetics, Men
Sweet drinks			

Figure Captions

Fig. 1 Biological processes (Gene Ontology terms) associated with saliva proteins in high consumers of sweet snacks. Gene Set Enrichment Analysis was conducted separately for diabetic participants or controls. A « positive » or « negative » effect refers to the situation where proteins attached to the GO term are over-expressed and under-expressed, respectively, in high consumers of sweet snacks. The size of the dots indicates the number of proteins attached to each GO term in the experimental dataset, and the colour of the dots translates the gene ratio, i.e. the number of proteins attached to each GO term in the experimental dataset divided by the total number of proteins linked to that GO category in the human genome. In addition, four biological processes are common to all high consumers of sweet snacks, regardless of their diabetic status : *regulation of cellular macromolecule biosynthetic process* (negative effect), *platelet degranulation*, *leukocyte migration involved in inflammatory response* and *regulation of immune system* (positive effect).

Fig. 2 Estimation of the performance of minimal models predicting the consumption level of vegetables, butter and sweet snacks : distribution of AUCs (Area under the Curve) of the ROC (receiver operating characteristic) curves for 10000 simulations. For each food group, results are presented for three models: minimal model selected as described in the Material & Methods section (upper panel), minimal model with systematic inclusion of age, sex, cohort, diabetic status (middle panel), minimal model without protein variables (lower panel).