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The SALAMANDER project: SALivAry bioMarkers of mediterraneAN Diet associated with long-tERm protection against type 2 diabetes


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Abstract

Saliva offers the advantages of simple and non-invasive sampling and a rich source of biomarkers thanks to the high diversity of its microbiome, proteome and metabolome. The main objective of the SALAMANDER project is to identify and validate salivary signatures indicative of healthy dietary choices (adoption of a Mediterranean diet) with a positive long-term health outcome (protection against type 2 diabetes) throughout adult life.

The project will utilize the UK Biobank, a bank of saliva samples with dietary, lifestyle and health associated data from 85000 donors, collected from 2006 to 2010. UK Biobank will enable SALAMANDER project researchers to relate past salivary composition and dietary...
patterns to health status several years after baseline. For biomarker discovery, subjects will be categorized as healthy at baseline, and either still healthy (H+) or currently diagnosed with type 2 diabetes (H-). Compliance to the Mediterranean diet (D+, D-) will also be evaluated with appropriate adjustment factors (physical activity, dietary supplements...). Within each of the four D/H groups, 50 subjects will be randomly selected and their salivary microbiome, proteome and metabolome will be analysed by 16SrDNA analyses, nano LC-MS/MS of tryptic digests and $^1$H NMR, respectively. Integration of analytical data will then be performed to define a multimarker signature of healthy diet associated with protection against type 2 diabetes. The data obtained utilizing UK Biobank samples will be validated using two other population-based cohorts of elderly subjects in Spain (ENRICA) and France (3City-Bordeaux), enabling verification of whether the identified signatures of biomarkers are conserved as ageing proceeds. The SALAMANDER project aims to advance the current knowledge and methods in nutritional epidemiology by proposing objective and non-invasive biomarkers of compliance to a beneficial diet associated with prevention of a diet-related disease. It will also provide information on the elderly population, the salivary profiles of which are poorly documented, especially in relation to diet and health despite the importance of nutrition in healthy ageing.

**Keywords:** Salivary biomarkers, microbiome, proteome, metabolome, dietary patterns, Mediterranean diet, type 2 diabetes, ageing
Introduction

Saliva is increasingly recognized as a valuable fluid for biomarker analysis, mainly because it offers the advantages of simple and non-invasive sampling. Whole saliva can be collected on subjects of various ages and physical conditions, while limiting concerns about pain and privacy associated with blood, urine or faeces sampling. Saliva proved a valuable fluid to detect or monitor oral and systemic diseases, such as periodontitis (Prakasam & Srinivasan, 2014), type 2 diabetes (Rao et al., 2009) or Parkinson’s disease (Masters et al., 2015), and there is mounting evidence that it can be applied to studies focusing on diet and nutrition. Saliva is a rich source of biomarkers thanks to the high diversity of its microbiome, proteome and metabolome. For example, among 18 sampling sites in five body habitats (gastrointestinal, urogenital, nasal, oral and skin habitats), saliva showed among the highest within-subject diversity measured as genus-level phylotypes or 16s RNA gene OTUs (Huttenhower et al., 2012). Recent reviews on the saliva proteome (Amado et al., 2013) and metabolome (Dame et al., 2015) reported more than 3000 proteoforms and 800 metabolites, respectively. The diversity of the salivary proteome exceeds that reported for urine (Froehlich et al., 2014) and the saliva metabolome is considered as comparable to that of serum in terms of chemical diversity and number of compounds (Dame et al., 2015), making it a fluid of choice for biomarker discovery.

Type 2 diabetes mellitus (T2DM) is a major public health concern, affecting more than 400 million adults worldwide, a figure that may rise to 642 million by 2040 (IDF Diabetes Atlas 2015). Effective strategies to prevent or delay the onset of the disease include modifiable factors such as physical activity and nutrition (Ley et al. 2014). Traditionally, among dietary factors, the beneficial effects of isolated foods or nutrients were studied, but the dietary pattern approach is appealing and allows consideration of the additive, synergistic or antagonist effects of nutrients consumed in combination (Alles et al., 2012). For example, the Mediterranean Diet (MeDi) combines different food groups that are beneficial for nutrition-related diseases. A MeDi pattern is characterized by high consumption of vegetables, legumes, fruits, cereals and olive oil, moderate-to-high intake of fish, low intake of dairy products and meat, and low-to-moderate intake of wine. Beyond dietary habits, the MeDi is also part of a lifestyle with traditional practices, skills and traditions (Bach-Faig et al., 2011). The MeDi has been consistently associated with a lower risk of T2DM over time in several longitudinal cohorts (around -20%). In the PREDIMED trial, a traditional MeDi
supplemented with extra-virgin olive oil or nuts reduced the risk of T2DM by 40% among adults at high cardiovascular disease risk, compared with a low-fat diet (Salas-Salvado et al., 2014). Adopting a MeDi is also effective on glycaemic control, i.e. the management of T2DM (Ajala et al. 2013).

In nutritional epidemiology, most studies rely on questionnaires and self-assessment of dietary intake, which are prone to recall and social desirability biases. Recent efforts have evaluated the validity of objective markers of diet, for example joint evaluation of faecal metabolites, minerals and microbiota (Misawa et al., 2015) in a small group of subjects (n=8) or serum metabolite markers (Guertin et al., 2014) in a larger cohort (n=502). The studies successfully identified markers of consumption of specific food groups (e.g. citrus fruit, coffee...) or global diet pattern (e.g. high protein diet). Saliva is also gaining interest in the broad field of dietary behaviour since its secretion and composition are altered in patients suffering from early childhood protein-energy malnutrition (Psoter et al., 2008) or anorexia (Paszynska et al., 2015). Macronutrient intake has also been linked to saliva characteristics: fat intake and salivary fatty acid profiles (Actis et al., 2005) or carbohydrate intake and salivary antioxidant capacity and amylase activity (Méjean et al., 2015). Concerning the qualitative aspects of diet (the type of food consumed), the transition from a milk-based to a diversified diet in infants induces modifications of protein (Morzel et al., 2011) and peptide (Morzel et al., 2012) profiles. In adults, the same microbiota but discriminant metabolome are reported in the saliva of omnivorous, vegetarian and vegan subjects (De Filippis et al. 2014). In a recent study comparing saliva of children with eating difficulties vs healthy children (Morzel et al., 2015), proteomics and metabolomics methods allowed discrimination of the two groups despite comparable energy and nutrient intake and in spite of within group heterogeneity (age, medication etc.). This study confirmed that diet diversity plays a role in shaping the composition of saliva.

Based on this scientific background, the SALAMANDER project aims at advancing knowledge and methods in nutritional epidemiology by proposing objective and easily accessible salivary biomarkers of compliance to a beneficial diet associated with the prevention of a diet-related disease. The project combines characterization of dietary patterns, measurement of the health outcome years after initial biological sampling and a multi-omics analytical strategy. The project includes four partners from three European countries (France, United Kingdom, and Spain). This European consortium offers the opportunity to
study subjects from different environments with different food cultures and practices, and thus will allow drawing of pan-European conclusions on the interplay between saliva, diet and health. The SALAMANDER project was elaborated as a response to a call from the Joint Programming Initiative (JPI) A Healthy Diet for a Healthy Life (HDHL).

Hypothesis and aims of the SALAMANDER Project

In SALAMANDER, the main hypothesis is that among healthy subjects, some salivary patterns may convey two types of information: adherence to MeDi and a future positive health trajectory (protection against T2DM in the following years). The overall objective of the project is to identify and validate salivary biomarkers that are indicative of healthy dietary choices with positive long-term health outcomes throughout adult life. The specific objectives are:

- To identify salivary profiles that are indicative of adherence to MeDi associated with protection against T2DM in the following years
- To verify that such biomarker signatures are conserved as ageing proceeds, by investigating the link between health, diet and salivary profiles in elderly subjects.

The project is based on the use of three population-based cohorts across Europe. Firstly, UK Biobank, with 500,000 subjects at baseline. Next, two cohorts of older adults, ENRICA Spain with 3200 subjects at baseline and 3-City Bordeaux France with 2100 subjects at baseline. Blood samples, questionnaires data (physical activity, diet, dietary supplements, clinical data and drug treatments and use...) and physical/clinical measurements (anthropometrics, blood pressure...) are available in the three cohorts. In addition, saliva samples have been taken form 85,000 subjects at baseline in the UK Biobank and salivas also will be collected on the Seniors ENRICA and 3-City Bordeaux participants at the beginning of the project (Figure 1).

SALAMANDER Project structure

A. Discovery phase.

1. Selection of individuals: adherence to MeDi/ incidence of T2DM.

Figure 1 near here
Data from the UK Biobank cohort will be analysed and subjects selected if they had no diet-related diseases at the time of saliva sampling (in 2009 and 2010) and either remained healthy or developed T2DM over the follow-up period. Four groups of subjects will be defined (D for adherence to MeDi at baseline, H for Health at follow-up):

1. High adherence to MeDi (D+), low adherence to MeDi (D-)
2. Absence of T2DM at follow-up (H+), T2DM diagnosed at follow-up (H-).

High adherence to MeDi will be estimated with a validated scale, as described performed previously (Féart et al., 2009), by presence or absence of presumed beneficial (e.g. olive oil) or detrimental (e.g. meat) food groups. Attention will be paid to confounders such as drugs and tobacco consumptions, co-morbidities, and lifestyle (such as dietary supplement intake and physical activity). T2DM status will be obtained through self-reporting in questionnaires filled at baseline and at follow-ups. Based on previous studies in the field, we propose to study 50 subjects in each group giving 200 (see Figure 2).


**Salivary Microbiome.** The composition of microorganisms in the salivary microbiome will be described by 16SrDNA gene analysis (Pramanik et al., 2012). Microbial genomic DNA will be extracted and 16S rRNA genes will be amplified by PCR using hypervariable V4 region specific primers together with unique ‘barcode’ sequences, as described by the Human Microbiome Project (http://www.hmpdacc.org/). The amplicons will be purified, checked for size and purity and quantified. Sequencing will be performed and microbial species identified using the QIIME (Quantitative Incites Into Microbial Ecology) open source suite of programs (qiime.org). Analyses will include descriptive statistics of species diversity and richness, phylogenetic identification of the bacterial taxa making up the samples and statistical comparisons of community composition.

**Salivary proteome.** Saliva samples will first be depleted of amylase in order to optimize the detection and quantification of low-abundance proteins (Deutsch, 2008). After trypsin digestion of the resulting proteins, the peptide hydrolysates will be analysed by shotgun sequencing using liquid nano-chromatography coupled on line to a high-resolution mass spectrometer (nano-LC MS/MS, Orbitrap, Thermo Scientific). After LC-MS/MS analysis, the
Thermo Proteome Discoverer (version 1.4) software will be used for raw data files processing and MASCOT will be used for database search. The acquired spectra will be loaded into the Progenesis LC-MS software (version 4.1, Nonlinear Dynamics) and label-free relative quantitation will be performed. The potential interaction and interconnected biological network between proteins of interest will also be appreciated by using the STRING software.

**Salivary metabolome.** $^1$H-NMR spectra will be obtained using a Bruker DRX-600 Avance NMR spectrometer operating at 600.13 MHz for a 1H resonance frequency. The $^1$H-NMR spectra will be acquired using the Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequence with presaturation and a total spin-echo delay (2ns) of 320 ms in order to attenuate broad signals from proteins and lipoproteins. All spectra will be phase- and baseline-corrected, and data reduced using AMIX (version 3.8, Bruker, Analytik). Approximately 600 NMR buckets are typically included in the data matrices. 2D $^1$H–$^1$H COSY (correlation spectroscopy) and 2D $^1$H–$^{13}$C HSQC (heteronuclear single quantum coherence spectroscopy) NMR spectra will also be registered for selected samples as an aid to spectral assignment, which is based on matching data to a home-made reference database, as well as with other databases (e.g. [http://www.bmrb.wisc.edu/metabolomics](http://www.bmrb.wisc.edu/metabolomics); [http://www.hmdb.ca](http://www.hmdb.ca)).

**Integration and analysis of omics data sets.** For each category of salivary constituents, data will be normalized using the classical procedures specific to the omics method: total read counts for microbiome, use of the scalar factor in Progenesis QI software for proteome and normalization by the total spectral area for metabolome. It should be noted that flow rates are not available for the UK Biobank subjects. For the purpose of simplicity, raw data will be normalized by total protein concentration (taken as a rough estimate of dry matter in saliva). Statistical analyses will be performed first by type of constituents in order to identify sub-set of markers per-type specific to D/H groups. Second, integration of the microbiome, proteome and metabolome analyses will be performed by relevant methods (e.g. logistic regression models built based on different combinations of biomarkers), allowing the definition of a salivary signature to be validated. Prediction accuracy will be estimated using the area under the ROC (Receiver Operating Characteristic) curve and robustly estimating the error rates by cross-validation.
Overall, the outcome of this discovery phase will be to identify a salivary multimarker pattern, indicative of adherence to MeDi associated with protection against T2DM in the following years.

B. Validation phase

1. Selection of individuals for validation and extrapolation.

Validation will be performed firstly on different subjects of the UK Biobank, randomly chosen in the D/H groups defined in the discovery phase of the project. Secondly, individuals from the two additional cohorts of elderly (Seniors ENRICA and 3-City Bordeaux) will be selected based on comparable criteria to those of the discovery phase (past adherence to MeDi/ prevalent cases of T2DM). T2DM is recorded by self-report (confirmed by medical treatment) or using biological data (fasting blood glucose $\geq 7.2$ mmol/L) depending on the cohort. As T2DM will be already diagnosed at the time of saliva sampling, the duration of diabetes will be considered as an adjustment factor in the diseased group. The inclusion of subjects from the aged cohorts will enable exploitation of the wide age range available (mainly 65-75 yr for Senior ENRICA, over 80 yr for 3-City Bordeaux) in order to determine whether positive salivary patterns identified previously are conserved along the ageing process.

2. Salivary analyses and validation of the multimarker signatures

In the planned next wave of data collection for the Senior ENRICA study and the 3-C Bordeaux study, at-rest saliva will be sampled on elderly subjects during at-home interviews where current dietary and clinical data will also be recorded. Special care will be taken to ensure that sampling is performed following the exact same protocol as in the UKBiobank, i.e. collection by passive drool, sample immediately placed on ice and stored at 4°C during transport, freezing at -80°C within a maximum of 24h. Saliva from subjects selected above will then be analysed. The sample size will be decided primarily based on quantitative results of the discovery phase. It is anticipated that 16SrDNA analyses will remain the method of choice for microbiome, while proteome and metabolome analyses will be done with higher-throughput targeted measurements of proteins or metabolites, provided that
relevant kits (ELISA or enzymatic kits for example) are available. The clinical validity of the multimarker signature will be assessed by the same statistical methods as used earlier.

**Update on the SALAMANDER project**

The consortium partners had an inaugural meeting in April 2017. Relevant dietary and disease related data have been obtained from UK Biobank for the 85,000 subjects who provided a full volume of saliva sample at baseline. These data are currently being analysed in order to determine the relationship between adherence to a MeDi and development of T2DM in the cohort. Saliva samples are also being collected from subjects in the Seniors ENRICA and the 3-C Bordeaux studies.
Acknowledgments

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Figure legends

Figure 1. Overview of the data and saliva samples relevant to the SALAMANDER project obtained from three cohorts: UK Biobank; ENCRICA and 3-C Bordeaux (3-C Bdx)

Figure 2. Biomarkers in saliva associated with high (D+) or low (D-) adherence to a Mediterranean diet and with (H-) or without (H+) type 2 diabetes (T2DM) will be investigated as part of the SALAMANDER project.
References.


Figure 1.

UK Biobank  \[N=500\,000, 40-69y\]  47-76y
- Diet  \[X\]
- Diabetes  \[X\]
- Saliva  \[X (N = 85\,000)\]

ENRICA  \[N=3200, 60+y\]  \(N \approx 1600, 68+y\)
- Diet  \[X\]
- Diabetes  \[X\]
- Saliva

3C-Bdx  \[N=2100, 65+y\]  \(N = 750, 82+y\)
- Diet  \[X\]
- Diabetes  \[X\]
- Saliva
Figure 2.

baseline  
follow-up (several years after baseline)

**Diabetic Subjects**
*not considered in SALAMANDER*

- **High adherence to MeDi**
  - Diagnosis of diabetes since baseline
  - No diagnosis of diabetes
  - D+H- group
  - D+H+ group

- **Low adherence to MeDi**
  - Diagnosis of diabetes since baseline
  - No diagnosis of diabetes
  - D-H- group
  - D-H+ group

**Non-diabetic Subjects**

*random selection of 50 saliva samples per group*