Abstract: Fatty acids (FAs) exert diverse biological functions in humans, influencing physiological responses and, ultimately, health and disease risk. The analysis of FAs in human samples has significant implications and attracts interest in diagnostics and research. The standard method for assessing FA profiles involves the collection of blood samples, which can be inconvenient, invasive, and potentially painful, particularly for young individuals outside hospital settings. Saliva emerged as a promising alternative for evaluating FA profiles in both clinical and research settings. However, to the best of our knowledge, an updated synthesis of the related evidence is unavailable. This comprehensive review aims to summarize data on FA analysis and highlight the potential of the use of salivary FAs as a biomarker in health and disease. Over the past decade, there has been a growing interest in studying salivary FAs in chronic diseases, and more recently, researchers have explored the prognostic value of FAs in acute conditions to check the availability of a non-invasive sampling methodology. A deeper understanding of salivary FAs could have relevant implications both for healthy individuals and patients, particularly in elucidating the correlation between the dietary lipidic content and salivary FA level. Finally, it is crucial to address the standardization of the methods as the sampling, processing, and analysis of saliva are heterogeneous among studies, and limited correlation between blood FAs and salivary FAs is available.

Keywords: fatty acids; omega-3 fatty acids; dietary fat; lipidomics; lipids; lipase

Introduction

Fatty acids (FAs) are ubiquitous organic acids consisting of “a repeating series of methylene groups that impact hydrophobic character” [1]. They mainly act as energetic substrates, structural components of cell membranes, and regulators of gene expression, affecting cell physiology and human health [2–4]. Investigating the status of FAs may provide information on multiple conditions, ranging from early development, growth, and occurrence or prevention of diseases during adulthood [2, 3]. FAs are classified into (i) short-chain (3–7 carbon atoms), medium-chain (8–13 carbon atoms), long-chain (14–20 carbon atoms) and very-long chain (20 or more carbon atoms) according to their length [5], and (ii) saturated and unsaturated in relation to the presence and number of double bonds [6]. While most FAs derive from both diet (with triglycerides being the main source) [1] and endogenous synthesis, linoleic acid (LA, 18:2 n−6) and α-linolenic acid (ALA, 18:3 n−3) are defined as essential FAs as they cannot be synthesized by the organism. As a result, they are the most correlated to nutritional intake. Together with the other polyunsaturated fatty acids (PUFAs), they are components of cell membrane phospholipids, precursors of mediators such as eicosanoids, and are involved in the activation of nuclear transcription factors [7].

In human tissues and bloodstream, FAs are mainly incorporated into triglycerides (49 % of total FAs), phospholipids (24 % of total FAs), and cholesterol esters (16 % of total FAs) [8], while a minor percentage known as non-esterified fatty acids (NEFAs) circulates bound to albumin or in the form of free fatty acids (FFAs) (Figure 1). The assessment of the FA profile in the context of circulating lipids is
routinely performed on samples of plasma, serum, whole blood, or erythrocytes, as a tool for evaluating nutritional status and physiologic and pathologic conditions involving FA metabolism [9, 10]. Albeit it was thought that the coagulation process and anti-coagulants introduced in samples could have an impact on the lipid profile [9], more recent studies confirm that the FA composition is highly comparable between serum and plasma [11]. As inequality in the levels of specific classes of lipids in the different blood cell pools may occur, the choice of the sample must reflect the research or clinical goal [8]. For instance, the erythrocyte turnover does not coincide with the half-life of PUFAs, leading to possible bias in the results [8]. The FA profile of whole blood provides a balanced picture of essential FAs and PUFAs in relation to physiological states and dietary habits, compared to other blood samples [12].

However, blood collection has some limitations outside hospital facilities. Also, the withdrawn procedure from the antecubital vein is invasive and potentially painful, especially in young subjects, although it has been almost in part substituted by the sampling of blood drops through a puncture of the fingertip (or from a heel prick in newborns).

Increasing data suggest that saliva might be a reliable specimen to assess FA profiles both in clinical and research settings [13–15]. Saliva is an easily accessible biological fluid with high potential in diagnostics [16, 17]. Among the advantages, the collection of saliva solves the difficulties of blood samples for both the healthcare worker and the patient. It reduces costs, requires less training effort and it overcomes the problem of blood clotting and the individual fear of needles when performing the sampling. Finally, saliva is a widely available biological fluid (the total amount produced at rest within a day counts an average of 1,000–1,500 mL [5]).

However, to the best of our knowledge, an updated synthesis of the evidence regarding the use of salivary FAs is unavailable. This comprehensive review aims to briefly illustrate techniques for FA analysis in saliva, address the relationship between diet and FAs, and report current evidence on the use of salivary FAs as a biomarker in health and disease.

### Fatty acids in human saliva

Data from the analysis of the lipid panel of saliva have shown the presence of mono-, di-, and triglycerides, FFAs, squalene, phospholipids, wax esters (WE), and cholesterol esters (CE) [18].

The quantitative studies of salivary lipids by Larsson et al. have revealed that the neutral lipids, such as cholesterol esters, cholesterol, triglycerides etc., accounted for 96–99 % of the total salivary lipids, while the polar fraction composed of phospholipids represents only a minor portion [19].

Regarding FFAs in saliva, their concentration is around 1 μg/mL [20]. In a recent study by Neyraud et al. [21], the levels of total FAs (non-esterified plus esterified fatty acids) as well as FFAs were examined in the resting saliva of 54 subjects. The findings revealed higher concentrations in whole saliva, with levels reaching 8.99 μg/mL for total fatty acids and 3.56 μg/mL for FFAs (representing around 50 % of total fatty acids), respectively [21]. Palmitic (16:0), oleic (18:1

![Figure 1: Lipidic profile in saliva. FA, fatty acid; FFA, free fatty acid.](image-url)
n−9), linoleic (18:2 n−6), and stearic (18:0) acids are the four major FFAs identified in resting saliva, with a concentration ranging from 0.5 to 2.5 μg/mL. Other twelve FFAs have been detected at levels less than 1 μM: lauric acid (12:0), myristic acid (14:0), myristoleic acid (14:1 n−5), pentadecanoic acid (15:0), pentadecenoic acid (15:1 n−5), palmitoleic acid (16:1 n−7), margaric acid (17:0), heptadecenoic acid (17:1 n−7), arachidic acid (20:0), eicosatrienoic acid (20:3 n−9), arachidonic acid (20:4 n−6), and behenic acid (22:0) [20].

**Measurement of salivary FAs**

**Collection**

Although there is no standardized technique to collect saliva for FA composition analysis, the collection procedure remains a critical step to maintain the reliability of the analysis, especially for specific FA classes such as SCFAs. Different strategies have been employed so far (Table 1). The collection of whole saliva is mostly performed in the morning before breakfast [14, 22–24]. In one study, samples were gathered at 6 pm [21] whereas in another trial, saliva samples were collected four times a day [22].

A variable period of fasting was requested: it varied between 30 min [23, 31] and 1 [18, 24], 2 [15, 20, 22, 32, 34], 6 [25], 8 [13], and up to 14 h [35], with the indication not to consume any drinks during that period [25].

In some studies, the participants were asked to avoid routine behaviors potentially impacting FAs content such as toothbrush [13, 25, 31, 34], use of hygiene products [20, 21, 27] and/or oral rinse, consumption of chewing-gum [26, 33] and physical activity practice [13, 25]. In one study, it was requested to avoid smoking within 1 h before collection. In one case, participants were asked to use the same non-antimicrobial fluoridated toothpaste [30].

Most studies analyzed unstimulated saliva, which was obtained using a Pasteur pipette placed under the tongue [22], by aspiration [34], by spitting [34] or letting the subject drool into a collection tube [31], a sterile plastic container [13, 23, 27] or glass tube/cup [25]. Subjects were instructed to avoid swallowing for 10 [21] or 5 min [34] during the sampling procedures. The volume of saliva collected ranged between 0.5 [27, 30] and 100 mL [35]. The salivary flow rate was recently evaluated as a parameter to consider during the collection phase [14]. In other studies, collection time was fixed at 5 [29, 33, 34] or 10 min [21, 23].

Stimulated saliva sampling was performed using a Salivette® [14, 24] chewing gum base with a Curby cup [26], or chewing on a piece of paraffin [29]. However, as recently observed in the analysis of 90 metabolites [33], stimulation of salivary production may introduce bias in the analysis of fatty acids. Notably, the concentrations among spitting, aspiration and Salivette® samples were equivalent only for palmitoleic acid, while the levels of palmitic, linoleic, oleic, and arachidonic acid were significantly different with Salivette® collection [33]. Neyraud et al. showed that the FAs concentration in stimulated saliva is higher than in resting collection [29]. The authors suggest that lipids mainly originate from the parotid glands, which have a higher impact on the secretions of whole saliva [29]. However, results from other studies do not confirm that secretions from parotid glands are superior in lipids than sub-mandibular salivary product [36]. Rinsing of the mouth may also influence salivary FA components, stearic acid in particular, suggesting how collection methods without rinsing are preferable [33].

**Sample preparation (centrifugation and storage)**

A few studies have centrifuged the saliva samples before the analysis or storage [14, 23, 24, 26, 31, 34], eventually immediately after the collection [20, 22, 30, 37]. Storage was performed at −80 °C immediately after the sampling [21, 30], after [14, 23, 24, 29, 32] or before [21] the centrifugation step. In a few cases, storage was performed at −20 or −14 °C [31] and then the sample was centrifugated to analyze the supernatant [13]. In one study, the sample was spiked with butylated hydroxytoluene to prevent oxidation before storage [32]. Eventually, samples were placed on ice and then stored at −80 °C [27, 32]. In some studies, the collection was performed in ice or the tube was pre-chilled [20]. In one trial, the frosting was not executed [22]. In some cases, details on storage or centrifugation were not reported [18, 35]. Apparently, refrigeration and freezing at −80 °C are required to effectively remove cellular or bacterial membranes that would alter the result. This represents a consistent limitation of the analysis of fatty acids from saliva. If this is impractical, the sample can be collected on ice and quickly proceed with the analysis.

As for SCFAs analysis, Lenzi and colleagues [38] conducted a stability study and confirmed a variation of lactic acid only, after storage at room temperature for up to 6 h. An increase in lactic acid was recorded, probably due to the presence of lactate dehydrogenase in saliva.

**Extraction and derivatization**

The choice of the lipid extraction technique must be made according to the sample matrix [1]. In the salivary samples
<table>
<thead>
<tr>
<th>Ref.</th>
<th>Analyzed fatty acids/lipids</th>
<th>Saliva collection: stimulated/unstimulated</th>
<th>Preparation before salivary collection</th>
<th>Saliva collection</th>
</tr>
</thead>
<tbody>
<tr>
<td>[25]</td>
<td>Fatty acid composition expressed as percentage of total fatty acids</td>
<td>Unstimulated whole saliva</td>
<td>Collection performed in the morning, after a minimum of 6 h of fasting, with no physical activity or toothbrush</td>
<td>Drooling</td>
</tr>
<tr>
<td>[26]</td>
<td>Free fatty acids profile (concentration in saliva) and percentage of single fatty acids among the total fatty acid content</td>
<td>Stimulated whole saliva</td>
<td>No information</td>
<td>Chewing gum base (whole saliva) and curby cup (parotid saliva)</td>
</tr>
<tr>
<td>[27]</td>
<td>Lysolipids, fatty acids, monoacylglycerols. Fold change reported</td>
<td>Unstimulated whole saliva</td>
<td>Collection performed in the morning, fasting and refrained from eating from 11 pm</td>
<td>No further information</td>
</tr>
<tr>
<td>[22]</td>
<td>Lipid phosphorus: composition of fatty acids of phosphatidylcholine, phosphatidylethanolamine and total lipid phosphorus (expressed as percentage of total fatty acids)</td>
<td>Unstimulated whole saliva</td>
<td>Saliva was collected 4 times per day for 5 consecutive days, before breakfast and at least 2 h after meals, with a Pasteur pipette placed under the tongue</td>
<td>4 times a day, 5 consecutive days (Pasteur)</td>
</tr>
<tr>
<td>[28]</td>
<td>Free fatty acids profile (concentration in saliva), cholesterol and phospholipids in saliva</td>
<td>Unstimulated whole saliva</td>
<td>Collection performed in the morning, on three occasions at intervals of one week. Fasting from food only and have cleared teeth and tongue at least 1 h before sampling. Rinsed mouth with water, and after a 5 min rest period, collection was performed</td>
<td>Saliva was accumulated in the mouth over 8–10 min without swallowing, then expelled in polypropylene containers</td>
</tr>
<tr>
<td>[29]</td>
<td>FFA e total FAs. Expressed as relative percentage of each fatty acid among the total</td>
<td>Stimulated and unstimulated</td>
<td>Collection performed in the morning, on three occasions at intervals of one week. Fasting from food only and have cleared teeth and tongue at least 1 h before sampling. Rinsed mouth with water, and after a 5 min rest period, collection was performed</td>
<td>Stimulated (chewing parafilm) and unstimulated (drol into container for 5 min)</td>
</tr>
<tr>
<td>[20]</td>
<td>NEFA expressed as concentrations</td>
<td>Unstimulated</td>
<td>Fasting from food and drinking except water. Refrain from using hygiene products within 2 h before collection. Tubes chilled in ice during the collection of saliva</td>
<td>Unstimulated</td>
</tr>
<tr>
<td>[23]</td>
<td>NEFA profiling in saliva and plasma (as FAMEs). Salivary FAs are expressed as nmol/mg protein</td>
<td>Unstimulated whole saliva</td>
<td>Collection performed at least 30 min after food or liquid ingestion. Mouth rinsed with water before collection</td>
<td>Drooling, 10 min</td>
</tr>
<tr>
<td>[30]</td>
<td>Metabolomic approach, referring only to fatty acids. Difference expressed in fold change</td>
<td>Unstimulated whole saliva</td>
<td>Standardized toothpaste. Subjects asked to fast to 11 pm of the previous night, avoid toothbrush in the morning</td>
<td>Drooling into sterile polypropylene tubes</td>
</tr>
<tr>
<td>[31]</td>
<td>EPA:AA concentration ratio</td>
<td>Unstimulated whole saliva</td>
<td>Fasting from food and drink within 30 min and avoid toothbrush within 1 h before collection</td>
<td>Drooling into collection tubes</td>
</tr>
<tr>
<td>[21]</td>
<td>FFA e total FAs: fatty acid composition for TFA and FFA is expressed in the mean ±SD relative percentage of each fatty acid among all the fatty acids detected and in the median of the concentration of each fatty acid</td>
<td>Unstimulated whole saliva</td>
<td>Collection on 2 different days at 6 pm. Fasting and avoid smoking within 1 h before the collection of saliva samples</td>
<td>Saliva drooling, avoiding swallowing, for 10 min</td>
</tr>
<tr>
<td>[13]</td>
<td>Salivary fatty acids profile: free fatty acids expressed as a percentage of total FAs</td>
<td>Unstimulated whole saliva</td>
<td>Collection performed in the morning, after a minimum of 6–8 h of fasting. Prior to saliva collection, the study subjects did not consume any drinks and were at rest with no previous physical activity, tooth brushing and/or oral rinse</td>
<td>Drooling into sterile plastic tubes</td>
</tr>
</tbody>
</table>
considered for this review, total lipids were extracted with Folch’s [20, 21, 25, 26, 28] or Moilanen and Nikkari methods [29, 34], which both consist of the employment of a mixture of chloroform:methanol that allows the successful recovery of lipids. Following the extraction, derivatization methods are chosen according to the analysis technique, the lipids of interest, and the objective of the study [1, 8]. For instance, fatty acids in some sphingolipids are linked also with amic bonds and require for transmethylation with an acidic reagent, i.e. HCl. Instead, for FFAs a mild reagent is sufficient. Huang and colleagues [23] applied the same protocol of plasma NEFA profiling to salivary NEFA by converting them into fatty acid methyl esters (FAMEs), which best allow the analysis with GC [1]. The sample was mixed with iso-propanol, hexane and methylation was performed with methanol and sulphuric acid. Afterward, hexane was used to extract FAMEs. A simultaneous quantification of sterols and FAs was developed using one-step tert-butylidimethylsilyl (TBDMS) derivatization of lipids extracted with tert-butyl methyl ether [15]. Additional details are available in Table 2.

### Analysis

FAs in saliva have been measured alone or in combination with other lipidic components. For both purposes, in some cases lipids were previously extracted and then separated by thin-layer chromatography [22, 26]. However, gas or liquid chromatography followed by mass spectrometry is nowadays the most commonly employed tool to assess salivary FAs. Moreover, gas chromatographic methods represent the best practice as they allow to obtaining a satisfactory separation of all the FAs types, including isomers – positional and geometrical – for unsaturated FAs [15, 20, 24, 30]. Variants include high-performance liquid chromatography coupled to time-of-flight mass spectrometry [13, 18], inductively
coupled plasma mass spectrometry [39], ultrahigh performance liquid chromatography–tandem mass spectrometry [14, 27], liquid chromatography/electrospray ionization–tandem mass spectrometry [31] and reversed-phased liquid chromatography and hydrophilic interaction liquid chromatography, coupled to high-resolution mass spectrometry [34], gas chromatography–flame ionization detection technique [21, 29, 32], eventually after sample lyophilization [21]. Spectrophotometric techniques were also used [35]. Nuclear magnetic resonance was adopted by Neyraud and colleagues [29]. Additional details are available in Table 2.

### Short-chain fatty acids (SCFAs)

They consist of a class of branched-chain saturated FAs with two to seven carbon atoms that are mainly produced by colonic bacteria [38, 40]. The sample preparation and analysis of SCFAs require precautions as they may be lost in the process due to their volatility and water solubility [38]. To prevent sample deterioration, storage at −20 °C or lower temperature is required. Meanwhile, the adoption of an acidifying agent to mitigate the partial hydrophilic nature of SCFAs can improve the extraction [41]. Few studies assessing SCFAs in saliva were conducted with different techniques, including nuclear magnetic resonance (NMR) [42], high-performance liquid chromatography (HPLC) [43], LC-MS [44], isotope dilution method using GC–MS [45] and enzyme-linked immunosorbent assay (ELISA) [46]. Considering their properties, derivatization is often performed in case GC-MS is adopted, although a pilot study quantified SCFAs with a solid-phase microextraction coupled to GC-MS, without the adoption of a derivative agent successfully [47]. Due to the methodological differences, the assessment of SCFAs will not be deepened.

### Table 2: Summary of the human studies analyzing fatty acids in saliva: analysis.

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Sample processing</th>
<th>Analysis</th>
<th>Results on salivary fatty acids</th>
</tr>
</thead>
<tbody>
<tr>
<td>[25]</td>
<td>Lipids extraction with Folch’s method. Methylation with F3B-methanol</td>
<td>GC-FID</td>
<td>Higher concentration of ALA and lower concentrations of AA in vegetarians compared to mixed dietary group. Significant correlation between dietary and salivary FAs was found for miristic acid in the mixed diet group and AA in vegetarians</td>
</tr>
<tr>
<td>[26]</td>
<td>Centrifugation after collection. Supernatant was added with EDTA and filtered through millipore filter. Dialized and lyophilized. Lipids extracted with Folch’s method. Storage and −80 °C until the analysis. Total lipids were methylated with methanol/HCl solution (5 %) and were heated at 100 °C for 1 h</td>
<td>GC-FID and GC-MS to detect esters</td>
<td>Free fatty acids were detected from neutral lipids in parotid saliva. The saturated fatty acids were higher than unsaturated fatty acids. The main saturated fatty acids were palmitic and stearic acids, and the main unsaturated fatty acids were oleic, linoleic, palmitoleic, arachidonic and docosahexaenoic acids between caries-susceptible subjects and caries-resistant subjects. And stearic, linoleic and docosahexaenoic acid concentrations were higher in caries-susceptible subjects</td>
</tr>
<tr>
<td>[27]</td>
<td>Storage at −80 °C immediately after collection</td>
<td>Untargeted metabolic profiling platform UHPLC/MS/MS</td>
<td>Increased of multiple fatty acid involved in inflammation and triacylglycerol degradation (probably due the upregulation of lipase activity) Decrease of unsaturated fatty acids in smokers’ phospholipids. Increase of salivary total lipid phosphorus in smokers in the evening</td>
</tr>
<tr>
<td>[22]</td>
<td>Centrifugation after collection, supernatants were used. Extraction with chloroform:methanol 2:1. Separation of lipids by TLC. The obtained phospholipid class was mixed with methanol and kept 1 h at 80 °C with BHT. Extraction of methyl esters with hexane</td>
<td>GC-FID</td>
<td>16 different fatty acids were retrieved. Even though the levels of multiple FFAs were comparable across the follicular, ovulatory and luteal phases, some differences in salivary FAs composition were observed. Oleic, acetic, palmitic, butyric and lauric acid are influenced by the levels of circulating steroidal hormones and their levels in saliva is high during ovulatory and luteal phase. Levels of stearic, myristic, butyric, caproic and linoleic acids were minimal in the ovulatory phase</td>
</tr>
<tr>
<td>[28]</td>
<td>Extraction with Folch’s method. Saponification of lipids in a water bath. Extraction with solvent. 2/3 of the organic phase containing FAMEs transferred for analysis</td>
<td>GC-FID</td>
<td></td>
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Table 2: (continued)

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<tbody>
<tr>
<td>[29]</td>
<td>Centrifugation immediately after collection. Two aliquotes: 1. NMR fatty acids and 2. Lipid extraction by Moilanen and Nikkari method. Separation of FFAs from TL by the use of aminopropyl columns/cartridge. Preparation of fatty acids methyl esters by BF3/methanol 7%</td>
<td>NMR (fatty acids) and GC-FID (FAMEs)</td>
<td>Fatty acids, together with some amino acids and N-acetyl-groups, were among the principal metabolites that discriminated between stimulated and resting saliva. NMR data revealed an over-representation of FFAs in stimulated saliva when compared to resting saliva</td>
</tr>
<tr>
<td>[20]</td>
<td>Centrifugation immediately after collection. Supernatant transferred and lyophilized. Lipid extraction with Folch’s method, dried under nitrogen. Derivatization with PFB, dried under nitrogen and dissolved in acetonitrile</td>
<td>GC-FID</td>
<td>16 NEFA identified in resting saliva, mainly palmitic, linoleic, oleic and stearic acids (concentrations ranging from 2 to 9 μM)</td>
</tr>
<tr>
<td>[23]</td>
<td>Centrifugation, collection of supernatant stored at −80 °C. Samples mixed with isopropanol, hexane, followed by methylation (methanol and sulfuric acid); extraction with hexane, evaporation under nitrogen, resuspended in isoctane</td>
<td>GC-MS</td>
<td>Saturated FAs detected in saliva. Significant decrease in lauric acid and myristic acid in periodontal group. Levels of PUFAs including AA were not significantly different in both groups</td>
</tr>
<tr>
<td>[30]</td>
<td>Samples immediately stored at −80 °C after collection. Extraction of FAs with unspecified method</td>
<td>GC-MS and LC-MS</td>
<td>LA 18:2 n6, ALA 18:3 n3, DPA 22:5 n3, AA 20:4 n6 increased in the saliva of subjects with diabetes and periodontal disease compared to diabetic subjects without oral illnesses</td>
</tr>
<tr>
<td>[31]</td>
<td>Centrifugation after collection. Supernatant added to acetonitrile (for deproteinization), passed through a cartridge for purification. DAPPZ-derivatization</td>
<td>LC-ESI, MS/MS</td>
<td>Successful measurement of the change in the salivary EPA/AA ratio owing to EPA supplementation. The salivary EPA/AA ratio in the healthy subjects was considerably lower than the plasma EPA/AA ratios reported in the literature</td>
</tr>
<tr>
<td>[21]</td>
<td>Storage at −80 °C immediately after collection. Centrifugation before biochemical analysis. Saliva samples were lyophilized and kept at −80 °C for 8 h. Total lipid extraction with Folch’s method. FFAs converted into PFB esters: total lipids treated with IPREA acetonitrile and PFB-Br in acetonitrile. TFA: transmethylation with BF3-MeOH. Dissolved in hexane</td>
<td>FFA: Analysis with GC-MS. TFA: Analysis with GC-FID</td>
<td>TFA concentration of 8.99 μg/mL of saliva. FFA concentration of 3.56 μg/mL of saliva. Lipolytic activity had significant positive correlation with FFA concentration</td>
</tr>
<tr>
<td>[13]</td>
<td>Storage at −80 °C until centrifuged. Total salivary lipids extracted with Folch’s method. FA methylation with sodium methoxide</td>
<td>GC</td>
<td>Salivary concentrations of ALA 18:3 n3 were significantly associated with the intake of nuts. Concentrations of LA 18:2 n6 and AA 20:4 n6 were associated with vegetable oils and with red meat, cold meat and viscera intakes, respectively</td>
</tr>
<tr>
<td>[24]</td>
<td>Immediate storage of sample at −80 °C. Extraction with tert-butyl methyl ether, derivatization with MTBSTFA, and pyridine</td>
<td>GC-MS/MS</td>
<td>Simultaneous determination of various FAs and sterols in human saliva samples, with good linearity, precision and accuracy</td>
</tr>
<tr>
<td>[24]</td>
<td>Centrifugation within 1 h, storage at −80 °C. Extraction, purification and derivatization with Mettobrep GC kit</td>
<td>Untargeted metabolomics analysis with Mettobrep GC kit. GC-MS</td>
<td>Among other metabolites, palmitic acid was higher in obese patients. Propanoic acid, butanoic acid and 2-hydroxybutyric acid were higher in obese patients without steatosis</td>
</tr>
<tr>
<td>[24]</td>
<td>Centrifugation, supernatant spiked with BHT, stored at −80 °C. NEFA spiked with 5-alfa-colestane and C15:0. After sample acidification, NEFAs were extracted twice with chloroform. Lower layers (lipophilic phase) washed with NaCl solution and dried under a nitrogen steam. FAs derivatization with boron trifluoride/methanol at 60 °C</td>
<td>Qualitative analysis with GC-MS and quantitative analysis with GC-FID</td>
<td>The total NEFA levels were significantly increased in CF patients compared to controls. The percentage of increase of unsaturated NEFAs were higher than those of saturated NEFAs. Higher U/S NEFA ratio in CF patients with severe lung disease than those with mild and moderate severity</td>
</tr>
<tr>
<td>[18]</td>
<td>Saliva samples were treated with isopropanol containing lipid standards. Centrifugation</td>
<td>Analysis with HPLC, coupled to TOF-MS</td>
<td>FFAs were absent in both stimulated and unstimulated whole saliva of patients and controls</td>
</tr>
<tr>
<td>[33]</td>
<td>Storage at −20 °C</td>
<td>HPLC (analysis with RP and HILIC columns), coupled with ESI-MS. Four methods of analysis in total</td>
<td>Spitting and aspiration sampling are equivalent for most of the 60 studied metabolites (including FAs), while Salivette sampling gives different results.</td>
</tr>
</tbody>
</table>
Factors associated with FA composition of saliva

Salivary FA profile could vary upon aging or exposure to environmental factors such as diet, physical activity, smoking, and other habits associated with inflammation [14].

Lingual lipase

Lipids composing the human diet are mainly represented by triglycerides. Their absorption in the small intestine is preceded by their digestion and hydrolysis in FFAs thanks primarily to the pancreatic lipases action.

The existence of salivary lipase has been debated for decades. Some observations point out that approximately half of the healthy individuals present lipase in the oral cavity [48, 49]. Such lipase might be particularly abundant in breastfed infants [50]. However, the secretion of lipase by human salivary glands has never been demonstrated [48]. It is currently assumed that the gastric or pancreatic lipase might reach the oral cavity by gastroesophageal reflux or duodenogastro-oesophageal reflux, respectively [48]. The lipase activity within the oral cavity is reduced due to the pH of the mouth, which is usually higher than the optimum of its action on lipolysis (pH 5.4) [51]. Studies conducted in periodontal disease-affected subjects suggest that lipolytic activity may be increased in this population, probably due to microbially produced enzymes and to the inflammatory environment [27]. It is currently speculated that lipase in the oral cavity is involved in fat taste sensitivity as the liberation of FFAs from dietary lipids increases their binding to receptors responsible for fat perception [52].

Smoking

Smoking is associated with inflammatory status and several pathological processes in the oral cavity. These effects might involve salivary FFAs. Palmerini et al. analyzed the salivary lipid levels of 7 smoking with those of 7 non-smoking subjects aged between 20 and 25 years [22]. Although free FFAs were not evaluated, an overall decrease in unsaturated FFAs (in fatty acyl chains of phospholipids) was observed in smoking as compared to non-smoking subjects [22].

Dietary intake

Unhealthy dietary habits are modifiable risk factors for chronic non-communicable diseases [55]. In fact, the health status may be affected by both the amounts and quality of some components of the diet, included lipids. Traditional tools such as food frequency questionnaires

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Table 2: (continued)

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<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>[34]</td>
<td>Centrifugation after collection. Storage at –20 °C. Lipid extraction (Moilanen and Nikkari)</td>
<td>LC/MS (analysis with RP and HILIC columns), coupled with detection by HRMS in positive or negative ionization modes</td>
<td>Rinsing the mouth before collecting the saliva induces effects for many compounds, therefore it is preferable to use methods without rinsing while waiting for studies on the effects of the parameters involved in the steps</td>
</tr>
<tr>
<td>[14]</td>
<td>Measure of sample pH. Calculation of salivary flow rate. Centrifugation, storage at –80 °C. Protocol extended from the analysis of plasma</td>
<td>MEPS-UHPLC MS/MS</td>
<td>Isovaleric acid and linoleic acid were overexpressed in the experimental group</td>
</tr>
</tbody>
</table>

BHT, butylated hydroxytoluene; DIPREA, diisopropylethylamine; DPA, docosapentaenoic acid; ESI-MS, electrospray ionization mass spectrometry; GC, gas-liquid chromatography; GLC, gas-liquid chromatography; GLC-FID, gas-liquid chromatography with flame ionization detection; HILIC, hydrophilic interaction liquid chromatography; MEPS-UHPLC MS/MS, microextraction by packed sorbent-ultra-high-performance liquid chromatography tandem mass spectrometry; MS, mass spectrometry; NMR, nuclear magnetic resonance; PFB, perfluorobutyric acid; RP-LC, reversed-phase liquid chromatography; TLC, thin-layer chromatography; TOF-MS, time-of-flight mass spectrometry; UHPLC, ultra-high-performance liquid chromatography.
Correlated with the dietary intake of omega-6 vegetable oils and viscera (Salivary concentrations of ALA were significantly correlated to the estimated intake through an FFQ of 20 adults following a mixed or vegetarian diet [25]. A significant correlation was found only for myristic acid (14:0) for the mixed diet group (p=0.03) and arachidonic acid (AA, 20:4 n-6) for vegetarians (p=0.05) [25]. A possible explanation provided by the authors is that the different FA profiles between the two groups may reflect the lipid nutritional status, which is different among omnivores and vegetarians. Moreover, significantly higher concentrations of α-linolenic acid (ALA, 18:3 n-3) were found in vegetarian subjects compared to the mixed dietary group [25]. In another study [13], salivary concentrations of eighteen FAs of the omega-3 and omega-6 classes were correlated with the dietary intake expressed as a percentage of total FAs from each food item of an FFQ of 20 adults following a mixed or vegetarian diet [25]. A significant correlation was found only for myristic acid (14:0) for the mixed diet group (p=0.03) and arachidonic acid (AA, 20:4 n-6) for vegetarians (p=0.05) [25].

A comprehensive analysis performed, as only palmitic acid, stearic acid, oleic and linoleic acids were measured [32]. In cystic fibrosis patients, especially in those with severe lung disease, compared to healthy controls [32]. However, an exhaustive quantification of FAs was not performed, as only palmitic acid, stearic acid, oleic and linoleic acids were measured [32]. A comprehensive analysis of the FAs, including the complete PUFA profile, is therefore necessary for patients affected by cystic fibrosis, where the role of unsaturated fatty acids may be a key factor in the course of the disease [59].

In pediatric patients, the analysis of salivary metabolic parameters was performed to evaluate the differences between obese with and without hepatic steatosis, and normal-weight subjects [24]. In obese patients, the level of palmitic acid was significantly higher than controls; obese patients with hepatic steatosis were characterized by a significant rise in palmitic acid and a decrease in oleic acid levels [24].

Diseases and inflammatory conditions

Sjögren’s syndrome is one of the first pathological conditions that was studied for its alterations in the lipidic content of saliva [56]. Sjögren’s syndrome is an autoimmune, inflammatory disease characterized by the infiltration of lymphocytes in exocrine glands, including salivary glands with implications for dryness in the mouth. Specific FAs, especially palmitic acid, have been studied to unravel and modulate the inflammatory mechanisms of this disease. Palmitic acid was found to enhance the expression of IL-6 and, consequently, the inflammatory response in salivary glands by promoting CD4+ cell differentiation [57]. It was also found to interfere with the activity of antigen-presenting cells and adhesion mechanisms [57]. However, a recent study did not reveal the presence of the FFAs component, neither in patients with Sjögren’s syndrome nor in healthy subjects [18].

The relationship between cystic fibrosis and FFAs has also been evaluated. As chronic inflammation and increased lipolytic activity are key metabolic aspects of cystic fibrosis, FFAs are potentially relevant markers of disease activity [58]. The level of FAs in the saliva of young cystic fibrosis patients was first studied by Slomiany and colleagues in 1980 when a significant increase in FFAs in stimulated saliva was reported in 10 young patients compared to controls [36]. The authors identified a significant rise in salivary total FFAs and cholesterol compared with healthy subjects [36] and a recent study confirmed these findings [32]. In cystic fibrosis adult patients, the total salivary FAs are inversely correlated with serum triglycerides, possibly due to the increase in lipolysis. The ratio of unsaturated to saturated non-esterified FAs was found to be higher in cystic fibrosis patients, especially in those with severe lung disease, compared to healthy controls [32]. However, an exhaustive quantification of FAs was not performed, as only palmitic acid, stearic acid, oleic and linoleic acids were measured [32]. A comprehensive analysis of the FAs, including the complete PUFA profile, is therefore necessary for patients affected by cystic fibrosis, where the role of unsaturated fatty acids may be a key factor in the course of the disease [59].

Women across the lifespan

The fatty acid profile in saliva varies according to the reproductive phase, as an evolutive mechanism of sexual recognition. Sixteen different fatty acids were retrieved by analyzing the saliva of 60 young women [28]. Even though the levels of multiple free fatty acids were comparable across the follicular, ovulatory and luteal phases, some differences in salivary FAs composition were observed. Oleic, acetic, palmitic, butyric and lauric acid are influenced by the levels of circulating steroidal hormones and their levels in saliva are high during the ovulatory and luteal phase. Stearic, myristic, butyric, caproic and linoleic acid levels were minimal in the ovulatory phase [28]. The magnitude of the secretory tissue of salivary glands decreases with age, with effects on the amount and composition of saliva. Recently, the metabolic components of saliva were compared between young and older women [34]. Among the lipid metabolites, isovaleric acid and linoleic acid were over-expressed in elderly [34].
with steatosis had higher levels of propanoic acid and 2-hydroxyisocaproic acid; obese patients without steatosis showed higher levels of butanoic acid and 2-hydroxybutyric acid [24].

Chronic oral inflammation and infection occur in periodontal disease, which is associated with dysbiosis, immunological alteration, and eventually connective tissue damage and bone loss. Dysbiotical alterations of the biofilm on the tooth surface, together with genetic and salivary factors, and exposition to sugar may lead to caries [60]. In both phenomena, bacteria are known to reshape the lipid composition of parotid saliva, and a possible role of the FAs composition in caries susceptibility was investigated [23, 26]. Huang et al. retrieved higher concentrations of lauric acid and myristic acid, while no significant change in the levels of polyunsaturated fatty acids and arachidonic acid was observed [23]. However, multiple metabolites derived from arachidonic acid were significantly increased in subjects with periodontitis [23]. A greater concentration of total FFAs, stearic, linoleic and docosahexaenoic acid were observed in subjects susceptible to caries compared to controls. In this context, SCFAs have to be explored in saliva due to their possible role in periodontitis [42, 43, 45], gastroesophageal reflux [44] and specific cancer types [46]. In fact, alterations in the production of SCFAs by the oral microorganisms may be implied in the proinflammatory response, alterations with the immune system and carcinogenesis [46].

Patients with diabetes mellitus (DM) frequently develop oral inflammatory conditions such as gingivitis and periodontitis. Barnes et al. [30] compared the metabolomic profile of 80 subjects affected by DM with the one from 81 controls. Among the 161 subjects, 53 suffered from gingivitis and 55 periodontal disease.

An increase in inflammation-associated FAs was observed in subjects with periodontal disease without DM, particularly 12-hydroxyeicosatetraenoic acid, linoleic acid, and arachidonic acid. A similar FAs profile was detected in DM patients with gingivitis as compared to DM patients without gingivitis or periodontal disease [30]. A significant increase in docosapentaenoic acid (DPA 22:5 n−3) was also retrieved recently in acute heart failure subjects compared to chronic heart failure, partly due to the lipid rearrangement that occurs in relapses of heart failure [14].

**Discussion**

FAs have a role as energy sources, especially in glucose deficiency. FAs influence the cell membrane composition, as part of phospholipids, with a direct effect on membrane biophysical properties as fluidity, membrane protein functionality and movement. They represent a significant component of salivary secretions, from parotid, submandibular and labial glands (Figure 1) [20, 61]. In this review, we summarized data on FAs analysis and highlighted the potential of the use of salivary FAs for several purposes.

In chronic diseases, the analysis of salivary FAs has been increasingly explored in the last decade focusing on conditions with chronic inflammation and salivary modifications, such as cystic fibrosis and Sjögren’s syndrome [18, 32, 57]. More recently, the prognostic value of FAs has been explored in acute conditions such as heart failure [14]. In all available studies, a correlation between the status or the severity of the disease and the alterations of salivary FAs has been observed. Nonetheless, available data are insufficient to introduce the routine use of FAs into day-to-day clinical practice.

The relation between salivary FAs and dietary intake is a further promising field of research. The composition of salivary FAs was found to vary upon nutritional habits [13, 25, 52] and it is known to influence fat perception, with a possible role in weight gain [52, 62]. However, evidence on this topic appears insufficient and might be affected by the overall nutritional status of the individuals. Further studies are required to have a deeper understanding, especially of the correlation between the dietary lipidic content and salivary FAs level. This evaluation might have relevant implications both for healthy individuals and patients.

Heterogeneity in the analysis of FAs is a key issue to address. First, the differentiation between free and esterified FAs was not systematically performed. Furthermore, differences in the methodology of collection and storage steps suggest how further efforts must be made to harmonize and compare results [63, 64]. Above all, the choice of acidic or basic reagent, the flow rate and the transesterification steps represent delicate points of the analysis of FAs in saliva, especially when SCFAs are evaluated. Overall, the comparability of available studies is limited by the differences in the sampling, process and analysis of saliva specimens. Neuer studies are necessary to develop reliable and standardized procedures for salivary FAs assessment.

It is necessary to attribute a diverse biological significance to lipids found in saliva, as the salivary gland metabolism is completely different from the cell membrane formation. With the diffusion of the lipidomic analysis, clarity is needed about what is the information retrieved from each type of specimen, according to a careful evaluation of its biological origin. The relationship between circulating and salivary FAs is poorly known. Only one study concurrently assessed the fatty acid composition in human
saliva and plasma [30]. However, no analysis of the correlation or comparison between the levels of fatty acids in saliva and plasma was performed [30]. A few studies assessed the levels of lipids, such as cholesterol, triglycerides and lipoproteins, in serum and saliva finding significant correlations [35, 65]. However, the relationship between serum and salivary FAs has never been investigated.

In conclusion, salivary FAs are a promising tool both in health and disease. Table 3 summarizes the unmet questions and future perspectives on this issue.

### Reference

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