Chapter 29

The salivary volatome in breast cancer

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Introduction

Saliva is a complex body fluid secreted in the oral cavity by three major salivary glands pairs, parotid, submandibular, and sublingual, along with hundreds of minor salivary glands located in the labial, buccal, palatal, lingual, and retromolar regions of the oral mucosa [1]. Its primary role involves the gastrointestinal functions associated with food digestion, such as mastication, swallowing, and taste perception [2]. Additionally, a correct supply of saliva is essential for the protection and preservation of the oral and gastrointestinal tissues against microbial and viral pathogens [3,4]. The articulation of speech is another function to which saliva is certainly important and often forgotten [4]. Biochemically, saliva is a clear, slightly acidic solution composed of approximately 99% of water with soluble analytes, such as nucleic acids, proteins, metabolites, and minerals [5]. It is well established that the salivary constituents interchange with blood and vice versa by various mechanisms, such as active transport, intracellular passive diffusion, and extracellular ultrafiltration [6]. Therefore, saliva shares many constituents with blood, like DNA, RNA, and proteins, and can be an excellent alternative to blood sampling to study the physiology of the body. In fact, despite the large repertoire of molecules it contains, saliva harbors unique molecular constituents that can be discriminatory for the screening and detection of oral and systemic diseases and can provide important clues about the pathophysiological condition of the human body [7]. Furthermore, saliva sampling possesses inherent qualities, such as a noninvasive and painless procedure and easy handling and storage. Together, these conditions make it imperative to profile, identify, and quantitate the salivary metabolites that can help not only in diagnostics but also in prognostics and therapeutic drug monitoring. In this context, different omics approaches, like transcriptomics, proteomics, and metabolomics, are being applied to profile saliva and unveil putative biomarkers [8–15] (Fig. 29.1). Particularly, with the advent of modern mass spectrometry in combination with robust separation techniques, the metabolomics approach is viewed as a valuable resource to pinpoint the role of metabolic dysregulation in many malignant diseases. Many metabolites generated in the cell are volatile in nature, and they are lost during the routine metabolomic preparation and analysis protocols, resulting in an incomplete metabolic landscape. This can be overcome through the specific characterization of the volatile fraction of the metabolome. Such analysis, known as volatomics, deals with the detection, identification, and quantitation of the volatile and semi-volatile organic metabolites (VOMs and semiVOMs) produced by the human body [16,17]. It is an attractive strategy to unveil putative diagnostic biomarkers, particularly when it involves noninvasive sampling procedures. Moreover, VOMs are highly amenable for the integration into sensor devices, often known as eNOSEs, which have a great potential for an easy translation into Point Of Care Testing (POCT) devices [18]. This represents a breakthrough for disease prevention and management, considerably improving the efficiency of the screening programs of high-risk populations or allowing more effective treatments through a more accurate therapeutic drug monitoring.

Volatile organic compounds as disease biomarkers

Interfaces with the human microbiome, genome, metabolome, and volatome

Saliva is being continuously produced along with moisture in the oral cavity and so, to a certain extent, it can be considered a mirror of the human metabolism interaction with the environment, allowing the obtention of near real-time snapshots of such interaction [19]. This is reflected

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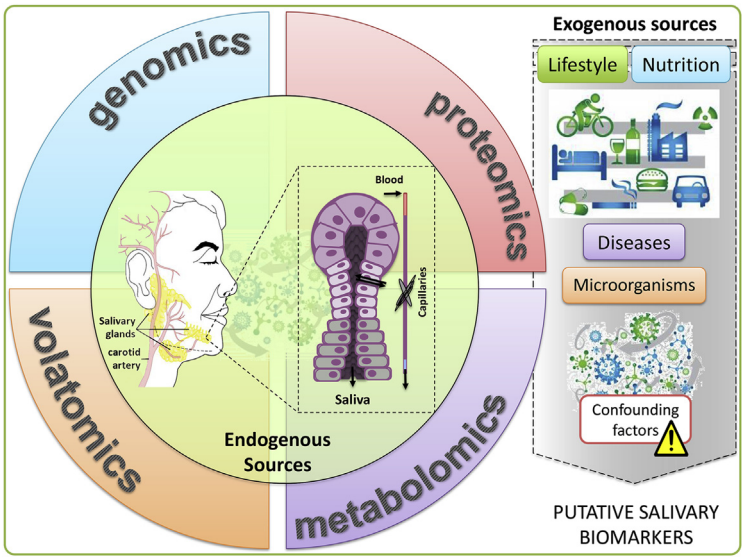

FIGURE 29.1 Overview of the metabolic interplay involving saliva sampling and the definition of putative salivary biomarkers.

in different levels, which include the human microbiome, genome, metabolome, and volatome. Saliva composition will be necessarily modulated by the overall metabolic fitness of each person but also integrates other endogenous and exogenous contributions. The mutual interaction between the individual oral microbiome and saliva composition, for instance, has been extensively studied in the context of oral hygiene. Regarding this [20], showed that chewing was essential to stimulate salivary flow and recover pH levels, thereby inhibiting tooth decay caused by Streptococcus mutants. In a recent study, [21] were able to identify unique VOCs signatures for bacteria species that often colonize our mouth, S. mutants, L. salivarius, and P. acidifaciens. This result shows the potential of a breath test to follow different clinical conditions in oral health. Food intake has an obvious interference in the oral microbiome and saliva composition, but this modulation also involves the genome itself. A recent study points to a correlation between fat mass, genomic composition, and oral microbiome in obese women from Croatia. This result is supported by the observation that there was a higher occurrence of Staphylococcus aureus, a major human pathogen, and a lower presence of Streptococcus oralis, Streptococcus mitis, and Serratia ureilytica in the saliva of these women when compared with the control group [22]. And these three strains are a very tiny part of the microbiome that each person hosts, as over 300 bacterial species have been already identified just in the oral cavity [23,24]. In fact, the volatile composition of saliva includes the VOCs produced by oral bacteria, as aliphatic amines, branched chain fatty acids, indole, phenol, and volatile sulfur-containing compounds [23,24], but also VOCs derived from many other sources, as the VOCs in inhaled and exhaled air, that dissolve differentially in saliva [24]. At a deeper level, the acinar cells that compose the salivary glands are vascularized, allowing VOMs transference from the blood through several mechanisms, as passive diffusion, ultrafiltration, and active diffusion [4,25]. Overall, this shows that the salivary volatile composition includes blood, gingival exudate, nasal cavity, gastrointestinal reflux, food debris, commercial products, and environmental pollution contributions (reviewed in Ref. [26]. In this way, the salivary volatile composition reflects both the oral compositions as biochemical and metabolic blood information, constituting a valuable diagnostic tool for myriad clinical conditions. This includes different local and systemic diseases, malignancy, infections, cardiovascular problems, and genetic disorders, among others, as reviewed by Ref. [27].

The salivary volatome and potential correlations with breast cancer

Previously, different studies have explored the suitability of saliva for cancer diagnosis, unveiling proteins differentially expressed in cancer patients. This includes, for instance, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), and carcinoembryonic antigen (CEA) that were significantly elevated in the salivary fluid of cancer patients [28]. Specifically, regarding breast cancer, augmented levels of CA15-3, and EGF receptor have been reported to be found in patients with this pathology [29]. In turn, [30] proposed a broader proteomic analysis of saliva to discriminate different phenotypes and stages, and consequent therapeutic interventions, therefore, saving patients at lower risk from unnecessary chemotherapy administration prior to surgery. More recently, significant

differences between BC patients at stages I–II and healthy controls were observed in the concentrations of 15 salivary free amino acid [31]. At the genomic level, higher number of detectable mitochondrial RNAs (miRNA) species have been detected in saliva, breast milk, and seminal fluid by comparison with urine, cerebrospinal fluid, and pleural fluid [7] and in fact [32] prevalidated eight mRNA biomarkers and one protein biomarker in saliva for breast cancer detection. Overall, it is becoming clear that different cancer-specific signatures are embedded in saliva [33] and a volatomic fingerprint able to discriminate BC is certainly feasible, as it has been already proposed by Refs. [16,34]. So far, over 350 salivary VOMs have been reported in different studies (reviewed in Ref. [35]. However, this number is very conservative, and the salivary volatome should be much more complex. This statement is greatly supported by the fact that more than 300 different bacterial species have been already identified in just the oral microbiome [23,24]. There is, therefore, a great complexity in the salivary volatome to unveil, but also a great potential to identify putative biomarkers for several diseases, including different forms of cancer and particularly BC.

Diagnostic implications for BC

Although BC diagnosis, screening programs, and treatments clearly improved in the last decade, the fact is that BC continues to figure among the main cause of death in women worldwide [36]. This is certainly a great motivation to continue to develop better and more reliable diagnosis tools, preferentially able to detect the disease in its early stages when the treatments are more efficient and the long-term survival achievable. Additionally, most of current diagnostic tools available are expensive, therefore, representing a severe burden to the developing countries and prohibitive or inexistent in the other countries. In this context, the identification of volatile biomarkers for BC using the patient's biofluids (saliva, urine, exhaled breath) is a breakthrough that ongoing research efforts are trying to achieve [16,34,37,38]. Among the human biofluids, saliva is certainly one of the easiest forms to obtain valuable snapshots of human metabolism in health and disease. Its noninvasive nature and easy to repeatedly collect under controlled parameters, without causing discomfort to patients, make this sample very relevant for volatomic research. Furthermore, saliva sampling is technically much easier than exhaled breath analysis, and its analysis is also facilitated by its lower volatile complexity, when compared with urine, for instance.

Techniques for volatome studies in cells, tissues, and fluids

The identification and quantification of VOMs emitted by biological samples of clinical importance, such as cells, tissues, and biofluids (mainly urine, saliva, exhaled breath) can be carried out using different analytical techniques. Broadly, such techniques can be classified according to the use of gas chromatography (GC) in the analytical layout. This technique, usually combined with mass spectrometry (MS) is the most often reported for volatile studies, allowing a comprehensive characterization of the volatile composition of different samples, but requires expensive and complex equipment (Fig. 29.2). In contrast, approaches involving different sensors sets and architectures, commonly known as *e-noses*, are more suitable for the

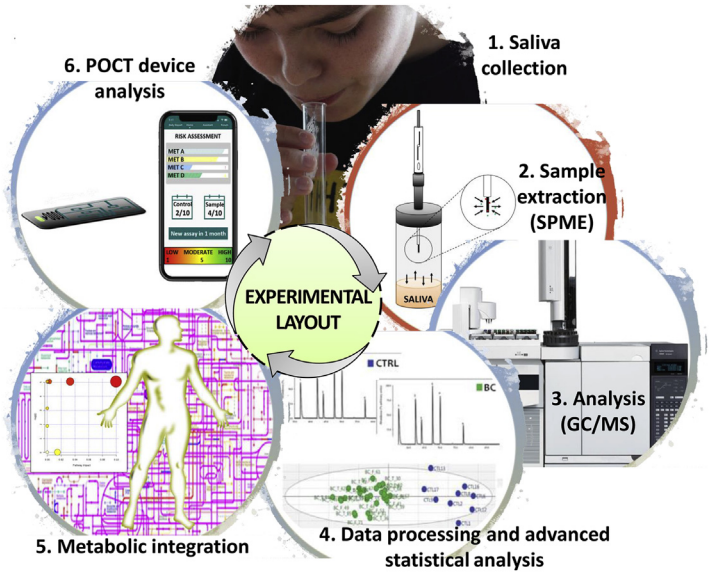

FIGURE 29.2 Experimental layout most often reported for the characterization of the volatile composition of different biofluids, including saliva.

clinical environment, but only target specific groups or family of compounds (reviewed in Ref. [39]).

GC-dependent techniques

GC/MS is the most often reported methodology in the field of volatile analysis. Essentially, the volatile and semi-volatile components of a mixture are injected in a capillary column and separated under a temperature gradient, being finally identified in the detector. In its most simple configuration, the GC apparatus is relatively affordable, in the range of $20.000, but adding a second dimension to the GC separation (GC×GC), as well as robust and powerful mass detection configuration (TOF-MS/MS), can increase the costs up to 20 times or more. Compared with this, the methodologies and equipment to perform sampling are inexpensive (<$100 each SPME fiber). The sampling step is required to trap the sample VOMs and inject them in the GC apparatus, being the solid-phase microextraction (SPME) the most popular sampling format for GC/MS analysis. SPME involves the adsorption/desorption of the sample VOMs in specific sorbents coated in the SPME fiber. These interactions are dependent of the equilibrium constants between the sample headspace and the stationary phase and can be modulated using several parameters, as the time, temperature, and form of exposure of the sample VOMs to the fiber (headspace, static, or dynamic sampling), the type and polarity of the sorbents and thickness of the fiber (reviewed in Refs. [40,41]). Following this sampling procedure, the trapped VOMs are thermally desorbed at high temperature from the SPME fiber (using a carrier gas) to the GC/MS inlet. Then, the VOMs will be separated (retained) along the capillary column and detected and quantified. Overall, SPME is quick, easy to use, is compatible with the preservation of the structure of the biological sample and allows high reproducibility and sensitivity of the following GC/MS analysis (limit of detection up to the ppbv range) [42]. For these reasons, SPME has been widely reported for the analysis of biofluids regarding the identification of putative diseases biomarkers [14,38,40,41,43]. Needle Trap Micro-Extraction (NTME) is a novel approach for the extraction of VOMs in which the sorbent material is packed inside a needle [44]. This format adds to the advantages described to SPME, an exhaustive character that allows an enhanced sensitivity and the ability to do sampling and storage in a single device [37,44,45]. Experimentally, NTME usage is quite simple, just involving the sample headspace pumping through the sorbents packed in the needle trap device by using a disposable 1 mL syringe. The following analytical steps are then performed as the regular SPME, including the same GC/MS configuration [37,45]. This certainly expands and facilitates the use of NTME to field and clinical applications. Thin film microextraction (TFME) can be particularly relevant for many clinical applications. Instead of a fiber, the TFME sorbents adopts a thin sheet format that enables a larger surface area to extraction phase volume ratio [46,47]. Consequently, the extraction efficiency will be largely improved, allowing the analysis of larger amounts of VOMs within shorter periods. TFME can be operated in a similar mode as SPME or NTME, requiring only a modified desorption chamber to transfer the trapped VOMs from the fiber to the GC/MS instrument [48].

GC-independent techniques

The most popular GC-independent techniques for VOCs analysis were essentially designed for real-time measurements of the whole volatile fraction of a given sample (untargeted analysis) or just for specific VOMs (targeted approach). In the first case, proton transfer reaction mass spectrometry (PTR-TOF-MS) is certainly in the forefront of real-time and noninvasive volatomic analysis. PTR-TOF-MS is a direct injection mass spectrometric analytical approach based on chemical ionization, which allows rapid determination of VOMs with high sensitivity and specificity [49]. Most importantly, it does not involve any sample preparation or sample destruction. PTR-MS, different from the conventional MS, use H_3O^+ generated by an external ion source to cause the soft chemical ionization of the sample VOMs [50]. This allows a very low response time and limit of detection (in the range of pg/g), making PTR-MS an excellent alternative to GC-MS. It is nevertheless, much more expensive, which explains why it was not already widely adopted for volatomic analysis. Regarding the targeted volatomic analysis, myriad different platforms using laser spectroscopy or chemical sensors designed for artificial olfaction, often known as *e-noses*, is already available and used in the clinical environment (reviewed in Ref. [51]). This technology offers many advantages over routine analytical platforms, notably its easier usage, not requiring high technical expertise, its lower equipment and operation costs, portability, and real-time measurements [52,53]. Finally, e-noses offer flexibility in choosing different sets of gas sensor arrays for customized applications. However, there are also drawbacks to be considered, namely the inability to identify individual analytes in the complex mixture of VOMs, the moisture interference, lower accuracy, and sensitivity as compared to most of the analytical setups and shorter life span [54].

Challenges and research opportunities in volatomic analysis using biofluids

The identification of putative volatile biomarkers for different diseases using human biofluids collected noninvasively is

an exciting strategy that is being pursued by several research groups around the world. In the last decades, major improvements are driving this strategy more closely to the clinical environment and real-time applications. In this context, eventually, one of the most notable works is the artificially intelligent nanoarray for breath analysis developed by Hossam Haick group [55]. This portable sampling device with the size of a small USB drive was used to diagnose and classify 17 different diseases using pattern analysis of the VOMs detected in the exhaled breath of the patients enrolled in the multicenter study [55]. Another approach is applying different forms of ion mobility spectrometry (IMS), notably high-field asymmetric waveform IMS (FAIMS), to gas analysis in medical diagnosis [56]. In this regard, Owlstone Medical Ltd. (Cambridge, UK) is exploring the concept of breath biopsy using FAIMS, and has already developed several solutions able to deliver comprehensive volatomic analysis for the clinical environment. Currently, Owlstone has several clinical trials ongoing on different forms of cancer and asthma, which will challenge the methodologies developed in large-scale studies.

There are still several important challenges to address in volatomic research. Novel extraction procedures and advanced detection systems and methodologies are continuously being developed, thereby producing a steady increase in the quality and coverage of the volatile fingerprints. Surprisingly, this is not being translated into proportional clinical biomarker identifications. There are several reasons that account for this high false discovery rate, with the complexity of human metabolism at the top. So far, over 1700 VOMs have been assigned to the human volatilome, but their origin and dependency from confounding factors as age, gender, diet, genetic background, environmental exposition, etc., has not been satisfactorily elucidated [26]. To address this problem, extensive longitudinal studies enrolling dozens of patients must be implemented. Furthermore, simultaneous characterization of the volatomic composition of different biofluids (saliva, urine, exhaled breath) from the same patient will be crucial to minimize the influence of confounding factors and achieve the full characterization of the metabolic routes of putative biomarkers. This is particularly relevant to discard the exogenous contribution from the volatile fingerprints and improve their utility as a diagnostic tool. Specifically, regarding the contribution of salivary VOMs to BC diagnosis, there are additional points that should be critically considered. As already mentioned, the salivary volatome contains valuable metabolic information about health and diseases, including for BC [16,34]. However, saliva can be easily contaminated with exogenous VOMs from different sources, particularly from the environment during normal breathing, from the diet, and from oral bacteria that colonize our mouth in an extension that depends on the oral hygiene of each subject. Furthermore, saliva flow rate and composition are affected by circadian rhythms. For minimizing these interferences and improve the quality of the volatomic data obtained, the most effective strategies are the design of consistent protocols for saliva collection and the recruitment of large cohorts, preferentially around or above 100 subjects by intervention group.

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