Usefulness of Saliva Samples for Biomarker Studies in Radiation Research

Eileen Pernot1,2,3, Elisabeth Cardis1,2,3, and Christophe Badie4

Abstract
Salivary biomarkers have important potential to facilitate breakthroughs in epidemiologic studies, management of emergency situations, and detection and surveillance of diseases by medical staff. During the last decade, an increasing number of studies on salivary biomarkers have been published as a consequence of the impressive development of new high-throughput technologies. Here, we present a review of salivary biomarkers potentially useful in ionizing radiation (IR) research, particularly in molecular epidemiologic studies. Although several salivary biomarkers of cancer and other IR-associated diseases have been identified, few salivary biomarkers of exposure and no biomarker of susceptibility or effects specific to IR have been reported so far. Further studies are therefore needed to fully assess the potential of saliva as a source of biomarkers in the radiation research field. Although the use of saliva samples is not without drawbacks, it could represent an ideal noninvasive alternative to blood, particularly in children and in the context of large molecular epidemiology studies on the effects of low doses of IR, where, given the expected limited magnitude of effects, an extensive number of samples is required to reach statistical significance.

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Introduction
Although the interest for biomarkers in saliva samples for screening, diagnostic, or research purposes is not new, an increasing number of studies assessing the possibilities of using such fluid have been reported during the last decade as a consequence of the constant development of molecular, high-throughput technologies (1). In fact, more than 50% of the available literature (as of July 2014) related to "salivary biomarkers" and "salivary diagnostic" in PubMed was published after 2007.

The advantages of saliva over other types of biosamples, such as blood, are numerous. First, saliva sampling is noninvasive, thus providing far less discomfort to subjects than blood collection. In epidemiologic and other research studies, this facilitates approval from ethical committees compared with blood collection, in particular in studies involving children (2), leads to higher acceptance by participants (e.g., 31% of participation for blood sampling vs. 72% of participation for saliva sampling in the Danish nurse cohort study; ref. 3), and allows repeated sampling even in younger participants. Furthermore, the logistics of sample collection are considerably simplified and more economical compared with venipuncture: the collection can be done by the subjects from their home without the presence of medical staff (4, 5), many types of samples can be stored at room temperature in appropriate tubes for days, weeks, or years (Table 1; ref. 6), and can be sent by mail to the investigator team, research laboratory, or hospital.

From a clinical point of view, saliva biomarkers, if validated, could provide fast and economical tools for diagnostic screening—possibly complementing analyses of serum biomarkers—and monitoring treatment performance (e.g., decreasing level of tumor-specific miRNA throughout the course of the cancer treatment). The use of saliva as a source of biomarkers is also of great interest in the case of emergency situations, in which large population screenings in a short time is needed (7, 8).

Ionizing radiation (IR) is a well-studied human carcinogen that can induce different biologic effects, depending on radiation type, exposure pattern, and dose levels. However, due to a lack of direct human evidence, further work is needed to better characterize health effects induced by exposure to low doses and low dose rates of IR (in medicine—particularly CT-scan

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patients—occupational settings, and accidental situations). DoReMi, a European Network of Excellence, aims to encourage multidisciplinary approaches to address key policy questions in radiation protection identified by the High Level Expert Group (HLEG) on low-dose risk research (9) including “The shape of dose–response for cancer, tissue sensitivities for cancer induction, individual variability in cancer risk, the effects of radiation quality, risks from internal radiation exposure, risks of—and dose–response relationships for—noncancer diseases, and hereditary effects.” Because the impact of low-dose IR exposure on health is expected to be modest, very large studies are required to adequately quantify risk (10, 11). As shown in Fig. 1, the power of a study relies heavily on cohort size, variability of data, length of follow-up, and frequency of the disease of interest (Fig. 1). Biomarkers are increasingly used in epidemiologic studies to increase the power of studies to adequately quantify the relationship between exposure and health effects (12–14). Biomarkers of exposure help improve exposure assessment, hence decreasing misclassification (an important source of statistical power reduction) and improving accuracy of health-risk estimates; biomarkers of susceptibility can identify radiosensitive patients and lead to the analysis of a more homogeneous population; biomarkers of early- or late-biologic effects can be used to reduce biases due to health outcome misclassification.

The majority of biomarkers relevant for low-dose IR research have been validated or investigated in blood (see for instance ref. 15) or blood components, cell lines, and sometimes tissues (12). The validation of IR biomarkers in saliva would greatly facilitate radiation research and help answering the aforementioned key questions (12).

Here, we review the literature on saliva biomarkers of exposure, susceptibility, and effect, potentially useful for IR research purposes.

### Materials and Methods

Salivary biomarker studies were identified through PubMed searches and publication bibliographies (see Supplementary Fig. S1 for search strategy). A PubMed search using the terms “saliva biomarkers” and “saliva biomarkers radiation” was performed on a weekly basis from the December 10, 2013 to June 1, 2014 (n = 2,339). Additional PubMed searches were performed: “salivary diagnostics” (n = 245) and “salivary biomarkers” (n = 2,241) on the January 28, 2014; “early cancer diagnosis biomarker in saliva” (n = 100) and “hormone AND salivary biomarkers AND cancer” (n = 83) on the July 9, 2014. Bibliographies from resulting publications were consulted to identify other relevant studies.

### Table 1. Collection and storage conditions of saliva samples

<table>
<thead>
<tr>
<th>Salivary biomarker type</th>
<th>Collection conditions</th>
<th>Storage condition and duration</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>Saliva collection kits commercially available</td>
<td>At least 5 y at RT or/and freeze at −20°C/−80°C indefinitely</td>
<td>(5, 29–39, 87, 88)</td>
</tr>
<tr>
<td></td>
<td>Unstimulated saliva collection into a cryovial with no preservative</td>
<td>Up to 5 d at RT or/and freeze at −20°C</td>
<td>(40)</td>
</tr>
<tr>
<td>RNA</td>
<td>Saliva collection kits commercially available</td>
<td>At least 48 h and up to 2 mos at RT or/and freeze at −20/−80°C indefinitely</td>
<td>(87, 89)</td>
</tr>
<tr>
<td></td>
<td>Stabilization of unstimulated whole saliva or cell-free saliva with commercial reagents</td>
<td>Variable, at least 1 wk at RT in certain conditions or/and freeze at −80°C indefinitely</td>
<td>(18, 19, 22, 90)</td>
</tr>
<tr>
<td>Protein</td>
<td>Variable, unstimulated saliva collection in plastic tube, optional preservative (no sodium azide)</td>
<td>Variable, freeze sample supernatant at −80°C</td>
<td>(7, 16, 52, 53, 72)</td>
</tr>
<tr>
<td>Hormones: variable collection methods (pads, drooling in cryovial, etc.), optional preservative, plastic tube with low affinity to hormones</td>
<td>Variable. Refrigerate and freeze (e.g., cortisol, testosterone, and estradiol at −20°C/−80°C, analyses within 28 d); few days at RT for glucocorticoids and androgens without preservative. Longer storage (weeks) at RT with preservative.</td>
<td>(84, 85, 91)</td>
<td></td>
</tr>
<tr>
<td>Metabolite</td>
<td>Unstimulated saliva collection, no preservative</td>
<td>Freeze immediately</td>
<td>(20)</td>
</tr>
</tbody>
</table>

Abbreviation: RT, room temperature.
We selected all published studies investigating salivary biomarkers of IR exposure, IR susceptibility, or early effects (late effects if no early effect biomarkers were available) of diseases relevant to IR exposure. In this review, we specifically covered the following fields: cancer, cardiovascular diseases, and diabetes.

Results

Composition and collection of saliva

The development of the so-called “salivaomics” during the last decade by several consortiums has led to the creation of different databases such as the Salivaomics Knowledge Base that includes datasets of proteomics, transcriptomics, miRNA, and metabolomics (1). Specific proteome analyses reported the identification of 2,340 salivary proteins with 20% of them also found in plasma (16). Recently, Wang and colleagues (17) have described a computational method for predicting salivary proteins that come from circulation and proposed 31 breast cancer salivary biomarkers.

Studies on the human saliva transcriptome have been published since 2004 (18), but more recently, a characterization of the human saliva transcriptome by massive parallel sequencing reported between 3,200 and 8,000 gene transcripts—depending on the saliva fraction—involved in most metabolic processes of the body (19). This study also reported that more than 90% of RNA content was mRNA while the rest was noncoding (e.g., 224 snoRNA). Finally, a metabolomic study has reported 57 salivary metabolites with the potential to predict oral, breast, and pancreatic cancer as well as periodontal diseases (20).

Although most molecules that are present in saliva are produced locally by the salivary glands, some of them pass from blood to saliva through salivary glands capillaries (17, 21). It became therefore evident that saliva could be an attractive noninvasive fluid for detecting effects occurring in other organs and tissues in the body. The collection of saliva is made easy by its abundance—it is produced in healthy adults at a rate of approximately 0.5 mL/min (21)—and the existence of several methods and commercially available kits [e.g., Biomatrix, Isohelix, DNA Genotek (Oragene), Norgen, etc.] or stabilizers (Table 1). The choice of preservation buffer is important given that it can modulate measurements or even inhibit assays, in particular, for non-DNA/RNA biomarkers (e.g., sodium azide in protein samples; ref. 22).

Salivary biomarkers of exposure

A recent study in saliva samples from patients with cancer reported the response of three proteins following multiple fractions of total body irradiation compared with preirradiation levels: MCP-1 (AUCmax, 0.93 at 4 hours), IL8, and ICAM-1 protein responses in cancer patient saliva upon IR exposure.
Salivary biomarkers of susceptibility

The use of saliva could greatly facilitate the analyses of genomic biomarkers of susceptibility. The quality and quantity of DNA retrieved from whole saliva, mouthwash, and buccal swabs have been largely investigated in comparative studies and it appears that saliva contains suitable DNA for high-throughput genomic analyses. We are not aware of published epidemiologic studies investigating IR susceptibility through salivary DNA analyses, though such analyses are foreseen in future studies investigating IR susceptibility classically studied in blood lymphocytes (see for instance ref. 24) as long as enough cells can be collected. In fact, a number of studies have investigated low-dose IR biomarkers in exfoliated buccal cells and reported a significant increase in micronucleus 10 days after exposure to panoramic radiography (25) or an increase in cytotoxicity after in vitro exposure to CT or radiography, with no difference in micronucleus frequency (26, 27). Another study reported a dose-response relationship in gH2AX induction after in vitro exposure of buccal cells up to 4 Gy (28).

Partial body exposure is a crucial aspect to consider when assessing a circulating biomarker of exposure in saliva after medical or accidental irradiation. In that case, having information about the distance between the part of the body exposed to IR and the head is important to assess the level of dose received at the level of the buccal cavity by scattering. It has to be noted that the observed biologic effects could reflect either direct local exposure at the level of the salivary gland or systemic effects from distant exposure—possibly modulated when passing from blood to saliva—or both. Those different exposure scenarios could have different dose-response curves, hence making extrapolation of dosimetry results difficult without information on the dosimetry.

Salivary biomarkers of effect in saliva

Systemic biomarkers of effect in saliva

To our knowledge, no other biomarker of exposure to IR present in whole saliva has been reported so far. Because saliva samples contain buccal cells and lymphocytes (23), it should theoretically be possible to investigate the proteomic and transcriptional biomarkers of exposure classically studied in blood lymphocytes (see for instance ref. 24) as long as enough cells can be collected. In fact, a number of studies have investigated low-dose IR biomarkers in exfoliated buccal cells and reported a significant increase in micronucleus 10 days after exposure to panoramic radiography (25) or an increase in cytotoxicity after in vitro exposure to CT or radiography, with no difference in micronucleus frequency (26, 27). Another study reported a dose-response relationship in gH2AX induction after in vitro exposure of buccal cells up to 4 Gy (28).

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It should be noted that most of the salivary biomarkers of cancer described above have been investigated in limited groups of patients (n < 100) and need validation. In addition, the usefulness of such biomarkers for early effects has yet to be confirmed because previous experiences were conducted in population of patients at an advanced stage of the disease.

Biomarkers for early detection of cancer in saliva were mainly investigated in oral cancer (among the 100...
PubMed references returned with the keywords “early cancer diagnosis biomarker in saliva” 59 referred to oral cancer, which is less relevant for IR-related cancer than breast, thyroid, leukemia, or lung cancer. A study on patients at risk of breast cancer assessed salivary HER2 but did not conclude on any significant differences between patients at risk and control groups (57). The current status of early detection biomarkers for lung cancer has been recently reviewed elsewhere (58) and reported no biomarkers for saliva (at most, sputum). Finally, we did not find any salivary biomarkers for early detection of thyroid cancer or leukemia.

**Noncancer diseases.** The Life Span Study of atomic bomb survivors and studies on patients treated with radiotherapy for peptic ulcer have demonstrated that IR exposure can significantly increase the risk of developing heart diseases years after irradiation (59). However, the effects of IR doses below 500 mGy on the heart have yet to be clarified; to that effect, the detection of salivary biomarker of early cardiac effects in large cohorts of patients exposed to low doses (for instance radiotherapy patients with distant exposure to the heart or workers with protracted internal contamination) could be useful. Most salivary biomarkers for cardiovascular diseases, such as creatin kinase or cardiac troponin for instance, were investigated as late effect biomarkers and are therefore more interesting for confirmatory diagnostic purposes than research (60, 61). Early predictive biomarkers, such as cystatin C, fibrinogen, and vitamin D, were reported to be strongly associated (although not necessarily causally related) with cardiovascular diseases (62); cystatin C has been characterized as a biomarker for Sjögren syndrome in saliva (63), vitamin D levels can be measured in saliva by ELISA (64) but varies during the day and depends on diet (65), while fibrinogen is absent from cell-free saliva (66). Salivary endothelin-1 was higher in patients with chronic heart failure, including those with mild symptoms, but the sensitivity was low (63% sensitivity; 92% specificity; AUC, 78%; ref. 67). Finally, C-reactive protein (CRP), an inflammation biomarker associated but not specific to atherosclerosis, has been detected at low concentration in saliva (previously reviewed in ref. 21).

Diabetes has been studied in several studies of patients with cancer treated with radiotherapy (68–70). More recently, a retrospective cohort study of childhood cancer survivors including a dose reconstruction at the level of the pancreas has concluded on a dose–response relationship between radiation exposure of pancreas and subsequent risk of diabetes (68). Diabetes of type II is the most common form of diabetes (around 90% of cases) and remains largely undetected during several years. The salivary transcriptional profiling of 13 patients with type II diabetes versus 13 healthy subjects reported that the use of four biomarkers (Kras, Sat1, Egfr, and Psmmb2) could identify patients with 100% sensitivity, 77% specificity, and AUC of 0.917 (71). Proteomic profiling in whole saliva of 10 patients diagnosed with type II diabetes, 20 patients with other preclinical glucose homeostasis anomaly, and 10 healthy subjects revealed 65 proteins differentially expressed in patients with diabetes versus healthy subjects (72). Finally, a three times higher ratio of MMP-8/TIMP-1 and a two times higher concentration of MMP-8, a biomarker of inflammation, has been observed in saliva of patients with diabetes (73).

Inflammation is associated with both cancer and noncancer diseases (21, 74, 75). Given that cytokines, such as IL8, MMP3, PtgS2/Cox2, IL6, TNF, IL1β, and IL33, are modulated by direct and/or indirect IR exposure (75–78) and that many cytokines have been previously described in saliva (7, 74), their validation as salivary biomarkers could be potentially useful for investigating IR-related diseases but also bystander effects (76).

**Limitations related to the use of saliva as a source of biomarkers**

At this stage, it has to be noted that saliva has several limitations, some of them shared with other sources of biomarkers such as blood and other more specific to salivary gland physiology.

Saliva composition and flow is affected by lifestyle factors such as smoking (73), circadian rhythm (79), or dietary habits (e.g., salivary CRP six times higher in obese children; ref. 74); in addition, tooth brushing can lead to blood leakage into saliva and subsequent molecule concentration increase (80). Therefore, it is important to homogenize time and conditions of collection within (repeated sampling) and between subjects. Oral diseases and saliva stimulation methods can also interfere with saliva composition and biomarker results [e.g., salivary estradiol level increased after chewing (4), negative effects of acid stimulation on CRP myoglobin and IgE (81)].

Salivary microbiome contribution should also be considered. A study reported that 68% of the total DNA obtained by the kit Oragene (DNA Genotek) was of human origin (8). Another study found no significant interferences from bacterial DNA in genomic analyses compared with blood DNA (5). RNAs of more than 600 different prokaryote species were found in saliva from healthy subjects (19). In contrast, a study sequencing 117 clones from a cDNA library from pooled supernatant saliva in 10 healthy donors showed that all these clones contained sequences of human origin, the absence of bacterial RNA being attributed to centrifugation of saliva (82). Overall, there is a need to better identify human RNA from microbial RNA (83). Bacteria can also contribute to salivary steroids degradation (84, 85).

The relationship between plasma biomarkers and saliva biomarkers may not be straightforward as salivary biomarkers could be produced locally at the level of the salivary glands or the gum. In addition, the concentration of molecules is much lower in saliva than in blood (e.g., due to binding to carrier proteins, only 1%–10% of plasma steroids can be found in saliva; ref. 86), and therefore sensitive analytic techniques can be necessary.
Discussion

Overall the studies reviewed here reveal that a very limited number of salivary biomarkers have been developed for IR research purposes so far, with very few exposure biomarker for moderate doses of IR, no genomic studies using saliva as a source of DNA and some biomarkers of late effects, but not specific to IR. In contrast, the high specificity, sensitivity, and/or accuracy of most salivary biomarkers for cancer and other IR-related diseases presented in this review open the way to a reliable classification of cases in epidemiologic studies. However, the usefulness of such biomarkers is limited by the fact that most of them were detected in symptomatic patients, sometime at an advanced stage of the disease, and considerations about how much knowledge will be gained by performing costly biomarker analyses of late effects in addition to available clinical data would probably be needed. With the dynamic development of salivary biomarkers for screening purposes, it is expected that in the future, more biomarkers of early effects (or risk) will be available to detect asymptomatic subjects. Furthermore, a specific IR signature may be achievable (for exposure, susceptibility, or effects) by using a combination of non-IR-specific genomic (DNA/RNA/miRNA) and proteic saliva biomarkers.

The simplicity of saliva collection and storage as described in Table 1 appears adapted to IR studies investigating modest risks. The cost of molecular epidemiologic studies, including blood collection, can easily approximate millions of Euros: an ideal cohort should have large sample size, reliable individual dosimetry, long follow-up, validated case ascertainment method, and should collect all relevant information about confounders and effect modifiers. In addition, repeated biosamples should ideally be collected and stored throughout the study in a standardized manner. The use of salivary biomarkers instead of blood biomarkers can be a cost-efficient alternative.

Finally, to validate salivary biomarker assays and decrease unnecessary data variability, further work is needed to better characterize the origin of salivary molecules, in particular, the contribution of the microbiome to the salivary transcriptome, proteome, or metabolome and to assess to what extent it contributes to intra- and interindividual variability in biomarker results.

Conclusion

During the last decade, salivary biomarkers have drawn much attention in the clinical and scientific communities given their potential to assess a broad range of environmental and infection exposures and predict health outcomes; in addition, saliva sampling is easier, more economical, less invasive, more accepted by study subjects, and ethical committees, and provides sample stability compared with blood while also providing DNA for genetic analyses. Despite those advantages, salivary biomarkers have been scarcely investigated and used for IR research purposes: very few biomarkers of exposure, and no biomarker of susceptibility or effects specific to IR, have been reported so far. This type of sample is certainly not without drawbacks, but could represent an ideal alternative to blood, in particular for large molecular epidemiology studies investigating effects of low doses of IR, where, given the limited magnitude of effects, a large number of samples is required to help answering key questions in radioprotection such as the shape of dose responses for cancer, individual radiation sensitivity, noncancer effects of IR, effects of radiation qualities, effects of internal emitters, and tissue sensitivities.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

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