MiniReview

Salivary DNA Methylation Profiling: Aspects to Consider for Biomarker Identification

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Abstract: Is it not more comfortable to spit saliva in a tube than to be pricked with a needle to draw blood to analyse your health and disease risk? Many patients, study participants and (parents of) young children undoubtedly prefer non-invasive and convenient procedures. Such procedures increase compliance rates especially for longitudinal prospective studies. Saliva is an attractive biofluid providing good quality DNA to study epigenetic mechanisms underlying disease across development. In this MiniReview, we will describe the different applications of saliva in the field of epigenetics, focusing on genomewide methylation analysis. Advantages of the use of saliva and its comparability with blood will be discussed, as will the challenges in data processing and interpretation. Knowledge gaps will be identified and suggestions given on how to improve the analysis, making saliva ‘the’ biofluid of choice for future biomarker initiatives in many different epidemiological and public health studies.

The field of molecular biomarkers of health and disease is rapidly evolving, requiring extensive research and insight in the pathways involved in early disease development. Epigenetic factors defined as relatively stable and to some extent heritable changes that do not modify the DNA sequence provide important information and are critical regulators of gene and protein expression. The most studied epigenetic mechanisms are DNA methylation, histone modifications and non-coding microRNA (miRNA). The dynamic epigenome is thought to capture and encode environmental changes, serving as an important pathway for the interaction between genes and environment. Although we are only beginning to understand the importance of the epigenome in health and disease, it is clear that epigenetic factors are likely mediating factors in the development of complex diseases across the life course [1,2].

Environmental epidemiology and public health researchers often do not have access to specific target tissues related to complex diseases; as such, they must rely on peripheral biological sources for study. The majority of epigenetic and biomarker research to date has relied on peripheral blood as the main surrogate tissue. The need of trained personnel and logistics can make blood sampling challenging when doing decentralized investigations. In addition, blood sampling in vulnerable individuals, such as children, should be avoided as much as possible to reduce discomfort, increase participation and improve overall feasibility. Saliva can be a good alternative surrogate tissue as it is easier to collect and it is a good source of high-quality DNA for use in (epi)genomic studies [3–8]. Saliva has recently attracted much attention because it also contains a broad range of other diagnostically relevant molecules (i.e. microRNA, RNA, inflammation markers and antibodies).

Salivary research began with the study of salivary analytes towards research into prediction/diagnosis of systemic diseases and health conditions [9,10]. For example, salivary cytokine profiles have been successfully used as biomarkers of respiratory and other immunological disorders in the field of clinical diagnostics [9,10]. Over the last decade, research examining the impact of stress on human health and disease has relied almost exclusively on the measurement of neuroendocrine markers, including cortisol, from saliva particularly after side-by-side studies demonstrated the equivalence of salivary and blood measurement [11]. The research field is now also focusing on the detection of time-sensitive and context-dependent epigenomic alterations in saliva, and it has embraced a wide range of biomolecular techniques, including bisulphite sequencing, methylation arrays, a range of PCR and qPCR-based techniques.
One of the most important advantages of saliva over blood is the ease of collection, facilitating multiple collections in 1 day, or even over shorter time periods. Apart from the fact that it is more comfortable to take a saliva sample, repeated sampling is usually more practical than blood as (i) there is no need for the addition of an anticoagulant to avoid clotting upon collection and (ii) the risk of disease transmission that can occur when in contact or via needle prick is lower than with blood sampling [12]. In addition, no specially trained (para)medical personnel is required and, with appropriate instructions and attention to sample collection, an individual is able to self-collect multiple samples for clinical and research purposes. As with all biological samples, collection, storage and transport of saliva should be carried out with care to preserve sample integrity. The importance of correct sample collection procedures and the available different analytes present in saliva was recently discussed [9,10] and will therefore not be dealt with in this MiniReview. Briefly, it is advisable to carefully choose your saliva collection method (e.g. stimulated versus unstimulated and use of suction) depending on the envisioned downstream analysis.

miRNA expression studies in saliva [9,13,14] have increased in the past decade, while research into salivary histone modifications is still in its infancy with only few reports on salivary proteomics (including histones), mainly in the field of oral diagnostics [15,16]. In the current MiniReview, we will be focusing on DNA methylation profiling, data processing and interpretation. Our aim is to highlight the utility of saliva for DNA methylation research and biomarker identification.

**Primer on the Methylome**

DNA methylation occurs through the covalent binding of a methyl group to the DNA, catalysed by DNA methyltransferases (DNMTs), mainly at cytosine residues within CpG dinucleotides. In mammalian genomes, the CpG dinucleotide is under-represented and is mainly observed as dense clusters of CpG dinucleotides called ‘CpG islands’ [17]. About 60% of these islands overlap promoter regions and are largely unmethylated. It is generally believed that this status of hypomethylation promotes gene expression. Nonetheless, about 80% of all CpG sites are said to be methylated, mostly in repetitive sequences made up of transposable elements (incl. long terminal repeats (LTR)-retrotransposons, long and short interspersed nuclear elements (LINE and SINE, respectively) and DNA transposons), which are silenced by methylation to support genome stability and integrity [17]. The removal of 5mC is catalysed by the ten eleven translocation (TET) enzymes and involves, as an intermediate step, the oxidation of 5mC into 5-hydroxymethylcytosine (5hmC). It is generally believed that 5hmC may have a more specific role in regulating transcription, most probably reactivating gene expression, while 5mC might have additional roles in maintaining genomic integrity and transposon stability [17].

Altered global DNA methylation content is a feature of several diseases; its occurrence in cancer was first highlighted by Feinberg and Volgelstein [18]. An important feature of cancer development and progression is the dysregulation of DNA methylation patterns, characterized by the hypermethylation of specific genes concurrent with an overall decrease in the abundance of 5mC. In particular, hypomethylation can lead to overexpression of oncogenes [19,20]. Hypermethylation of the promoter regions of tumour suppressor and DNA repair genes causes gene silencing and contributes to tumorigenesis. Furthermore, studies with monozygotic twins show that environmental factors influence DNA methylation [21,22]. Age has also been shown to have a multifaceted effect on DNA methylation [23,24]. Interestingly, the within-tissue epigenetic correlation was reported to be higher earlier in development and diverges more later in development. In this respect, the DNA methylation status of approximately 50 epigenetic clock-associated CpG sites allows to calculate accelerated or decelerated epigenetic ageing as compared to biological ageing [25–27]. Although there is evidence of changes in methylation across development, ageing and with different environmental exposures recent data suggest that more dynamic changes in methylation, even over the course of a social stressor, may occur although studies carefully accounting for cell type changes and other factors are needed to determine the validity of these changes in either blood or saliva [28]. For example, acute psychosocial stress was observed to increase DNA methylation of the stress-associated gene OXTR already 10 min. after applying the stressor, returning to initial pre-stress DNA methylation levels 90 min. after the stress was applied [29]. This work underlines the dynamic nature of DNA methylation profiles. Unternaehrer et al. demonstrated the effect of acute psychosocial stress on DNA methylation in blood. We propose the investigation into the epigenetic effects of stress using saliva as biofluid because multiple saliva samples can be collected in a short time period and do not cause any additional stress as may be the case with blood sampling.

**Sample Quality**

Given the increased interest to use saliva for (epi)genomics, a range of DNA collection kits (available from, e.g., DNA Genotek, Ottawa, Canada, Norgen Biotek Corp. Thorold, Canada and Isohelix, Kent, United Kingdom), stabilizing reagents and purification procedures have been developed [30]. The kits ensure nucleic acid stabilization and isolation of high-quality and high molecular weight DNA. An increasing number of reports confirm that the yield and quality of saliva is high, as assessed by purity and feasibility of downstream applications including sequencing, genotyping, PCR amplification and genomewide arrays [31–33]. In our hands, saliva yielded good quality DNA as determined by the UV absorption profiles (median 260/280 = 1.8, median 260/230 = 1.1, median yield 54.4 µg from 2 mL saliva), which is in line with previous reports [34,35]. The samples passed the different steps for genomewide DNA methylation analysis using the Illumina Methylation 450K BeadChip [36]. To further confirm that salivary DNA is of high quality, in our recent birth cohort study, we observed that saliva of 99 children, collected at 11 years of age, yielded high
amounts of good quality DNA (median yield 79.6 μg from 2 mL saliva with median 260/280 = 1.81 and median 260/230 = 1.11) [37].

An additional advantage of salivary DNA collection kits claimed by the manufacturers is the possibility of sample storage at room temperature for 5 years or more without any detectable DNA degradation [38]. Independent researchers performed similar tests and reported that saliva can be stored at temperatures up to 37°C for between 6 and 18 months without compromising the DNA quantity, quality and applicability for a range of different genetic analyses [32,39,40]. Consequently, individuals can collect their saliva at home and either transport it to their doctor or the research site, or mail the sample directly via standard postal systems to the research institute, clinic or hospital. Ng et al. [40] even reported that storage of saliva collection kits at air-conditioned room temperature (20°C) for 6 months or at −80°C for 6 months did not affect DNA quality (OD260/280 values were comparable) in real-time PCR experiments and genotyping fidelity remained undiminished.

Concern about the presence of bacterial DNA in saliva has been raised by some researchers, particularly as current quantification methodologies cannot distinguish between bacterial and human DNA, resulting in a potential overestimation of DNA quantity. For instance, Rylander-Rudqvist et al. [33] observed PicoGreen and UV absorbance measurements (detecting bacterial and human DNA) to overestimate the yield of human DNA from salivary cells by ~1.6-fold compared to real-time PCR measurements (detecting only human DNA). Nonetheless, significant correlations were seen between the various DNA quantification methods. Bacterial DNA also contains 5mC, and concerns about the presence of bacterial DNA leading to an overestimation of global genomic 5mC levels in human saliva DNA have been discussed. For instance, ~0.75% of all cytosines in the DNA of E. coli are methylated, compared to ~4% of all cytosine residues in the human genome [17,41]. To the best of our knowledge, there have been no reports indicating that bacterial DNA competes with human DNA in the hybridization steps of genomewide DNA methylation screening approaches. Abraham et al. [34] reported DNA from saliva and blood samples to be of comparable quality when genotyping using either TaqMan assays or genomewide chip arrays. Moreover, in a recent study [36], we observed all our saliva samples to pass quality controls and behave in the same way as blood samples taken from the same individuals. In addition, results from our Illumina arrays were confirmed by gene-specific bisulphite pyrosequencing, which is also an indication that the presence of bacterial DNA was probably not an issue when using proper collection kits for genomewide analyses [36]. One approach to address this would be to perform a series of experiments where bacterial DNA at varying concentrations was spiked into the reaction and assess changes in genomewide DNA methylation analysis. Alternatively, manufactures of genomewide methylation chips could build in probes tagged to bacterial DNA. Analyses of these specific probes could be used to determine the amount of bacterial DNA contamination and examined for overall differences across the array. Noteworthy, to minimize any bacterial interference, the manufactures of the above-mentioned saliva DNA collection kits add an antibacterial agent to the stabilizing fluid, which prevents the growth of bacteria. For instance, it has been shown that the saliva collected with the Oragene DNA collection kits contain only 11.8% bacterial DNA, while about 5–8 times higher amounts are obtained from mouthwashes (median bacterial DNA content 60%) or buccal swabs (median bacterial DNA content 90%) [42]. In an independent study, Rylander-Rudqvist et al. [33] showed 68% of the total DNA to be of human origin when using the Oragene DNA collection kit. Taken together, the ability to obtain significant amounts of high-quality and high molecular weight DNA from saliva samples, despite some manageable concerns related to bacterial contamination when coupled with the improved efficiency of collection, supports the use of salivary DNA as an alternative to blood DNA in molecular epidemiological studies.

**Salivary DNA Methylation**

A handful of studies have compared DNA methylation patterns in blood and saliva [6–8,36,43]. However, these studies differ in experimental set-up and type of sample (whole blood versus more homogenous isolated cell subpopulations) that was used for the comparison, making generalization difficult at this moment. When looking at genomewide methylation patterns, Smith et al. [6] observed the saliva methylome to be positively correlated with methylation in blood for 88.5% of the CpG sites studied on the Illumina 450K arrays. Thompson and colleagues [7] generated genomewide DNA methylation profiles of whole blood and saliva samples of healthy adults on an Illumina 27K platform, observing 1.8% of the probes to be differentially methylated when applying a Benjamini–Hochberg-adjusted p-value (p adj < 0.001 and DiffScore > 30) as cut-offs. The methylation difference score (DiffScore) for a CpG sites is a parameter that Illumina’s Genome Studio Software provides in their output, which takes into account background noise and sample variability [44]. Langie et al. [36] applied the same cut-offs as Thompson et al. [7] (p adj < 0.001 and DiffScore > 30) to their Illumina 450K Beadchip data and also observed 1.8% of the CpG sites to be differentially methylated when considering the 27K probes. When using cut-offs of p adj < 0.001 and |Δβ| > 0.2, Langie et al. [36] showed 4% of the CpG sites on the Illumina 450K Beadchip to be differentially methylated. Overall, these data indicate that the majority of CpG sites were similarly methylated in blood and saliva.

The studies mentioned above were performed with adult volunteers. As DNA methylation patterns can be dependent on age and lifestyle, blood and saliva methylomes of young adolescents were recently compared. Langie et al. [37] recruited 11-year-old children, among which 20 with doctor-diagnosed respiratory allergy (having Phadiatop IgE ≥ 0.35 kU/L) and 20 healthy controls (no self-reported/diagnosed respiratory allergy, Phadiatop IgE < 0.35 kU/L). DNA was isolated from peripheral blood mononuclear cells (PBMC) (from 10 mL.
et al. [37]. However, based on analysis of global DNA methylation comparable to blood, both in adults [6,7,36] and in adolescents DNA methylation profiles of saliva are more than 90% comparable to each other, but the methylation status of about 96.5% of the CpG sites was highly comparable (72%) hypomethylated. Saliva and blood methylation patterns of the 20 healthy 11-year-old controls, 16,735 CpG sites (3.5%) showed differential methylation in saliva versus PBMC; of which, 4630 (28%) were hyper- and 12,105 (72%) hypomethylated in saliva DNA compared to PBMC. When considering only the methylation profiles of the 20 healthy 11-year-old controls, 16,735 CpG sites (3.5%) showed differential methylation in saliva versus PBMC; of which, 4630 (28%) were hyper- and 12,105 (72%) hypomethylated. Saliva and blood methylation patterns could be distinguished from each other, but the methylation status of about 96.5% of the CpG sites was highly comparable between PBMC and saliva (fig. 1).

A number of independent studies confirm that genomewide DNA methylation profiles of saliva are more than 90% comparable to blood, both in adults [6,7,36] and in adolescents [37]. However, based on analysis of global DNA methylation patterns, Godderis et al. could not completely confirm this. The authors compared global DNA methylation and hydroxymethylation (5hmC) in whole blood and saliva from 14 healthy volunteers [43]. Global DNA methylation was assessed as 5-methyl-cytidine (5mC) by LC–MS/MS and as the percentages of methylation of DNA repetitive elements LINE1 and Alu via bisulphite pyrosequencing. While methylation percentages were significantly lower in saliva samples compared to blood samples (e.g. for 5mC; 4.61 ± 0.80% versus 5.70 ± 0.22%, respectively), levels of 5hmC were significantly higher (0.036 ± 0.011% in saliva versus 0.027 ± 0.004% in blood). Levels of 5mC and 5mC in saliva showed a significant positive correlation, which was not observed for blood. No significant correlations between saliva and blood samples were observed for global methylation levels (either as 5mC, LINE1 or Alu), nor for 5hmC. In contrast, Wu et al. [8] observed salivary methylation levels in the repetitive elements LINE1 and Sat2 to be significantly positively correlated with those in DNA from WBC fraction. Apart from sample size, the main differences between the study from Wu et al. and Godderis et al. are as follows: (i) the sex of the volunteers (all girls versus four males and 10 females, respectively), (ii) the age range (6–15 years versus 22–43 years), (iii) ethnicity (N-Americans with different family backgrounds versus Caucasians, respectively) and (iv) use of DNA from isolated WBC versus whole blood. As mentioned above, DNA methylation is a dynamic process that is easily affected by environmental factors and can differ between people from various age groups, physical fitness (exercise, oxidative stress), opposite sexes or different ethnicity [27,45,46]. In addition, the different observations by Wu et al. and Godderis et al. could be due to the different cell composition of the WBC fraction as compared to whole blood. Genomewide methylation analysis of whole blood versus subfractions has shown clear differences induced by the different cell lineages [47–50]. Moreover, tissue’s cell composition can vary in response to various factors including age, gender genetic variations and health status (reviewed by ref. [12]).

**Accounting for Differences in Cell Composition**

DNA methylation patterns vary between different tissues because specific phenotypic features are controlled by epigenetic marks and tissues are composed of different cell types that also can have different epigenetic features. Blood samples typically contain leucocytes (granulocytes, lymphocytes and monocytes), whereas saliva also contains (dead) exfoliated epithelial cells in addition to leucocytes [6,51]. Such differences in heterogeneity and viability of the cells can result in interindividual variation of the salivary as well as blood DNA methylation profiles and affect the outcome of genomewide analysis. Therefore, researchers need to be cautious when selecting biomarker candidates as they may simply reflect variable proportion of each cell type, when this aspect is not taken into consideration [52,53]. For example, varying cell composition may explain apparent age-associated differences [54] or affect differential methylation associated with inflammatory diseases [50]. Thus, cell type heterogeneity and external or internal factors affecting this heterogeneity may influence genomewide results.

Various methods have been developed to correct epigenomewide methylation data for differences in cell composition (reviewed by ref. [49]). The most widely applied method is the reference-based deconvolution method originally described by Houseman et al. [48]. This method permits the estimation of the proportion of various cell types within a sample based on

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**Fig. 1. The distribution of difference in DNA methylation ($\Delta$B) between peripheral blood mononuclear cells and saliva from 11-year-old children.** The majority of the CpG sites (96.5%) show <20% difference in methylation between blood and saliva.
existing reference data sets, which are available from open-source databases such as the Gene Expression Omnibus (GEO) and the Genomic Data Commons Data Portal (specific for cancer genomic data sets, including The Cancer Genome Atlas (TCGA) data) [55]. For instance, for blood, several studies have analysed the methylation profile of the specific cell types present in whole blood [48–50], which can serve as reference data for correction of genomewide methylation analysis. On the contrary, detailed salivary cell composition and reference methylation profiling has not been performed systematically. Few studies have shown the presence of leucocytes, granulocytes, epithelial cells and bacterial cells in saliva [51,56], but as far as we know, none have quantified the various cell subtypes (e.g. CD8+ T-cell, CD4+ T-cell, B-cell, NK-cell, monocytes, granulocytes, buccal cells), nor studied the methylation profile of the salivary cell subtypes. To apply the Houseman deconvolution method on salivary genomewide DNA methylation data, reference methylomes from leucocyte subtypes (GEO GSE35069; [50]) were recently combined with buccal epithelial cells reference methylomes (GEO GSE46573; [57]) in a select number of studies [6,36]. When applying this, Houseman deconvolution method to the genomewide DNA methylation profiles of both salivary DNA and DNA from the PBMC fraction of 11-year-old children, saliva turned out to be less heterogenic compared to blood (fig. 2). However, there is a caveat when selecting reference data sets. This was observed in our recent methylome study [37] with saliva samples of 46 Flemish children aged 11 years, among which 26 with doctor-diagnosed respiratory allergy (having Phadiatop IgE ≥ 0.35 kU/L) and 20 healthy controls (no self-reported/diagnosed respiratory allergy, Phadiatop IgE < 0.35 kU/L). To apply the reference-based deconvolution method described by Houseman [48], we identified two possible reference data sets for buccal cells: (i) GEO GSE46573 [57], including Illumina 450K methylation data from three replicates of the same buccal epithelial cell sample originating from a single male volunteer, and (ii) GEO GSE48472 [58], including Illumina 450K methylation data from buccal epithelial cells of five volunteers (three females/two males; aged 22–40 years). To estimate the relative proportions of each cell type in our saliva samples, we used either of the buccal reference data sets in combination with reference methylomes from leucocyte subtypes (GEO GSE35069). We observed that the choice of reference data set can significantly affect the estimated proportions of expected salivary cell types (fig. 3). Hence, it is possible that the choice of reference data has a significant impact on downstream data analysis and the discovery of differentially methylated probes. The availability of a comprehensive reference data set could help increase robustness of salivary methylation analysis. However, we are not aware of such a publicly available data set.

In the meantime, a solution for this issue could be the use of reference-free deconvolution methods (reviewed by ref. [49]). This is essentially the unsupervised adjustment of DNA methylation profiles for cell type distribution. Most of these reference-free methods give only limited information of underlying cell types, which is often hard to interpret. Houseman et al. [49] recently published a reference-free deconvolution approach that can partly overcome these shortcomings, providing both proportions of putative cell types based on their underlying methylation as well as a way to evaluate to which extent the underlying profiles reflect specific cell types. To compare the application of the reference-based and reference-free method published by Houseman et al. on salivary DNA methylation profiles, we applied both methods on saliva
samples of 46 Flemish children aged 11 years. The reference-free method estimated an optimal of two underlying cell types in saliva, which is in accordance with the reference-based method (using GEO GSE35069 as leucocyte subtypes (GEO GSE35069) in combination with either of the following two possible reference data sets for buccal cells: (A) GEO GSE46573, including Illumina 450K methylation data from three replicates of buccal epithelial cells from a single male volunteer [57], and (B) GEO GSE48472, including Illumina 450K methylation data from buccal epithelial cells of five volunteers [58].

Although it is still not possible to retrieve the exact biologically relevant cell types, with this new approach or any other unsupervised method, the reference-free approach can be valuable for biofluids and tissues that lack proper reference data. However, when data sets of specific tissue methylomes are available, we advise to adhere to the reference-based correction method. As such, we propose to determine cell populations in saliva aliquots and perform epigenomic profiling of specific salivary cell subpopulations to allow for better data correction and identification of robust epigenetic biomarkers.
Biomarkers of Disease

Ideally, epigenetic modifications should be studied in target tissue. Unfortunately, for most diseases, it is impossible to obtain target tissue in humans and most studies into epigenetic biomarkers for complex diseases have used blood samples. Although Wren et al. [9] indicated that blood and saliva molecular profiles overlap, in the end the utilization of one source over another should depend on careful consideration of target outcome, analytes and practical aspects of sample collection and processing. To date, no study has definitely confirmed that for all applications one source of DNA is superior over another.

In the field of psychiatric disorders, Smith et al. [6] compared saliva and blood methylomes with methylation patterns in different brain tissues. They observed the salivary methylome to be more similar to methylation patterns in each of the brain regions than methylation in blood and suggested that DNA methylation profiling using saliva may offer distinct opportunities for epidemiological and longitudinal studies of psychiatric traits. This may be due to the finding that a significant number of cells in saliva are of buccal epithelial origin, a tissue that is derived from the ectoderm – the same embryonic tissue from which the central nervous system develops. In agreement, correspondence between brain and saliva methylation profiles has been observed in young, healthy adult individuals as well as in clinical populations (reviewed by ref. [9]). Early adversity, characterized by chronic violence or neglect, has been reported to influence the methylation state of important neuropsychiatric gene targets. Using saliva samples from children (5–14 years), 2868 CpG sites were found to be differentially methylated and three differentially methylated stress/neurodevelopment-related genes were identified as significant predictors of depression (reviewed by ref. [9]). Illumina 450 K Human Methylation analysis of salivary DNA from children (7–12 years) diagnosed with ADHD revealed altered DNA methylation in VIPR2 [59].

Recently, Langie et al. [36] used a case–control design to analyse and compare DNA methylation patterns in blood and saliva in individuals with respiratory allergies. When comparing respiratory allergy cases with healthy controls, 485 and 437 differentially methylated sites were identified in saliva and PBMC, respectively, of which 216 were in common and showed the same polarity in blood and saliva. Pyrosequencing analysis of three selected cg-sites confirmed the array data. In contrast, Godderis et al. [43] observed individuals with allergy to have slightly but significantly higher ($p = 0.042$) levels of hydroxymethylation ($0.029 \pm 0.002\%$, $0.027-0.032\%$) in DNA from blood compared to non-allergic participants ($0.025 \pm 0.004\%$, $0.021-0.031\%$), which was not observed in saliva.

Furthermore, salivary DNA has been used to identify differentially methylated genes in relation to systemic conditions, such as diabetes and a facioscapulohumeral muscular dystrophy [9]. In addition, salivary analysis has been shown to be a useful diagnostic tool in the field of cancer research [10,60,61]. Viet and Schmidt [62] used Illumina GoldenGate Methylation Arrays to study 807 cancer-associated genes in the saliva of patients with oral cancer and reported that methylation array analysis of saliva can produce a set of cancer-related genes that can be used as a composite biomarker for the early detection of oral cancer. Moreover, gene promoter methylation analysis of a test panel in salivary DNA was able to detect the early stages of head and neck squamous cell carcinoma [63,64]. Salivary DNA methylation analyses have also been applied for distant malignancies such as breast cancer [60,61]. Several salivary DNA methylation markers have been identified in breast cancer-related genes and were associated with risk factors for breast cancer development [65–67]. Overall, these data indicate that saliva is a useful source of DNA for detecting differential methylation marks in a non-invasive manner in vulnerable groups.

Conclusion

We conclude that certain DNA methylation marks are comparable between blood and saliva, both at a gene-specific as well as at a global(hydroxyl)methylation level. Not surprisingly, there are also marked differences between both biofluids. Much attention has been focused on blood as a surrogate tissue, and this MiniReview highlights the potential of saliva as surrogate tissue for epigenetic biomarker screening.

Saliva offers key advantages for DNA methylation studies, particularly studies that enrol vulnerable populations or seek to collect repeated samples. Saliva is easy to collect, and there are few constraints to storage and processing. Several studies indicate that high-quality methylation profiles can be generated from saliva. Furthermore, differentially methylated CpG sites/gene regions were identified in salivary DNA by several independent studies, and they are promising candidate biomarkers. The vast increasing development of bioinformatics tools (including cell composition correction methods) has boosted epigenome-wide data analysis. However, there is still a need for extensive profiling of the salivary cell composition and proper reference data sets for each of the specific salivary cell subpopulations. Table 1 gives an overview of the advantages of the use of saliva for DNA methylation studies, as well as the areas that require further research. The use of different

| Advantages of saliva | Areas of further research |
|---------------------|--------------------------|
| Non-invasive collection | Evaluate the effect of the target outcome, analytes and practical aspects of sample collection and processing. To date, no study has definitely confirmed that for all applications one source of DNA is superior over another. |
| Decentralized and multiple collections | Control probes on arrays to assess bacterial DNA contamination |
| Stable storage at room temperature | Full characterization of salivary cell composition |
| High yield and quality of high molecular weight DNA | Profiling of the methylome of salivary cell subtypes, to be used as reference data |
| Less heterogeneous compared to blood | Can capture acute and chronic effects of exposures and stressors |
| Useable biomarker discovery |

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non-invasive biospecimens, and saliva specifically, in epigenomics studies will provide new levels of insight in the molecular mechanisms through which environmental factors and interventions can alter an individual’s risk of complex diseases.

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