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Saliva, a molecular reflection of the human body? Implications for diagnosis and treatment

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ABSTRACT **For many diseases, and cancer in particular, early diagnosis allows a wider range of therapies and a better disease management. This has led to improvements in diagnostic procedures, most often based on tissue biopsies or blood samples. Other biological fluids have been used to diagnose disease, and among them saliva offers a number of advantages because it can be collected non-invasively from large populations at relatively low cost. To what extent might saliva content reveal the presence of a tumour located at a distance from the oral cavity and the molecular information obtained from saliva be used to establish a diagnosis are current questions. This review focuses primarily on the content of saliva and shows how it potentially offers a source of diagnosis, possibly at an early stage, for pathologies such as cancers or endometriosis.**

doi: 10.15698/cst2024.05.297
Received originally: 14.03.2024;
in revised form: 02.05.2024,
Accepted: 03.05.2024
Published: 27.05.2024

Keywords: saliva, miRNA, diagnosis, cancer, endometriosis

Abbreviations:
circRNA - circular RNA,
EV - extracellular vesicle,
gDNA - genomic DNA,
lncRNA - long ncRNA,
mRNA - messenger RNA,
miRNA - micro RNA,
ncRNA - non-coding RNA,
piRNA - protein-interacting RNA.

INTRODUCTION

The possibility of using biological fluids such as blood, urine, saliva and other biofluids to diagnose pathologies is a relatively old idea that has taken on a new lease of life in recent years. Thanks to a combination of technological advances, the first major progress was observed in genomics, proteomics and bioinformatics techniques, now including artificial intelligence, making it possible to establish observed differences between analysed samples with greater sensitivity and a higher degree of confidence. The second is the standardization of sample collection, processing, and storage, minimizing variations linked to sample collection and analysis, as well as to inter-individual variability. Finally, improved knowledge, particularly in molecular and cellular biology, makes it possible to give biological meaning to variations between samples. This is particularly decisive for the analysis of a particular class of RNAs, known as non-coding RNAs (ncRNAs). In recent years, understanding of the role and regulation of ncRNAs has progressed exponentially, particularly in terms of how ncRNAs respond to genetic and epigenetic perturbations, especially in pathological situations [1]. A crucial element in the use of biological fluids for the discovery of disease-associated biomarkers is the characterization and understanding of a mode of intercellular communication based on the exchange of vesicles between vesicle-secreting cells, called extracellular vesicles (EVs), and receptor cells within a target tissue [2, 3]. These EVs can act close to their site of secretion, promoting signaling in neighboring cells, or circulate in the blood or lymph to affect distant tissues. Their effect at distance depends on the concentration of the EVs and their content. A growing number of examples show that EVs found in biological fluids, and particularly in saliva, can reflect acute or chronic pathology located outside a patient's oral cavity [4]. In this review, we will describe the potential of saliva as a biological fluid, not only for the diagnosis of various diseases, but also for the establishment of possible therapeutic solutions.

EXTRACELLULAR VESICLES AND THEIR CONTENTS

As mentioned above, some of the differences in saliva content between healthy people and patients with a given pathology could be due to different concentrations of EVs, themselves of a different nature in patients. EVs differ in size, physical properties, mode of biosynthesis and content, which depends on the secreting cell [5]. EVs are generated from invaginations of the cell membrane which, following various maturation processes, form multivesicular bodies containing intraluminal vesicles [6]. These in turn can be degraded or secreted into the extracellular space notably as exosomes [7]. The orientation of transmembrane proteins on the surface of exosomes is identical to that observed in the cell membrane, albeit with different steric constraints due to the greater radius of curvature in exosomes. Other, larger types of EVs can also form from

buds on the cell surface, or from cells undergoing apoptosis, generating so-called apoptotic bodies [6]. Over the past decade, considerable progress has been made in clarifying the content of the molecules transported by EVs, known as "cargoes", and the role of EVs. Cargoes include proteins such as membrane receptors, key signalling proteins, major histocompatibility complexes, intracellular trafficking regulators and various RNA-binding proteins [8]. Remarkably, many nucleic acids, particularly ncRNAs, are found within EVs, as are amino acids and metabolites. It is now accepted that the biogenesis mechanism of EVs can serve to actively capture and concentrate cargoes through the action of effectors [6, 9]. Conversely, other cargoes can also be passively encapsulated within EVs. The secretion of EVs constitutes a genuine mechanism for the exchange of molecules and the transmission of signals between a secretory cell and the receptor cell, through paracrine effects [10]. As mentioned above, the content of EVs depends on the cell type secreting them, and this content can be disrupted in stressful or pathological situations such as cancer [11, 12]. Numerous studies have shown that in pathological contexts, notably cancers, the rate of secretion of EVs by tumour cells is higher than in normal cells [13]. EVs in biological fluids may therefore reflect cell or tissue dysfunction.

To what extent EVs are found in different biological fluids and how their distribution in these fluids provides specific answers to their use as a tool for diagnosis are open questions.

SALIVA, A BIOLOGICAL FLUID FOR BIOMARKER DETECTION

Saliva is a biological fluid secreted by various salivary glands, which once poured into the oral cavity, is mixed with secretions from the epithelial cells of the oral wall. It also contains cellular infiltrates and many micro-organisms, including bacteria and viruses [14, 15]. It is an abundant saliva secretion, estimated to a liter per day for a healthy person [16]. Saliva, made up of 98% water, contains a variety of proteins (mucins, lactoferrin, histatins, various proteases, immunoglobulins) involved in protecting the oral cavity and in the initial stages of digestion [17], as well as growth factors, hormones, cytokines and metabolites [18]. Proteomic studies have shown that most proteins are synthesized in the salivary glands [19], although some are transported from the blood or lymph into the saliva [20]. Saliva also contains nucleic acids: nuclear DNA [21] and, above all, many different classes of ncRNA [22]. More than a decade ago, the question was whether saliva vesicles and their contents originated solely from the salivary glands, possibly revealing oral pathologies, or whether they could be derived from cells belonging, for example, to a tumour (or their microenvironment) located at a distance from the oral cavity. Numerous studies have now shown that small ncRNAs found in the saliva of cancer patients are associated with tumour-derived cancer cells [23]. The question then arose as to how EVs originating from tumour cells or associated with tumours find their way into the patient's saliva. The prevailing idea is that EVs secreted by tumour or tumour-associated cells reach the salivary glands via the general circulation, or via the lymph. EVs are then taken up by the acinar cells of the salivary glands by membrane fusion or by an invagination mechanism (endocytosis). The salivary glands then secrete mixed EVs into the saliva, originating from both acinar cells and EVs derived from the tumour located at a distance from the salivary glands [23]. This model explains why ncRNAs detected in saliva, particularly in pathological situations, will have a different profile to those found in other biological fluids, notably blood [24].

For certain pathologies, saliva can therefore serve as a biomarker of disease. The higher the number of samples, the more robust the biomarker. The fact that saliva sampling is easy, non-invasive, easy to handle, inexpensive and can be used on a large scale of patients [25] opens new prospects not only for the discovery of biomarkers for the diagnosis of pathologies, but also for the understanding of the pathologies studied.

DNA IN THE SALIVA

Saliva contains many different types of nucleic acid. Much of the DNA extracted from saliva comes from bacteria, but mainly from leukocytes and epithelial cells in the oral cavity [26]. The proportion of cellular DNA will depend on how the saliva is collected, influencing the collection of cells in the saliva sample. Sequenced genomic DNA (gDNA) will provide information on the patient's genetic identity but cannot be used to determine somatic alterations in the cells involved in the disease. In this sense, gDNA purified from saliva can be substituted for gDNA purified from blood leukocytes to perform whole genome sequencing [27] or large-scale analysis of gDNA methylation profiles [28]. Average telomere length can even be measured by PCR from saliva samples [29]. Nevertheless, in diagnostic terms, gDNA from oral cavity cells will not be informative, except for familial cancers [30], and cancers forming in the upper aerodigestive tract for which saliva contains cancer cells [31].

In cancer patients, saliva is likely to contain circular DNA consisting of short DNA sequences wrapped around one or more nucleosomes [32]. Nevertheless, a significant fraction of saliva's extracellular DNA is protected from nuclease action, suggesting that it is contained within secreted EVs, either by cells of the oral cavity or, in the case of cancers, by more distal tumour cells [33]. The origin of extracellular DNA and gDNA-containing EVs is not fully elucidated. It could come from apoptotic bodies generated by cells undergoing apoptosis [6]. Studies have shown that nuclear DNA is not found in small, highly purified EVs (exosomes) [8]. However, in ovarian cancer patients, it has been shown that 10% of exosomes contain genomic DNA. Its presence in EVs is thought to be linked to micronuclei that characterize the genetic instability of cancer cells [34]. Other mechanisms linked to the genetic instability of cancer cells may also explain the leakage of gDNA into the cytoplasm [35]. These results are in line with various studies which have shown that mutations (KRAS, TP53, EGFR, and PIK3CA) associated with different types of cancer (pancreas, breast, lung) could be detected from EVs purified from patient blood, notably thanks to the use of digital droplet PCR enabling a region of DNA to be amplified with high sensitivity [36–39]. In saliva, extracellular DNA is present at lower concentrations than in blood [40]. A better understanding of the origin of the extracellular DNA present in saliva is needed to consider the enrichment of EVs containing tumour DNA, and to evaluate the potential of saliva for the detection of cancer-associated mutations.

There are also stress or pathological conditions that cause mitochondrial DNA [41] to leak into the cytoplasm, inducing

inflammatory-type responses initiated by the recognition of DNA found in the cytoplasm by specific sensors [42]. Under these conditions, cytoplasmic mitochondrial DNA can be incorporated into EVs, contributing to the propagation of the inflammatory response [43]. It has been shown that mitochondrial DNA amplicons can be directly amplified and quantified by qRT-PCR from saliva independently of the cellular content of the saliva [44]. In head and neck cancers, the normalized amount of extracellular mitochondrial DNA present in saliva correlated with poorer patient survival indicating that the level of extracellular mitochondrial DNA in saliva can be used as a robust predictor of overall patient survival [45]. It will be of interest to study whether quantification of mitochondrial DNA, and possibly mitochondrial gene sequence, established from saliva constitutes a novel biomarker for cancers [46].

miRNAs IN SALIVA

As aforementioned, saliva can reflect systemic processes, mainly through the EVs contained in it. Like other EVs, salivary EVs contain a variable proportion of ncRNAs corresponding to RNA molecules that are not translated into proteins. ncRNAs include small RNAs of around 22 nucleotides (nt) called microRNAs (miRNAs), long non-coding RNAs (lncRNAs) whose size exceeds 200 nt, and a particular class of Piwi protein-interacting RNAs (piRNAs) notably involved in the repression of transposable elements [4, 33]. These ncRNAs are accompanied by single-stranded circular RNAs (circRNAs) produced by mRNA by back-splicing of the upstream 5′ splice donor site with the upstream 3′splice acceptor [47]. CircRNAs, whose abundance varies with tissue and age, are particularly stable and have been detected in plasma and saliva [47]. They act as miRNA sponges, and as such represent a new source of biomarkers, notably in many cancers [48].

miRNAs were first identified in the *C. elegans* worm [49, 50] and then in vertebrates [51]. Numerous studies have since clarified the mode of biosynthesis and mechanism of action of miRNAs [52]. They are synthesized as precursors by RNA polymerase II and undergo an initial maturation step in the nucleus, generating miRNAs of around 70 nt in size, which are then exported to the cytoplasm [52]. In the cytoplasm, these stem-loop miRNAs undergo the action of the DICER endoribonuclease, producing double-stranded RNA duplexes of around 22 nucleotides. One strand of the miRNA duplex (the other strand is degraded) forms a complex with the Argonaute protein (AGO), called RISC, whose action will switch off the expression of genes with sequences complementary to miRNA [53], either by destabilizing the RNA targeted by the miRNA, or by inhibiting its translation, depending on the degree of homology of the miRNA with its target RNA [54, 55].

It has been established that most mammalian messenger RNAs (mRNAs) can be targets for miRNAs [56]. Over 2,600 different miRNAs have been identified, of which more than 1000 are annotated in databases [57]. However, the number of protein-coding genes whose expression is effectively regulated by miRNAs remains to be determined. It is estimated that 60% of coding genes are regulated by miRNAs.

Although miRNAs found in biofluids are not necessarily EV-associated, it is now recognized that miRNAs secreted into EVs are key players in EV-mediated intercellular communication [58]. It should be noted that the population of miRNAs released into EVs is distinct from the cellular population by the presence of export sequences (Exomotifs) favoring their concentration in EVs [59]. miRNAs can also be secreted into biological fluids by binding to RNA-affine proteins such as Ago2 and lipoproteins [60]. Extracellular miRNAs have been found in all biological fluids, including saliva [33]. miRNAs are highly stable in biological fluids, both because of their structure and because they are released from EVs [61]. A large number of studies have exploited the use of blood miRNAs as biomarkers of disease [62], but fewer have explored the utility of salivary miRNAs. Nevertheless, recent studies indicate that biomarkers based on salivary EVs could predict several pathologies other than those of the oral cavity [63].

SALIVARY miRNAs: BIOMARKERS OF CANCER

Although saliva has advantages over blood as a biological fluid for diagnosis, variations in its potential contamination and composition have been obstacles to its use. This limitation can be partially overcome by the standardization of samples and by the purification of EVs, which constitutes a step in the purification and enrichment of ncRNA contained in saliva [47]. In addition to head and neck cancers, miRNAs from salivary EVs have recently been shown to be biomarkers for oesophageal cancers [64], colorectal cancers [65, 66], lung carcinomas [67, 68], hepatocarcinomas [69] and as potential biomarkers for pancreatic cancer [70, 71]. Salivary miRNAs are also thought to be biomarkers for ovarian cancer, for which several miRNAs purified from patient blood have been associated with the proliferation and migration of epithelial cancer cells [72–74].

Various protocols have now been developed to purify EVs from saliva, including exosomes, opening new horizons for the use of saliva in diagnosis. In particular, salivary EVs can be concentrated by immuno-purification using antibodies directed against four-domain transmembrane proteins called tetraspanins (CD9, CD63, CD81) found on the surface of most exosomes [7]. However, there are now several evidences in the EV-field that there may be a significant population of EVs that are negative for those markers [75]. Mass spectrometry studies have also shown that exosomes derived from pancreatic tumours may contain CD151, another tetraspanin [76], making it possible to enrich specific exosomes associated with a particular type of cancer from saliva. Similarly, ovarian cancers overexpress a family of proteins called syndecans [77, 78] consisting of an extracellular domain, a transmembrane domain and a cytoplasmic domain which communicate with tetraspanins to regulate vesicular trafficking and the activity of associated receptors [79]. It is conceivable that syndecans overexpressed in ovarian cancer could escape proteolytic cleavage of their extracellular domain, enabling the purification of specific exosomes with appropriate antibodies to facilitate diagnosis.

COMPLEXITY OF miRNA EXPRESSION REGULATION

While the mechanism of miRNA-mediated repression of gene expression has been extensively studied, the mechanisms leading to changes in miRNA expression, particularly in cancer cells, are much less well understood [80]. miRNAs can be classified into intragenic and intergenic miRNAs. Most of the miRNAs deregulated in cancer (onco-miRNAs) are integrated into the introns of host genes. Using multidimensional omics

databases to simultaneously study the expression of intragenic miRNAs and their host genes, it has been shown that, in several types of cancer, the expression of onco-miRNAs is often correlated with that of related host genes [81]. It is reasonable to assume that this co-regulation may be linked to epigenetic modifications of either the host gene promoter or the microRNA promoter [82]. There are numerous examples showing that, in cancer, the expression of onco-miRNAs can be derepressed via hypomethylation of the CpG islands of their promoter, while conversely, the expression of miRNAs with a tumour suppressor role is inhibited via hypermethylation of the CpG islands [83]. Nevertheless, analysis of the links between onco-miRNAs and host genes is still far from complete. Nevertheless, the analysis of the links between DNA methylation and miRNA expression remains complicated because miRNAs, themselves regulated by epigenetic modifications, control the expression of genes encoding key DNA methylation factors, thus creating feedback loops [83]. Moreover, it has recently been shown that methylation of host gene DNA regions bordering the miRNA facilitates ribonuclease-mediated maturation of pre-miRNAs [84]. This highlights the complexity of miRNA expression regulation, which depends on transcriptional, epigenetic and post-transcriptional mechanisms involved in miRNA maturation and stability. This underscores the difficulty of establishing causal links between miRNA expression variations. Nevertheless, the co-regulation of several miRNAs may be indicative of an epigenetic modification with a causal link to a given cancer.

miRNAs: FROM MECHANISMS TO THERAPEUTIC SOLUTIONS

Beyond the diagnostic use of the signatures constituted by the expression profile of salivary miRNAs, the aberrant expression of miRNAs associated with cancers can be used to glimpse therapeutic solutions [85]. As the functions of miRNAs are essentially defined by the identity of their targets, the action of miRNAs must be considered in the context of gene regulatory networks [86]. In some cases, variation in the expression of one or more miRNAs promotes tumour transformation by influencing the expression of a key mediator of a signalling pathway [87] or a tumour suppressor [88]. In other cases, a single miRNA may promote tumour development by altering anti-tumour immunity [85, 89]or by stimulating angiogenesis [90]. Numerous strategies based on the inhibition of miRNA expression, or conversely on their restoration, have been envisioned [91]. Recent progress, linked to the development of RNA vaccines, suggests that the introduction of miRNAs could be exploited therapeutically [92]. In cases where cancer is accompanied by the extinction of expression of a key miRNA, it is conceivable to introduce, using lipid nanoparticles, synthetic double-stranded miRNAs (mimicking the miRNA) capable of forming a complex with RISC and targeting the mRNA encoding the overexpressed oncogene. Very recently, a new approach called RiboStrike was developed to identify molecules capable of inhibiting the activity of miRNA-21, which plays a key role in breast cancer development [93]. This screen, based on different datasets and using cell-based screens and artificial intelligence, identified from millions of molecules three compounds with miRNA-21 inhibitory activity whose efficacy was validated in mouse models of breast cancer [93]. These results demonstrate the power of new therapeutic approaches targeting miRNA activity.

lncRNAs AND piRNAs IN SALIVA: NOVEL BIOMARKERS?

Other non-coding RNAs, apart from miRNAs, have been identified in saliva, broadening the potential spectrum of biomarkers [47]. Among these, piRNAs, which range in size from 26 to 31 nucleotides [94], associate after maturation with Piwi proteins to form piRISC complexes involved in target gene silencing through transcriptional and post-transcriptional mechanisms [95]. piRNAs are predominantly expressed in germ lines to protect their genome against mobilization of transposable elements by repressing transposon-encoded proteins [96]. Their expression is controlled by genetic and epigenetic mechanisms at specific loci, which restrict their expression mainly to germ lines [97]. Nevertheless, piRNAs are also expressed in somatic cells, particularly in tumours of different cancer types, in which their expression is correlated with aberrant DNA methylation profiles [98]. As such, aberrant expression of piRNAs in cancers can also serve as a biomarker for diagnosis, and potentially some piRNAs could be targeted for therapeutic purposes [99, 100]. The most abundant piRNAs are found in all biological fluids and circulate mainly in EVs [47]. The concentration of specific piRNAs is particularly high in gastric, colorectal, renal, and prostate cancers [101]. Each fluid has a specific piRNA signature indicating that cohorts can potentially be identified based on several classes of small ncRNAs.

LncRNAs are another class of RNAs whose expression is deregulated in cancers [1], yet their concentration is lower in saliva. They are transcribed, modified, and matured in the cell using the canonical mechanisms of mRNA maturation [102]. They act on gene transcription in multiple ways, notably by regulating the recruitment of factors associated with transcription, or by directly or indirectly modifying the state of chromatin or the architecture of the nucleus [103]. They also act at the post-transcriptional level through multiple mechanisms, including regulating alternative splicing of mRNAs [104], serving as a platform to create molecular scaffolds [105] or acting as sponges to titrate miRNAs [106]. They can also take part in phase separation processes to concentrate macromolecular machineries at their site of action [107].

Certain lncRNAs, such as HOTAIR, MALAT1, NEAT1, H19 and XIST1 [101] are found deregulated in multiple cancers and are prognostic markers [108–115]. The H19 lncRNA, located on the H19 locus close to the IGF2 (Insulin-like growth factor 2) gene, is particularly interesting since this locus is subject to imprinting: the H19 lncRNA is expressed by the maternal allele, while IGF2 is transcribed by the paternal allele [116]. Expression of the two genes is closely coordinated and controlled by methylation of an intergenic region located between the two genes [117]. H19 lncRNA has been shown to be deregulated in gynecological cancers and also in endometriosis, suggesting a common etiology between these cancers and endometriosis. The presence of lncRNAs in saliva has not been explored in detail as in the case of miRNAs, notably because of their lower abundance and stability. In saliva, HOTAIR is found overexpressed in pancreatic cancers [118], while MALAT1 is a salivary biomarker of oral epithelial lining cancer [119]. Further studies are needed to determine whether lncRNAs in saliva can

be biomarkers of cancer or other pathologies.

USE OF SALIVA IN THE DIAGNOSIS OF ENDOMETRIOSIS

Endometriosis is a systemic, heterogeneous disease characterized by the presence of endometrial like tissue outside the uterus, causing intense pelvic pain and a high risk of infertility [120]. Although this disease affects 5-10% of women of reproductive age, it is not diagnosed early, complicating the implementation of treatments, which are themselves limited. Endometriosis is classified into three subtypes: superficial peritoneal endometriosis, deep infiltrating endometriosis and endometrial cyst of the ovary [121]. It causes systemic inflammation with consequences extending beyond the areas infiltrated by transformed endometrial cells [122]. Various models have been proposed to explain the ectopic localization of endometrial cells, which must acquire new properties to migrate and establish themselves in a new environment [123]. Endometriosis was initially associated with retrograde menstruation [124], but this condition is clearly not sufficient [122, 123]. It is generally proposed that during retrograde menstruation, endometrial stem cells, along with other cells, are deposited on the peritoneum, ovaries and fallopian tubes [125]. Genetic or epigenetic alterations in these stem cells [126, 127], combined with hormonal stimulation and environmental cues, contribute to their survival, proliferation, altered differentiation and adhesion to their new environment [128]. Cells establish new interactions enabling the growth of endometriosis lesions [128]. The proliferation of precursor cells and their differentiation in endometriosis depots is favored by an imbalance in hormonal regulation associated with elevated estrogen expression [120–122] and various mechanisms affecting the action of progesterone, which normally inhibits estrogen-dependent proliferation of endometrial cells, induces endometrial decidualization and acts against inflammation [129]. These mechanisms may be genetic or epigenetic [130, 131]. Several environmental signals may contribute to the physiopathology of endometriosis. For example, periodic and repeated bleeding drains erythrocytes whose macrophage-mediated lysis is likely to release heme and iron from hemoglobin degradation into the peritoneal environment [132, 133] thereby inducing the formation of reactive oxygen species leading to oxidative damage of macromolecules contributing to chronic inflammation [134]. Notably, local excess of iron can induce ferroptosis, a form of cell death associated with lipid peroxidation [135]. Like cancer cells, it is suggested that stromal cells in endometriosis lesions have increased resistance to ferroptosis [136, 137].

All this reveals the complexity of the mechanisms behind endometriosis lesions. Very recently, single-cell experiments allowing to assess cell types in normal endometrium [138, 139] and endometriosis lesions [140, 141] have shed new light on this pathology. These studies provide an insight into the specific microenvironment associated with endometrial lesions, consisting of epithelial and stromal cells, infiltrates of immune cells, fibroblasts, vascular and mesothelial cells, illustrating the fact that endometrial lesions are often fibrotic with increased angiogenic capacity [140, 141]. They also reveal variations in gene expression between epithelial and stromal cells in endometriosis lesions and those of the normal endometrium [140, 141]. Interestingly, stromal cells in endometriosis lesions are more responsive to estrogen than stromal cells in normal endometrium, whereas this difference has not been observed in epithelial cells [141]. Moreover, for each cell type within the lesions, specific subtypes can be identified, with proportions varying between normal endometrium and endometriosis lesions [140, 141]. For example, the relative abundance of monocyte and macrophage subpopulations and natural killer (NK) subtypes differs between normal endometrium and endometrial lesions, which may explain the tolerance of endometriosis lesions to the action of the immune system and the inflammatory responses associated with endometriosis [140, 141]. Similarly, ectopic endometrial deposits show distinct subpopulations of venous endothelial cells and myofibroblasts [140].

It is quite clear that in many respects, endometriosis lesions have characteristics like those of tumours, and as such present biomarkers detectable in biological fluids. In normal endometrial function, stromal cells secrete EVs that contain multiple cargos including miRNA that mediate intercellular communication within the endometrium and condition physiological changes in the uterine environment [142]. Not surprisingly, EVs, including exosomes, have been purified from plasma and peritoneal fluid in endometriosis patients and healthy controls that reveal unique EV/miRNA signatures in endometriosis patients [143]. Variations in miRNA expression have since been observed in the endometrial tissues and various biological fluids of women with endometriosis [144]. Several reviews summarize the function of miRNAS that are differentially regulated in samples from patients with endometriosis [145, 146]. Although the biological role of certain miRNAs in endometriosis can be addressed [147], the question arises as to their use in diagnosis and as therapeutic targets.

A multicentric study with intermediate validation involving a cohort of 200 patients with confirmed endometriosis was conducted in the form of a collaboration between several endometriosis reference centers in France and the Core Facility (iGenSeq) of the Brain and Spinal Cord Institute (ICM, Paris) [148]. Using deep-sequencing and machine-based learning algorithm [149] from saliva samples, this study identified a panel of 109 miRNAs constituting a signature with a positive predictive value of 95.1% and a negative predictive value of 86.7% [146]. This study has led to the commercialization of a saliva test whose large-scale exploitation could constitute a decisive advance in the diagnosis of endometriosis(https://ziwig.com/endometriose/). Several concerns have been expressed regarding the large-scale use of this test for diagnostic purposes, for example, the consequences of a positive test in an asymptomatic patient, or conversely, a negative test in a symptomatic patient presenting a highly suggestive clinical picture [150]. Clearly, the test is not intended for asymptomatic patients, and has a low false-negative rate compared with other diagnostic methods [151]. Another interesting question concerns the acceleration of referral of patients with a positive test to specialized services [150]. The answer to this question is that a positive test could immediately authorize second-line treatment by primary care physicians (midwives, general physicians, and gynecologists), thereby reducing the need for referral to an expert center and holistic patient management.

Another issue is the applicability of the test outside France [150]. Rather, the miRNA signature should reflect the consequence of endometriosis depots, irrespective of polymorphisms linked to different ethnicities. Nevertheless, the question is legitimate and deserves attention.

CONCLUSIONS

Saliva is a liquid biopsy with many diagnostic potentialities and advantages. It is easy to collect and store, non-invasive, and contains a large number of molecules, including many miRNAs that have been validated as biomarkers in numerous pathologies. Nevertheless, beyond endometriosis, salivary diagnostics is still in its infancy, and many challenges remain to be overcome, notably to increase the concentration and specificity of biomarker molecules and the sensitivity of molecule detection. Moreover, there is a need to integrate and cross-reference saliva data from patients with different cancers on a large scale, combine several salivary biomarkers, and reduce the heterogeneity associated with the study of the same disease, which sometimes leads to disparate results for the same type of cancer from saliva samples. To increase specificity, the researchers set out to analyze the biomarkers contained in saliva EVs. A growing body of evidence indicates that EVs associated with cancer cells have specific characteristics, and that these EVs are found in saliva. Clinically applicable purification of EVs may represent a decisive step forward in the use of saliva for diagnosis. Finally, beyond diagnosis, the greatest challenge remains to transform omics data from saliva into biological information to understand the mechanisms of pathological situations and identify targets for therapeutic purposes.

ACKNOWLEDGEMENTS

We would like to thank Professor Emile Darai for his critical reading of the manuscript and his advice.

CONFLICT OF INTEREST

From December 2023 to April 2024, V.G. was employed by the Ziwig Biotech company.

Please cite this article as: Vincent Géli, Norbert Nabey (**2024**). **Saliva, a molecular reflection of the human body? Implications for diagnosis and treatment**. **Cell Stress** 8: 59-68. doi: 10.15698/cst2024.05.297

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