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Beyond liquid biopsy: Toward non-invasive assays for distanced cancer diagnostics in pandemics

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ABSTRACT

Liquid biopsy technologies have seen a significant improvement in the last decade, offering the possibility of reliable analysis and diagnosis from several biological fluids. The use of these technologies can overcome the limits of standard clinical methods, related to invasiveness and poor patient compliance. Along with this there are now mature examples of lab-on-chips (LOC) which are available and could be an emerging and breakthrough technology for the present and near-future clinical demands that provide sample treatment, reagent addition and analysis in a *sample-in/answer-out* approach. The possibility of combining non-invasive liquid biopsy and LOC technologies could greatly assist in the current need for minimizing exposure and transmission risks. The recent and ongoing pandemic outbreak of SARS-CoV-2, indeed, has heavily influenced all aspects of life worldwide. Ordinary tasks have been forced to switch from “in presence” to “distanced”, limiting the possibilities for a large number of activities in all fields of life outside of the home. Unfortunately, one of the settings in which physical distancing has assumed noteworthy consequences is the screening, diagnosis and follow-up of diseases.

In this review, we analyse biological fluids that are easily collected without the intervention of specialized personnel and the possibility that they may be used -or not-for innovative diagnostic assays. We consider their advantages and limitations, mainly due to stability and storage and their integration into Point-of-Care diagnostics, demonstrating that technologies in some cases are mature enough to meet current clinical needs.

1. Introduction

Since December 2019, the COVID-19 pandemic has affected all aspects of our lives worldwide. This unprecedented crisis, for which no healthcare system was fully prepared, caused a tremendous strain on health services, affecting directly and indirectly the course and treatment of many common illnesses (https://www.uptodate.com/contents/coronavirus-disease-2019-covid-19-cancer-screening-diagnosis-treatment-and-posttreatment-surveillance-in-uninfected-patients-during-the-pandemic). All pathologies, of course, require the same care and consideration, and many diseases have been neglected during Sar-CoV-2 pandemic (El Moussaoui et al., 2021; Wu et al., 2021), but what caught our attention and motivated our review’s focus was the dramatic decrease in newly diagnosed cancers from the beginning of emergency status (Campbell et al., 2021; Jacob et al., 2021; Jacome et al., 2021; Jazieh et al., 2020). Oncology patients, indeed, have been particularly impacted, since they represent a highly vulnerable group, for their immunocompromised status caused by both cancer and anticancer treatments. Delivering cancer care during COVID-19 era is challenging, given the competing risks of death from neglecting cancer versus serious complications from infection, and the likely higher lethality of COVID-19 in weaker hosts (Tsamakis et al., 2020). Other actions include cancellations of visits, delays in routine screening with a decline in the number of newly identified cancers during the pandemic, surgery postponements or cancellations, physical distancing in medical offices and infusion rooms. Even just the delay gathered in one year of

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missed screening, follow-ups and treatments, calls for new compelling tools able to perform diagnostic assays without recurring face-to-face meetings with clinicians. In this scenario liquid biopsy, above all using non-blood fluids such as saliva or urine, represents a method still little explored but potentially very useful. Moreover, their collection does not require any contact with medical personnel, limiting interactions and contamination.

The concept of *distanced diagnostics* is still a challenging area which has been addressed over many years to find attractive solutions, but times and technologies are sufficiently mature to provide instruments to fight a battle which realistically will last for a long time. So, the occasion is propitious to drive a productive push toward the finalization of *lab on a chip* tools which are ready to go through the market but fall into the death valley of *"chip in a lab"* (Hochstetter, 2020; Primiceri et al., 2018). Therefore, in this paper we aim to give an overview of the opportunity coming from the investigation of non-blood, easily accessible body fluids, which have the potential to radically modify current methods of diagnostics based on invasive assays. Furthermore, the possibility to shift toward non-blood specimens will be investigated from the point-of-view of available technologies, which would provide the tools for the self-collection, storage, and in some cases analysis of collected samples. Our current difficult period could allow a performance boost for technology and the final push towards the market exploitation of many of the tools and instruments. Our paper connects ideas to provide reliable analysis with great compliance, with growing patient needs, and newly established rules and requirements.

2. Liquid biopsy

The currently accepted definition of liquid biopsy identifies the collection, storage and analysis of peripheral blood to be tested for the presence of a panel of established cancer or disease-associated biomarkers. This kind of analysis is of crucial importance as some indicators can be found in blood at the very early stage of the disease when treatments are often the most successful. Liquid biopsy aims to replace the invasiveness of standard methods for tissue biopsies which may be uncomfortable and painful for patients, as well as costly and potentially risky.

Indeed, tissue-based tumour profiles usually provide only a snapshot which gives a little information compared to a condition, which is typical of cancer, heterogeneous by definition (Dagogo-Jack and Shaw, 2018; Johnson et al., 2014). To identify this dynamic scenario, cancer monitoring could often require repeated biopsies, thus lowering patient's compliance. In other cases, tumours are inaccessible for difficult localization or the general condition of a patient which does not allow the procedure. On the other hand, personalised medicine strategies for cancer patients require to follow in time the molecular makeup of a patient's tumor, thus enabling stratification of patients toward the best treatment, as well as monitoring response and resistance mechanisms (Brock et al., 2015). In order to tackle these unmet issues, liquid biopsy intended as blood investigation has been explored over the last few decades. Far greater interest in liquid biopsy has arisen since 2013 when it was proposed as a disruptive innovative method to early identify cancer pathologies from circulating biomarker, mainly considering, at that time, circulating tumour cells (CTC) (Alix-Panabieres and Pantel, 2013; Hodgkinson et al., 2014) and successively including cell-free tumor nucleic acids (cfNA), released in bloodstream from tumor primary site. Among these, we can list circulating cell-free DNA (cfDNA) (Alix-Panabieres and Pantel, 2016; Mihaly et al., 2018), circulating gene transcripts (cfRNA) (Chang et al., 2020; Dunkel et al., 2018), microRNAs (miRNAs) (Larrea et al., 2016; Ono et al., 2015), other noncoding RNAs (ncRNAs) (Arnaiz et al., 2019; Zhang et al., 2017b). The analysis of circulating tumor nucleic acid is useful in monitoring the progression of disease, response to clinical treatment and the emergence of drug resistance (Siravegna et al., 2017a). Circulating and cell-free nucleic acid can be also found in blood embedded into small extracellular vesicles (EVs), released by cells as membranous submicron entities abundant in tetraspanin proteins mirroring the type and condition of the cells they shed from. EVs are naturally secreted by almost all cell types to transport bioactive molecules intercellularly and a role in serving as vehicles for transfer between cells of membrane and cytosolic proteins, lipids, and RNA has been also largely demonstrated (Raposo and Stoorvogel, 2013). Among the EVs, exosomes, extracellular vesicles which fall in the range of 40–150 nm exhibit a more homogenous size distribution and sediment at 100,000 g by differential centrifugation (Xu et al., 2016). They are extremely interesting as they potentially influence cancer progression via different mechanisms involving their formation and trafficking in the microenvironment. They contain and protect the integrity of various proteins, lipids, nucleic acids (mRNA and miRNA) which would otherwise be damaged by enzymatic or hydrolytical reactions in the extracellular environment (Kalluri, 2016). Moreover, they seem to have a crucial role in supporting metastatic mechanisms together with tumour secretoma (Ogawa et al., 2020) by promoting the formation of pre-metastatic niche, acting in metabolic reprogramming, recruiting of immune and non-immune stromal cells and facilitating metastatic lesions outgrowth (Lobb et al., 2017). Recently, applying asymmetric-flow field-flow fractionation (AF4) technology (Zhang and Lyden, 2019), the category of exosomes was divided into three subgroups, including large exosome vesicles (120-90 nm), small exosome vesicles (80-60 nm) and an abundant population of non-membranous nanoparticles, called "exomeres" with a diameter of around 35 nm, whose biological function is still unknown (Zhang et al., 2019b). The proteomics of exomeres revealed an enrichment in metabolic enzymes and hypoxia, microtubule and coagulation proteins as well as specific pathways, such as glycolysis and mTOR signalling. Moreover, two distinct roles in hypersialylation of surface proteins and prolonged EGFR stimulation in recipient cells have been investigated (Fura et al., 2003).

Blood may contain all of the biomarkers that have been previously described along with constitutively circulating proteins, nucleic acids, metabolites. Humans circulate quadrillions of exosomes at all times (Kalluri, 2016), that arise from both healthy and diseased cells (independently from the considered disease) from all the body districts. To find and analyse the aliquot of extracellular vesicles carrying the information for diagnosis and prognosis among this huge quantity of exosomes is like looking for a needle in a haystack and several studies are focused on blood sample enrichment and selection of disease-derived exosomes (Bernardi et al., 2019; Chiriaco et al., 2018). It should also be noted that altered protein expression in body fluids originating from the diseased tissue/organ constitutes molecular signatures or "disease fingerprints" mirroring the original perturbation of cells. Evidence suggests that the concentration of these disease signatures is diluted 1000-1500-fold, during their passage from tissue interstitial fluids to the circulatory system (Ahn and Simpson, 2007). Moreover, there are well-established protocols to handle blood samples including the depletion of high molecular weight proteins, such as albumin or immunoglobulins before proceeding with proteomic assays. This will, however, also result in deletion of low-abundance proteins, which hinders the identification of significant biomarker molecules and decreases the sample loading capacity of analytical methods (Granger et al., 2005). A number of approaches are being proposed to address this issue but one advantageous strategy considers the use of molecularly imprinted polymers embedded into purification supports, able to selectively bind these big proteins into artificial antibodies traps (Andac et al., 2013; Yang et al., 2013).

A key consideration for liquid biopsy from blood is that drawing blood may not always be easy and some issues may arise which depend on the age and conditions of patients. Particular issues might arise with children, patients with small, fragile veins and paper-thin skin (elderly people, long-term corticosteroid treatments, chemotherapy, repeated testing) (Miller, 2012); dehydration and poor nutrition; presence of skin tears (LeBlanc et al., 2019); patients with disability (i.e. autism, schizophrenia) (McLenon and Rogers, 2019; Meindl et al., 2019); and

iatrophobia (fear of doctors, or of medical care) (Hollander and Greene, 2019). Needle phobia is estimated to affect roughly 10% of the population and this, in the form of a wide range of different reactions, can represent an obstacle to patients' testing and treatments and often require coercive actions like sedation, general anaesthesia or physical constraint to allow medical procedures (Meindl et al., 2019).

Such a complex scenario highlights that the existence and availability of different and alternative approaches to the detection of biomarkers from blood could be very useful in a range of conditions ranging from children to the elderly, to disabled patients' management. There are several other body fluids, more easily accessible than blood including saliva, urine, stool, sweat, breath, seminal fluid and even earwax for gathering disease-derived genetic information, protein signature, or micro- and nanovesicles directly shedding from tumor tissue. In addition, fluids originating from the same body district of the primary site of the disease and not diluted along the way to reach blood may represent a sample already enriched in the biomarker of the same disease.

On the other hand, the detection of cancer biomarkers from non-blood body fluid is still far from clinical practice in disease diagnosis and monitoring with only few exceptions, such as the specific tumor marker FDA-approved, PCA3, an indicator of prostate cancer risk from urine (Crawford et al., 2016). Other diagnostic tests currently run in diagnostic analysis laboratories include urinalysis, that considers physicochemical parameters referring to active infections or urinary tract disfunctions, faecal occult blood test (FOBT) in stool to indirectly identify lesions in the terminal tract of bowel (together with calprotectin detection) (Mowat et al., 2016), breath analysis to diagnose food intolerances or *Helycobacter pylori* infections (Miftahussurur and Yamaoka, 2016).

One of the known drawbacks in the use of biological fluids is the stability of molecules. Storage temperatures and times, physical stress due to the routine processing of samples (centrifugation, mixing, concentration steps) cause, for example, changes in pH, which may affect the chemical stability of compounds and induce their degradation (Fura et al., 2003). Other than the immediate addition of stabilising reagents, or storage in strict conditions, it is important to fix the weak points for the sample integrity and to keep in mind what conditions each kind of sample would require in order to allow a reliable liquid biopsy from unconventional body fluids. Innovative tools such as Lab On Chips (LOCs) can offer a bridge to overcome the problem of preserving samples from collection to analysis by allowing quick results in a *sample-in/answer-out* configuration (Chiriaco et al., 2016b; Volpe et al., 2020). Tools for self-diagnosis and/or sample storage have been increasingly in demand during the period of the pandemic emergency where access to healthcare institutions is restricted not only for Covid diagnostics but also for all the other kinds of diseases which require continuous monitoring and follow-up. The possibility of introducing LOCs that are able to process, store or analyse body fluids would represent a disruptive turning point and there is a need for effort to turn their potential into reality. Consideration also needs to be given to the overall cost of the LOC device and materials used. Exploiting polymeric/plastic devices and innovative microfabrication methods instead of common research lab materials could improve the possibility of an opening towards market penetration (Chiriaco et al., 2016a; Volpe et al., 2020; Zoupanou et al., 2021b). Lakey et al. have described an impedimetric array within a polymer microfluidic cartridge that can be produced using high volume approaches - such as microinjection moulding, hot embossing and roll-2-roll (R2R) processing - for low cost Point-Of-Care (POC) diagnostics (Lakey et al., 2019).

Despite the presence on the market of some kits for self-diagnostics from blood (glucose tests) or urine (pregnancy tests, ketone test strips), only few examples of LOCs with body fluids came out from research laboratories, despite the fact that several examples of unconventional fluids and their on-chip handling and analysis are available in the literature (Anderson et al., 2006; Karami et al., 2019; Sha et al., 2019).

In the following sections we will investigate all the above mentioned aspects from a multidisciplinary point of view, analysing the most promising body fluids and highlighting their potential in the new perspective of distanced diagnostics and suitability in non-invasive home collection. Fig. 1 summarizes the topics which are elucidated in this review paper.

2.1. Saliva

Whole saliva is a complex fluid derived from major and minor salivary glands and it is considered as a plasma ultra-filtrate (Esser et al., 2008). Whole saliva contains gingival cervicular fluid, expectorated bronchial and nasal secretions. The principal functions of saliva are lubrication to protect the oral mucosa, beginning food digestion, cleansing of the oral cavity, antimicrobial action, coadjuvant for speech, buffering action on mouth pH and maintenance of supersaturated calcium phosphate concentrations (Chiappin et al., 2007). Saliva has often been defined as a mirror of the body's health as it contains the serum constituents that are measured in standard blood tests to monitor health and diseases. Furthermore, whole saliva can be collected non-invasively, and by individuals with modest training.

The analysis of saliva biomarkers can give information about the diagnosis, detection and screening of certain malignant tumours. One of the most promising use of saliva in diagnosis of tumor disease is the identification of Oral Squamous Cell Carcinoma (OSCC), as saliva would contain molecules, cells and extracellular vesicles shedding directly from a tumor primary site and directly constitutes the closer tumor tissue microenvironment of the primary site. This disease is the most common among head and neck cancers, and is usually diagnosed at an advanced stage, where highly invasive surgery and chemotherapy are required, heavily compromising life quality and survival (Gupta et al., 2016; Tenore et al., 2020). Early detection of OSCC is the only way to limit consequences of the disease and the main challenge in prevention is large-scale screening, which could only be practically achieved through a non-invasive assay. Performing tests on saliva would be an attractive strategy that would be suitable for a wide-scale patient testing. Moreover, a saliva test would aid in monitoring the efficacy of treatment and avoiding repeated biopsies. A high number of molecules – including nucleic acids, proteins, metabolites and extracellular vesicles – have been investigated as novel and promising candidate biomarkers from saliva and related diseases (Table 1). The screening and monitoring of other pathologies could take advantage of the presence of biomarkers in saliva. Recently, indeed, some of the discovered biomarkers which can be used for early cancer detection from saliva, are being mentioned in the literature with increasing frequency. Wang et al. focused their work on viral-HPV16 DNA related to oropharyngeal cancer detection with observed LOD of 47.8 copies of HPV16 DNA/ml (Wang et al., 2020b). Ishikawa et al. propose ornithine, o-hydroxybenzoate and ribose 5-phosphate metabolites as biomarkers for distinguishing patients with OSCC and oral epithelial dysplasia (OED) from those with persistent suspicious oral mucosal lesions (PSOML), with observed AUC of 0,871 and 95% confidence interval (Ishikawa et al., 2019). A recent report has established miR412-3p and miR-512-3p as effective biomarkers over-expressed in OSCC patient samples (Gai et al., 2018). Salivary biomarkers for lung cancer prediction, such as the expression levels of GREB1 and FRS2, was the focus point of a study for a different group. The authors found that these mRNA biomarkers were significantly up-regulated in Non-small cell lung cancer (NSCLC) patients in comparison to healthy controls (Gu et al., 2020). Finally, early colorectal cancer detection from saliva was the aim for González and coworkers using salivary microRNAs as diagnostic values (Rapado-Gonzalez et al., 2019).

2.1.1. Saliva sampling and stability

A salivary sample has the advantage that it can be collected with

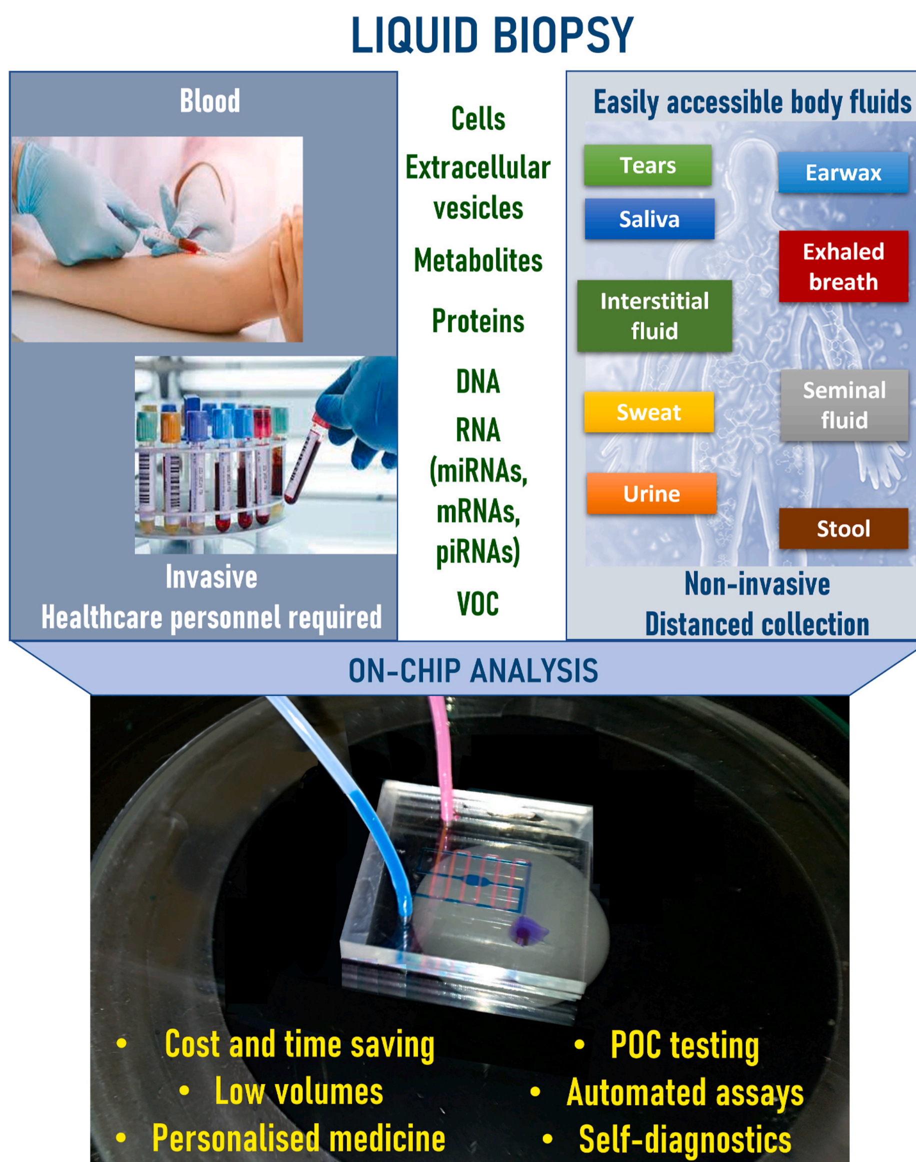

Fig. 1. Liquid biopsy from blood and other biological fluid of easy access. The Lab-On-Chip analysis would improve accessibility in distanced diagnostic times.

simple equipment, and hence provides a cost-effective approach for screening a larger population. Recently, due to the need for lowering risks related to biological fluid sampling and provide an expansion in the testing capability for COVID-19 (Valentine-Graves et al., 2020), the FDA has released the permission to self-collect saliva samples at home (https://www.fda.gov/news-events/press-announcements/coronavirus-covid-19-update-fda-authorizes-first-diagnostic-test-using-home-collection-saliva). There are different kits for saliva specimen collection. One of these, recommended by FDA, is the ORE-100 product(https://www.dnagenotek.com/ROW/products/collection-human/oracollect-rna/ORE-100.html).

This possibility opens the way to a plethora of tasks, as saliva could in principle be stored and treated before its use in clinical diagnostics. Moreover, an interesting strategy proposed by Lohse and co-workers lies in the pooling of saliva samples. That's an easy method to implement and has the potential to expand the laboratory and test kits capacity, when it is required to screen a large number of asymptomatic people. RT-PCR amplification is performed on the pooled sample and only in the case of positive results further analysis will be performed, considerably reducing the number of tests needed (Lohse et al., 2020). For all these reasons, saliva seems to represent a precious candidate for monitoring of patients.

Sampling stimulated and non-stimulated saliva will depend on the analyte searched but can be easy to perform, including: passively drooling through lower lip or directly spitting into a vial; using a polystyrene swab; chewing a cotton roll, a polyester or a paraffin wax piece (Chiappin et al., 2007). However, of the principal drawbacks to the use of saliva, is the rapid degradation which starts already during sample collection and occurs in 30 min. To overcome this problem, the immediate treatment with protease inhibitors together with a 4 °C storage may partially prevent the complete degradation, allowing the use of the sample in the first 4 h from collection, while the inhibition of bacterial metabolism seems to have no effect (Esser et al., 2008). The recovery of some species like s-IgA or catecholamines can be obtained if the sample is quickly stored at -20 °C or mixed with enzyme inhibitors (i.e. leupeptin or aprotinin), glycerol buffers, denaturing agents (trifluoracetate) or sodium azide (Groschl et al., 2001; Nurkka et al., 2003). Longer time storage is recommended at −80 °C. Moreover, it is possible to check the quality of the saliva specimens by considering the amount of three protein degradation products (with masses of 2937 Da, 3370 Da and 4132 Da) which increased 2 to 7-fold after 4 h storage at room temperature and are indicated as stable breakdown products of larger

Table 1
Biomarkers from saliva and methods of detection.

Type of cancer	Type of biomarker	Method of detection/Targeted Biomarkers/Ref.
Oral, Neck, Head	Metabolites	• capillary electrophoresis mass spectrometry - Ornithine, o-hydroxybenzoate & ribose 5-phosphate (Ishikawa et al., 2019)
		• RT-methylation-specific PCR - CD44v, SYNE1 & miR34a (Shah et al., 2018)
		• flame photometry – spectrophotometry - Albumin, LDH, IgA, IGF, MMP-2, MMP-9 & electrolytes (Na, Ca, P, Mg, K) (Shpitzer et al., 2007)
		• liquid chromatography/ELISA - Carbonyls, lactate dehydrogenase, MMP-9, Ki67 & Cyclin D1 (Shpitzer et al., 2009)
	Nucleic acid	• Acoustofluidics - RT-PCR/47.8 copies of HPV16 DNA/mL (Wang et al., 2020b)
		• miRNA seq on total RNA - 134 Exosomal miRNA differentially expressed (Langevin et al., 2017)
		• RT-PCR – ELISA - mRNA, IL-8, IL-1B & SAT (Elashoff et al., 2012)
		• PCR-TaqMan micro RNA assays - miRNAs (Yoshizawa and Wong, 2013)
		• multiplex PCR and massively parallel sequencing - Somatic mutations & tumor DNA (Wang et al., 2015)
		• qRT-PCR array and qRT-PCR - miR-512, miR-412, miR-302b, miR-517b, miR-27a & miR-494 (Gai et al., 2018)
		• quantitative methylation-specific PCR (Q-MSP) - DAPK, DCC, MINT-31, TIMP-3, p16, MGMT & CCNA1 (Lopes Carvalho et al., 2011)
	Proteins expression	• MALDI-TOF mass spectrometry - Peptide fragments Lom, OSCC, SENP7 & TLR4 (Ploypetch et al., 2019)
		• liquid chromatography-laser induced fluorescence (HPLC-LIF) - Glycoprotein, epithelial keratins, immunosuppressive cytokines & cell surface antigens (Venkatakrishna et al., 2003)
		• Monoclonal immunoradiometric assay - Cyfra 21-1, CA125, CA19-9, SCC & CEA3 (Nagler et al., 2006)
		• Proteomics- reversed-phase liquid chromatog. TOF mass spectrome. -Mascot sequence db searching -/M2BP, MRP14, CD59, profilin & catalase (Hu et al., 2008)
		• two-round linear amplification with T7 RNA polymerase - IL8, IL1B, DUSP1, HA3, OAZ1, S100P & SAT transcripts (Li et al., 2004)
		• 2D electrophoresis (2DE) and matrix-assisted laser desorption- MALDI-TOF MS - Salivary transferrin (Jou et al., 2010)
	Vesicle/exosomes	• RT-qPCR - CEP55 (Qadir et al., 2018)
Lung cancer	Nucleic acid	• qRT-PCR/CCNI, EGFR, FGF19, FRS2 & GREB1 (Shang et al., 2019)
		• electric field-induced release and measurement-electrochemical biosensor - exon 19 & p.L858 mutations (Pu et al., 2016)
		• qPCR - TURBO DNase treatment - mRNA, GREB1 & FRS2 (Gu et al., 2020)
	Vesicle/exosomes	• Affinity chromatography column combined with filter system (ACCF)//LC-MS/MS - Annexin, NPRL2, CEACAM1, MUC1, PROM1, HIST1H4A & TNFAIP3 (Sun et al., 2016)
Colorectal	Nucleic acid	• RT-qPCR - miR-185-5p, miR-29a-3p, miR-29c-3p, miR-766-3p & miR-491-5p (Rapado-Gonzalez et al., 2019)
Nasopharyngeal	Nucleic acid	• qRT-PCR - miR-937-5p, miR-4478, miR-1321, miR-3714 & mir-3612 (Wu et al., 2019a)
Oesophageal	Metabolites	• Raman Spectroscopy; chemometric analysis; genetic algorithm quadratic discriminant analysis (GA-QDA) model - C-O stretching of ribose, Amide III and CH2 wagging vibrations from glycine backbone, methylene deformation (Maitra et al., 2020)
Thyroid	Protein expression	• 2D electrophoresis coupled to nano-liquid chromatography electrospray ionization tandem mass spectrometry - ANXA1, MSN, CRNN, ENO1, LDH & MDH (Ciregia et al., 2016)
Pancreatic	Nucleid acid	• (qPCR)-logistic regression (LR) - KRAS, MBD3L2, ACRV1 & DPM1 (Zhang et al., 2010)

proteins (Esser et al., 2008).

2.1.2. LOC for saliva

Handling saliva in a safe manner and contemporary preserving its integrity for use as a fluid for liquid biopsy within LOC devices has been a challenging topic since a failure point of salivary diagnostics is the lack of appropriate sampling methods.

The currently available kit for saliva sampling is cumbersome since there is a requirement for vials and separate buffers to allow sample pretreatment and storage to prevent degradation of the biological specimen. The need to overcome these limitations and make saliva sampling a more patient compliant approach is necessary and some attempts in this direction have been made in recent years by various research groups. One of the latest examples focuses on a microfluidic polymer platform loaded with dry reagents to perform an on-chip lateral flow immunoassay, to be detected by fluorescent integrated reader (T. U et al., 2020).

One of the first demonstrations (2004) of realizing a microdroplet reactor into microfluidic lab-on-chip deals with a multiple assay on physiological human fluids by Srinivasan et al. who used the method of electrowetting. Their lab-on-chip architecture is a combination of sample injection, reservoirs, droplet formation structures, fluidic pathways, mixing areas and optical detection sites. In their work, they performed a glucose assay to confirm the efficiency of the device in real samples and the values were found to be in agreement with the reference measurements (Srinivasan et al., 2004). In a different work, the authors proposed a method of determining the subject's gender from saliva samples coupling of on-chip lysing and PCR. Specifically, the steps included amplifying and detecting the DYZ1 repeated region in the Y-chromosome. A simple microfluidic device having a serpentine channel was then created in order to perform the aforementioned process and achieved completion of 42 PCR cycles in less than 15 min. The fluorescence signature of each sample was then analysed and since they observed strong fluorescence from male saliva, they concluded that male and female samples can be clearly distinguished (Pjescic and Crews, 2012). It is also noteworthy that, in recent years, several works have been published that deal with polyacrylamide gel electrophoresis for salivary biomarkers detection (Herr et al., 2007; Krapfenbauer et al., 2014).

A large number of innovative detection tools for saliva biomarkers have been developed over a number of years that make use of different transducer methods such as organic photodetectors (OPDs) and an immunogold-silver assay (IGSA) resulting in LOD at picomol concentration levels (Dong and Pires, 2017) (Fig. 2A–B). Tian et al. have also realized a microfluidic paper-based electrochemical DNA biosensor for the detection of epidermal growth factor receptor (EGFR) mutations in patients with non-small cell lung cancer (NSCLC). This was achieved by analyzing the DNA hybridization reaction from saliva with a device that

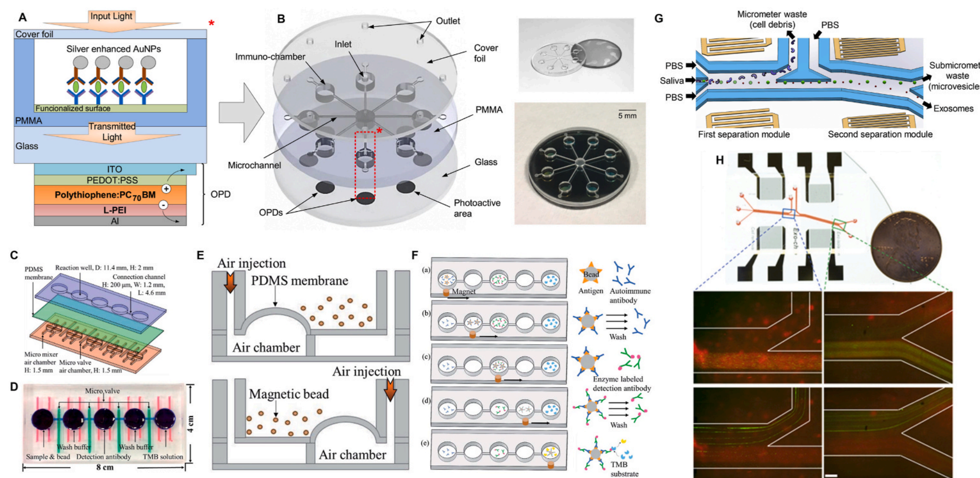

Fig. 2. A. Demonstration of the optical microfluidic biosensor based on a multilayered transducer and detection scheme based on IGSA. **B.** Illustration of the device's layers including the eight OPDs aligned with immuno-reaction chambers and optical images of the device. **C.** Multilayered PDMS structure of the microfluidic chip for detection of p53 in the work of Lin et al. (2018). The top layer contains reaction wells and microchannels, the intermediate layer holds a 100 μm membrane, the bottom layer provides mixers and microvalve components. **D.** Picture of the assembled chip mixers are filled with red ink, valves with green ink, reaction wells and channels with blue ink. **E.** Principle of micromixers and valves workflow. If compressed air is injected into the four independent air chambers, the membrane on the top rises up and a vortex-like disturbance is created in the liquid above the membrane. When compressed air is pumped into the air channel of the microvalves, the membrane above the air channels rose and obstructed the channels, so the liquid proceeds in the different microfluidic sections. **F.** Sequence of the immunomagnetic assay for p53 autoantibodies from samples. A magnet allows the sample movements to the next reaction well (washing/binding/measurements into a microplate reader). **G.** Architecture of SAW-based device containing two separation modules. **H.** Picture of the acoustofluidic platform. Blue and green boxed areas magnifications showing a mixture of particles running in the two separation areas of the device with (top) and without (bottom) SAW application. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

contains a three-electrode system, with a modified paperwork electrode (PWE), a screen-printed carbon electrode and a Ag/AgCl reference electrode. This device was able to detect target DNA (tDNA) with LOD of 0.167 nM for both tDNA1 and tDNA2 (Tian et al., 2017).

Images in the panel reproduced and adapted with Copyright permissions from references (Dong and Pires, 2017; Lin et al., 2018; Wang et al., 2020b).

Another approach worth mentioning and comparing with the above involves an automated microfluidic system designed by Lin and collaborators. The chip consists of three PDMS layers, the top containing the reaction wells and microchannels and the bottom containing the pneumatic mixers and microvalves (Fig. 2C–F). The combination of the microfluidic chip with the magnetic immunoassay allows detection of autoantibodies anti-p53, as useful oral cancer biomarkers, with a very short assay time and an LOD of 4 ng/ml (Lin et al., 2018). An acoustofluidic platform was recently developed for size-based exosome isolation and HPV16 DNA detection of HPV-associated oropharyngeal cancer, from saliva. This method, in combination with digital droplet PCR (ddPCR) was able to detect HPV 16 DNA in 80% of cases (Wang et al., 2020b) (Fig. 2G–H).

2.2. Urine

Urine is one of the simplest biological samples to access: risk-free, with the highest compliance for patients, lower matrix complexity when compared to blood (Daulton et al., 2021) and with the right potential for large-scale screening application. Moreover, one individual produces a large volume of urine daily through 6–7 urinations reaching 400–2000 mL. Urine is a precious source of biomarkers which reflect the entire body state of health, as the kidneys are continuously filtering, secreting, and reabsorbing substances coming from the whole body. Urinalysis (the use of urine for a plethora of tests) is probably the most widespread kind of non-blood liquid biopsy *ante litteram* as the most common biological fluid used for diagnosis. Pregnancy, infections, hypertension and diabetes are well-known conditions which urine analysis can reveal in common practice clinical tests.

The identification of biomarkers from the urine includes also some recent tests for cancers and clearly the most promising candidate diseases to be detected from urine are within the urological field. One of the most widespread investigated disease is prostate cancer which continues to affect male patients all over the world and is still one of the three most diagnosed tumours according to the 2018 World Health Organization statistics (Mattiuzzi and Lippi, 2019). Moreover, the age group most affected by prostate cancer coincides with that in which the coronavirus is more dangerous and has claimed more victims. The diagnosis of this disease usually relies on clinical examinations which are normally very uncomfortable for patients and require prostate biopsies (even repeated) which induce themselves an elevation of blood biomarkers related to prostate conditions, namely Prostate Specific Antigen (PSA), detected in its circulating forms (free or conjugated PSA) (Draisma et al., 2009; Kelly et al., 2018). A urinary assay for PCA3, an mRNA that is highly overexpressed in prostate cancer cells, has emerged in the last decade as a promising biomarker showing its usefulness as a diagnostic test for this common disease. In particular, it is able to give information about patients stratification, predicting the biopsy outcome and reflecting prostatectomy tumor volume and Gleason score (a parameter based on the histological analysis of biopsy). It may have clinical applicability in selecting men who have low volume/low grade cancer, being independent of prostate volume, serum PSA level and the number of prior biopsies (Deras et al., 2008).

It is possible to identify exosomes from urine towards biomarkers for prostate cancer. Widmark and co-workers in 2009 identified two types of microvesicles in prostate secretions: (i) prostasomes with a diameter of 150–500 nm and (ii) exosomes, nanovesicles with dimensions of 30–100 nm (Nilsson et al., 2009). The first were produced by prostatic ductal epithelial cells, involved in the production of seminal fluid and playing a role in male fertility; the second class, showing a cup-shaped morphology, are actively secreted by both normal and tumour cells. An elevated exosome secretion was found in cancer tissue secretome, serum and urine of patients (Mitchell et al., 2009). More recently, the proteomics of urine extracellular vesicles, including exosomes was further investigated for prostate and bladder cancers (Wang et al., 2020a) and some promising candidate biomarkers confirming the presence of PCA3 and TMPRSS2-ERG in urine exosomes open the way to a more accurate liquid biopsy with the ambitious goal to avoid unnecessary biopsies (Fujita and Nonomura, 2018).

The presence of cell-free DNA (cfDNA) in urine also known as transrenal DNA (tr-DNA) as a result of renal clearance of cfDNA from the blood, has recently emerged as a useful source of tumour-derived DNA (transrenal-tumour DNA - trtDNA), also considering that glomerular

filtration of plasma is highly selective and only molecular complexes with diameter below 6.4 nm and molecular weight of <70 kDa (corresponding to around 100 bp of DNA) can reach the lumen of nephrons (Siravegna et al., 2017a) and thus proceeding to ureters and bladder. This implies that bigger molecules found in urine derives from the shedding of tumour cells or their breakdown products of the latter urinary tract. Apart from foetal tr-DNA fragments found in mother's urine (Shekhtman et al., 2009), limited data are available about the possibility of biomarkers from non-urological origin. One of the most investigated cancers is colorectal cancer (CRC), which seems to release short DNA fragments (around 100 bp) that are able to reach urine with mutation related to BRAF and KRAS (Crisafulli et al., 2019b). This discovery offers new options to follow clinical treatment in metastatic CRC with high prognostic value together with the identification of newly acquired mutations in strong association to therapy resistance (Chen et al., 2019).

Because of the role of kidneys within filtration, the presence of proteins in urine is very strictly controlled and limited to some with very low molecular weight. The presence of bigger proteins like albumin identifies a well-known condition, called proteinuria or albuminuria, which refers to chronical kidney misfunction as a consequence of severe cardiovascular diseases, diabetes and high blood pressure with related risk factors (Gerstein et al., 2001). It is possible, instead, to find some shorter proteic entities like Galectin-3 (35-kDa), a member of the β-galactoside-binding lectins family or N-terminal pro-brain natriuretic peptide (NTproBNP, 8.5 kDa) which have been recently demonstrated as biomarkers respectively of heart failure (Dong et al., 2018) and bronchopulmonary dysplasia (Iliodromiti et al., 2020). The search for cancer-related protein biomarker in urine seems to be promising for bladder cancer (BCa): a list of this kind of molecule has been provided in the recent work of Mohan and collaborators who reviewed a series of urinary biomarkers which could be particularly attractive thanks to the direct contact of urine with urothelial tumor cells and the ease of sample collection (Chakraborty et al., 2019). Among them, they mention two FDA approved tests, both focused on the use of NMP22 (Nuclear matrix protein 22) (Soloway et al., 1996) and a list of protein molecules, which alone or in combination with others may serve in the detection, follow-up, prognosis and prediction of treatments for BCa. In particular, they underline the role of BLCA-4 (a nuclear transcription factor found in bladder tumours in the early stages of disease) and the enzyme Hyaluronidase, a promising validated biomarker that showed a sensitivity and specificity ranged from 87 to 100% and 89–98% according to Pham studies (Lokeshwar et al., 2000).

Cervical cancer has also been investigated through urine samples. Snoek et al. for example, optimized a test searching for DNA methylation of 6 DNA methylation markers (FAM19A4, GHSR, PHACTR3, PRDM14, SST and ZIC1) and high-risk HPV fractions and this resulted in a strong to near-perfect agreement with the standard cervical scrapes analysis (Snoek et al., 2019). EGFR mutation testing in urinary cell free DNA has been revealed to be a promising non-small cell lung cancer (NSCLC) biomarker, demonstrating an affordable correlation if compared with plasma ctDNA and primary tissue biopsies. It also accurately tracks the dynamic changes in mutational status during treatment with EGFR and Tyrosine kinase inhibitors (Chen et al., 2017). A summary of the literature on the detection of biomarkers from urine sample is provided in Table 2.

2.2.1. Urine sampling and storage

The collection of urine specimens is very easy but at the same time is a large source of preanalytical variability. The common practice of self-collecting morning or 24 h urine makes it easier to perform clinical tests but, as a double-edged sword, it can be a large cause of errors depending on each of the steps from sampling, transport to the laboratory, preparation of sample for testing and storage. In addition, if urine is needed for bacteriological culture, then this should allow easy sterile collection and avoid contamination by non-self microorganisms (Coppens et al., 2010). It is worth considering the features that a container should

Table 2
Biomarkers from urine and methods of detection.

Type of cancer	Type of biomarker	Method of detection/Targeted Biomarkers/Ref.
Prostate	Nucleic acid	• ExoMeth sequencing - GAP1 (Connell et al., 2020) • Next-Generation Sequencing-ddPCR - FTH1, BRPF1, OSBP, PHC3 & UACA (Sole et al., 2020) • D'amico criteria & Carpa - PCA3, TMPRSS2-ERG, HOXC6 & DLX1 (Connell et al., 2019) • Methylation-specifc PCR - HOXA11as, KLK10, GPR147, GPR62, HOXD4rc, HOXD3c, FRZB, GRASPrc, HOXBAS3, HOXCrcAS3, HOXD8rc, RASSF1 & SLC16A5rc (Brikun et al., 2019) • Digital droplet PCR - AR-V7 & AR-FL (Woo et al., 2019) • Mass Spectrometry and quantitative PCR - ADCY4, AOX1, APC, CXCL14, EPHX3, GFRA2, GSTP1, HEMK1, KIFC2, MOXD1, HOXA7, HOXB5, HOXD3HOXD9, XOXD10, NEUROG3, NODAL & RASSF5 (Brikun et al., 2018) • Real time-quantitative PCR - miR-196a-5p & miR-143-3p (Rodriguez et al., 2017), (Botchkina et al., 2005) • One-step RT-qPCR - HOXC5 & DLX1 (Van Neste et al., 2016) after DRE (digital rectal examination) • Whole-genome sequencing – cfDNA: TMPRSS2-ERG fusion, PTEN gene deletion, NOTCH1 locus amplification (Xia et al., 2016) • Real Time PCR and sequencing - miR-21, miR-204 & miR-375 (Koppers-Lalic et al., 2016) • Quantitative PCR - PCA3, TMPRSS2 & ERG (Cao et al., 2011a) • Real time-PCR - UCF DNA (Casadio et al., 2013) • Quantitative PCR - GSTP1, SFRP2, IGFBP3, IGFBP7 & PTGS2 (O'Reilly et al., 2019) • Infinium MethylationEPIC BeadChip (Illumina) - cfDNA & ZMIZ1 (Silva et al., 2020) • ExoDx Prostate IntelliScore (EPI) test - Circulating miRNAs (Tutrone et al., 2020)
	Protein expression	• Next-generation Sequencing and RNA-seq - 5510 differentially expressed transcripts; 37 genes upregulated in PCa (Lee et al., 2020) • Mass Spectrometry - Urinary exosomal proteins ADSV & TMG4 (Erozenci et al., 2019) • Liquid Chromatography-Mass Spectrometry - FABP5 (Fujita et al., 2017) • Ultra-performance liquid chromatography (UPLC)- Mass spectrometry - putrescine (Put), spermidine (Spd) and spermine (Spm) (Tsoi et al., 2016) • Liquid Chromatography/tandem Mass Spectrometry - Sarcosine (Cao et al., 2011b) • Liquid Chromatography-Mass Spectrometry - Annexin A3 & Sarcosine (Cao et al., 2011a) • Liquid Chromatography–tandem Mass Spectrometry - Urinary diacetylspermine, plasma arginine & ornithine (Selvi et al., 2019)
	Vesicle/exosomes	• Mass Spectrometry – Urinary exosomes (Erozenci et al., 2019) • PCR - CD6, EpCAM & EGFR (Cho et al., 2019) • ExoDx Prostate IntelliScore (EPI) test – urine exosome gene expression assay (Urabe et al., 2019)
	Cells	• Spiral microfluidic-fluorescent antibodies – Pca cells n > 8 cells (Rzhevskiy et al., 2020)
Bladder	Nucleic acid	• Next-Generation Sequencing - TERT, FGFRE, TP53, PIKECA, HRAS, ERBB2 & KRAS (Ou et al., 2020) • Ion Proton Sequencer and PCR – 168 somatic mutations (Hirotsu et al., 2019) • Targeted sequencing measured mutant allele frequencies MAFs/PCR - MAFs/PCR/SMs, TERT, FGFR3, PIK3CA, TP53, ERCC2, RHOB, ERBB2, HRAS, RXRA, ELF3, CDKN1A, KRAS, KDM6A, AKT1, FBXW7, ERBB3, SF3B1, CTNNB1, BRAF,

(continued on next page)

Table 2 (*continued*)

Type of cancer	Type of biomarker	Method of detection/Targeted Biomarkers/Ref.
		C3orf70, CREBBP, CDKN2A & NRAS (Ward et al., 2019)
		• Next generation sequencing - FGFR3-1, TP53, PIK3CA, ERBB3-2, TSC1, NF1, ERBB2,CDKN2A, ARID1A, STAG2, KTM2D, CREBBP & HRAS (Zhu et al., 2019)
		• Real-time PCR - IQGAP3, BMP4 & FAM1 (Xu et al., 2019)
		• Droplet digital PCR - TERT & FGR3 (Hayashi et al., 2019)
		• Next generation sequencing" (NGS) - Mutations, TERT & PLEKHS1 (Ward and Bryan, 2019)
		• Shallow whole genome sequencing (sWGS) - cfDNA & exoDNA; MDM2, ERBB2, CCND1 and CCNE1, CDKN2A, PTEN and RB1 (Lee et al., 2018)
		• Quantitative – Real time PCR (qRT-PCR) - MALAT1, PCAT-1 & SPRY4-IT1 (Zhan et al., 2018)
		• Quantitative methylation specific PCR (qMSP) - ACTB & RASSF1A (Bosschieter et al., 2018)
		• Next-generation sequencing and quantitative PCR - miR-30a-5p, miR-21-5p & miR-105b-3p (Pardini et al., 2018)
		• dPCR and targeted amplification (Tam-Seq) - MutDNA (Patel et al., 2017)
		• Next-generation sequencing - genomic profiles of urinary cellular DNA and cell-free DNA (cfDNA) (Togneri et al., 2016)
		• Next generation sequencing and digital droplet PCR (ddPCR) - Somatic genomic variants in tumor DNA; levels of cell free tumour DNA in plasma and urine (Birkenkamp-Demtroder et al., 2016)
		• Digital droplet PCR – ctDNA copies (Birkenkamp-Demtroder et al., 2018)
		• PCR amplification and sequencing - above 1 ng DNA cfDNA & sDNA (Stasik et al., 2019)
		• Quantitative PCR - IncRNAs (Yu et al., 2020)
	Protein expression	• Liquid Chromatography–tandem Mass Spectrometry - Tau, L-Phe, Hip & Cre (Gamagedara et al., 2012)
	Cells	• TCR sequencing - UDLs cells (Wong et al., 2018)
Lung	Nucleic acid	• Droplet digital PCR(ddPCR) - KRAS (Xie et al., 2018)
		• Mutation enrichment PCR coupled with next-generation sequencing detection (Trovera) - T790M (Goldman et al., 2018)
	Molecules	• Next-generation sequencing - TP53 (Wu et al., 2019b)
Breast	Nucleic acid	• Agilent human miRNA microarray chip and RT-qPCR - miRNA (Ritter et al., 2020)
	Molecules	• Liquid Chromatography-electrospray ionization tandem Mass Spectrometry - 8-oxodG (Guo et al., 2017)
Cervical	Nucleic acid	• Quantitative PCR – HPV DNA (Van Keer et al., 2018)
		• Next-generation sequencing - ZNF516, FKBP6 & INTS1 (Guerrero-Preston et al., 2016)
Colorectal	Nucleic acid	• Digital droplet PCR - KRAS or BRAF (Crisafulli et al., 2019a)
Urothelial	Nucleic acid	• Single-plex assay (UroMuTERT) - TERT (Avogbe et al., 2019)
Gastric	Nucleic acid	• Quantitative stem-loop PCR - miR-21-5p (Kao et al., 2017)

incorporate to allow optimal transport conditions and preservation of sample analytes (for example, proteins during the 24 h collection in preservative-coated vials). The stability of biological molecules in urine may undergo some stressful conditions which could give misleading test results. The storage temperature is a critical factor and has been identified as being crucial for the protein concentration, potassium, amylase integrity and bacterial growth, whilst sodium, urea, albumin, creatinine and uric acid concentrations are stable for up to 72 h regardless of the storage temperature (Van Berkel et al., 2010).

Myoglobin, the primary muscle constituent, may be investigated in urine (myoglobinuria). Although it was primarily indicated for diagnosis and risk assessment of kidney injury in patients with rhabdomyolysis (Zimmerman and Shen, 2013), it serves as a biomarker also in case of crush injuries and trauma and other pathologies involving striatum muscle, as in example of heart injury, and renal dysfunction (Loun et al., 1996). Its use is recommended before 24 h from collection and so, prompt analysis of fresh urine is needed; if not possible, storage under alkaline conditions using sodium carbonate as a preservative, is vital (Giesen and Lieske, 2016). Centrifugation, which is another common practice to analyse urinary sediments before storage, it causes a variable loss of erythrocytes and leukocytes between 20 and 80%, thus making correct quantification impossible (Coppens et al., 2010).

Nucleic acids in urine seem to be an interesting tool for urinalysis. As mentioned in the previous paragraph, small nucleic acid strands are emerging as biomarkers of both urological (Kutwin et al., 2018) and non-urological diseases (Nedaeinia et al., 2017). Indeed, due to the high concentration of RNA-hydrolysing enzymes in urine, mRNA is not preserved while miRNAs are more resistant to nucleases thanks to their small size (20–25 nucleotides) (Siravegna et al., 2017b). Mall and co-workers in 2013 tested a range of clinically relevant conditions: room temperature, refrigerated storage at 4 °C, and serial freeze–thaw cycles. Surprisingly, urinary miRNAs were relatively stable under all of the conditions evaluated. While there is some degradation over time, such degradation resulted in miRNAs levels within the upper limit of qPCR detection, thus confirming their potential as urine biomarkers (Mall et al., 2013). Moreover, the stability of miRNAs seems to be preserved also thanks to their inclusion into Evs, such as exosomes found in the sediments of urine. Due to these and many other elements which could be damaged, increase in the length of time occurring between sampling and analysis, lack of temperature control and use of specimens without preservatives within 2 h from collection, can affect the reliability of the analysis.

2.2.2. LOC for urine

Given the problems with urine storage, the possibility of analysing the sample in a safe and correct manner as soon as it is collected, is very appealing both in clinical practice and research studies. In this sense, microfluidic technologies are an attractive alternative to conventional analysis methods. Paper-based microfluidics, for example, and lateral-flow "pee-sticks," for pregnancy, ovulation, and menopause are a common everyday experience.

A recent report demonstrates a spiral microfluidic chip developed for isolating the PCa (prostate cancer) circulating tumor cells from the urine of patients with localized prostate cancer. The device isolates the CTC from urine as the cells experience inertial lift and Dean drag forces in the channel. The collected cells were then labelled with fluorescent anti-Glypican-1 (GPC-1), showing 79% sensitivity and equal specificity, if compared to the clinical samples analysed for the GPC-1+ putative tumour cells. The device was evaluated by using DU-145 cells, achieving 85% (±6) separation efficiency at 1.7 ml/min flow rate (Rzhevskiy et al., 2020) (Fig. 3A–B).

Another new cancer diagnosis technique focuses on citrate detection. To this end, the authors developed a device based on a colorimetric paper sensor using cysteine-capped gold nanoclusters (Cys-AuNCs) as peroxidase mimetic, and a Y-shaped microfluidic device which was designed for improving the biosensing performance of the paper assay. The discoloration of reagents is what makes this method successful, i.e. the higher the concentration of citrate the lower the blue density. Citrate levels, indeed, have been demonstrated to be lower in people with PCa (2 mM or less) and determining its concentration might be the key to the early detection of PCa since the LOD of this method is 0.4 μM. It is worth noting that the biosensor recovery falls into the range 86.0–98.0% (Abarghoei et al., 2019).

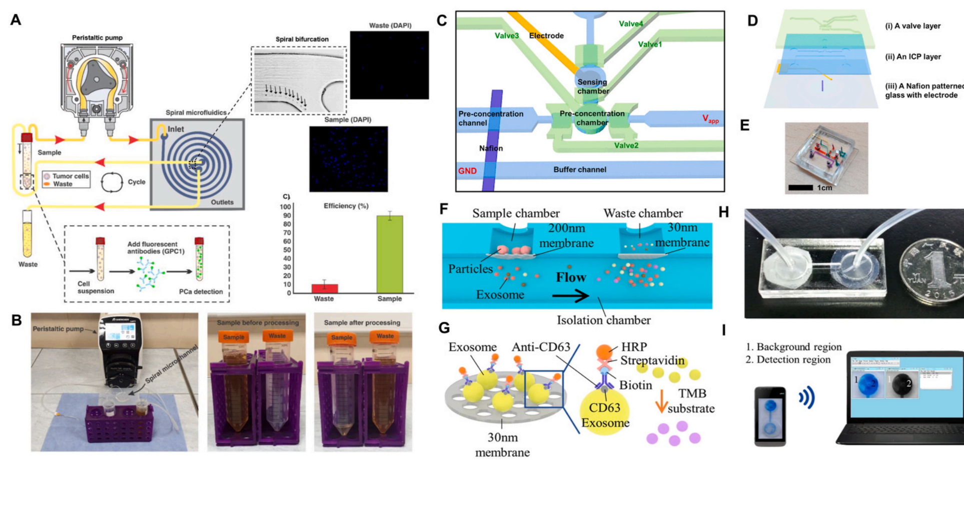

Fig. 3. A. Urine sample on chip manipulation for separation of PCa cells from waste through a spiral microfluidic chip containing a bifurcation at its end. **Inset:** visualization of the separated cells both optically and through fluorescence microscope. The efficiency of this method is reported in the bar chart. B. Experimental setup and urine sample before and after processing. **C.** Architecture of the microfluidic chip, including the pre-concentration and the electrochemical detection areas aligned with the sensing chamber. **D.** Schematic illustration of the device's layers **E.** Optical image of the microfluidic device. **F.** Scheme of the device realized by Liang and co-workers for isolation and detection of EVs including a double-filtration tool, based on size-exclusion. EVs with a size between 30 and 200 nm are isolated and enriched in the isolation chamber. **G.** Schematic of immunoassay steps for EV detection on-chip. **H.** Picture of the assembled PDMS/membranes double-filtration device. **I.** The test result is imaged using a smartphone and transferred to remote data analysis. Pictures in the panel adapted with Copyright permissions from references (Hong et al., 2018a; Liang et al., 2017; Rzhevskiy et al., 2020).

A third technique utilizes a POC microfluidic chip to investigate the level of methylation of glutathione-S transferase-P1 (GSTP1) and EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1) in urine as effective and promising biomarkers for the early detection of PCa. In principle, this method combines two different techniques - the nanostructured gold electrode increases the electrochemically active surface area which in turn improves the detection of methylated DNA, and the ion concentration polarization (ICP) phenomenon pre-concentrates the methylated DNA. This combination, has achieved an LOD of 7.9pM for GSTP1 and 11.8pM for EFEMP1, relative standard deviation (RSD) of less than 7% and detectable methylation level ranging from 10%–100% (Hong et al., 2018a) (Fig. 3C–E).

The isolation of EVs and in particular the detection of exosomes seems to be (as in all the biofluids) a promising approach for diagnosis. The standard method for isolation of EVs (i.e. ultracentrifugation) requires a long time (6–8 h), is labour-intensive and often instrument-dependent. To overcome these drawbacks, Liang et al. designed a microfluidic device made of four PMMA layers joined by double-sided adhesive tape. The architecture of the device included two points of separation for EVs, one obtained by the integration of a membrane with pores of 200 nm and another with pores of 30 nm (Fig. 3F–I). Based on this size-exclusion design, particles with a diameter larger than 200 nm are excluded by the first filtration system, whereas particles smaller than 30 nm pass through the double-filtration device. EVs with a size between 30 and 200 nm are instead isolated into another device compartment. By analysing urine samples from bladder cancer patients, they claim that the fraction with EVs size between 30 and 200 nm was found to notably increase if compared with healthy controls (Liang et al., 2017).

Despite the preserving function of EVs, standard protocols for miRNA recovery are quite laborious and time-consuming. The most commonly used method of ultracentrifugation suffers from the disadvantage that we have described previously. To find a solution to this, Samsonov and collaborators in 2016 described a protocol for collection of exosomes from urine, taking advantage on lectine-induced agglutination, subsequent centrifugation at relatively low *G-force* and analysis by western blotting and quantitative reverse transcription PCR (RT-qPCR) (Samsonov et al., 2016). Innovative method to translate this technology on chip are available in the panorama of tools for amplification and detection of nucleic acids. Among these, it is worth noting some recent works using this kind of technology, which is not limited to analysis of urine but suitable for a larger spectrum of applications. One example comes from the work by Cereda et al. who developed a system for the easy and automatized on-chip quantitative real-time PCR (qPCR). The platform is made of a non-disposable unit including all the tools for temperature switch, excitation sources, filters and optical readout and disposable cartridges, containing preloaded reagent mixtures organized into multiwell chambers. All the reactions and thermal cycling occur in the system and conditions change over time, without the need for moving solutions and reagents around the microfluidic chip. Control software allows the analysis to be performed, uploading of protocols and processing of results (Cereda et al., 2018). The same compact system has been translated to a series of on-field diagnostic assays for the easy and rapid detection of other pathogens (Giangaspero et al., 2019).

2.3. *Seminal fluid*

Seminal fluid (SF) is composed of secretions from glands in the male urogenital tract: approximately 40% of SF is prostatic material, released following global smooth muscle contraction and expulsion into the urethra, with the remaining contributed by the seminal vesicles and testes. SF is a miscellaneous and abundant molecular milieu, including nucleic acids, lipids, sugars, proteins, small metabolites and ions. Being mainly originated from the whole or bilateral male reproductive organs, it contains tissue specific mRNAs and miRNAs, more representative than tissue biopsy, which is a procedure not easily accepted and applied for male reproductive organs investigations.(Li et al., 2012). Its composition is mainly driven by prostatic activity, which is the reason why SF is highly enriched in PCa biomarkers, even more than urine and blood.

PSA, was discovered in SF, with a concentration which is approximately 5–6 orders of magnitude higher than in serum. Moreover, cells and secretion from both normal and malignant prostatic glands are naturally secreted into SF, thus indicating that they can be detected earlier with comparison to blood, where they arrive after crossing the barrier transition from tissue to the blood. This also implies that patients undergoing prostate biopsy could have had early detection of malignant cells from SF (Roberts et al., 2015).

The majority of patients with testicular germ cell tumours (TGCTs) also known as intratubular germ cell neoplasia, undergo surgical unilateral or bilateral biopsies, which are also performed in selected patients at risk of *Carcinoma in situ* (CIS), for example, those with a history of cryptorchidism. Hoei-Hansen and co-workers identified two important markers investigating by immunohistochemistry cells of probable CIS origin in the ejaculate: AP-2γ, expressed in fetal gonocytes, but not in cells related to ejaculatory pathway or in adult germ cells, and OCT-3/4, a transcription factor that is also not expressed in the adult testis and genital tract and is a marker of CIS, seminoma and embryonal carcinoma

(Hoei-Hansen et al., 2007).

Not only protein but also other kinds of biomarkers from SF have been associated with PCa. As an example, products of inflammatory metabolism were found to be unbalanced in PCa patients and this involved in particular citrate and zinc and other molecules like spermine and myo-inositol, as well as miRNA markers such as PCA3 and Hepsin (Roberts et al., 2011). The potential of miRNA diagnostics from SF is of great interest also for other tumours like testicular neoplasia or for pathologies related to male infertility. Altered expression of miRNA could be an excellent indicator of male reproductive health and capacity. This would be based on the fact that circulating miRNAs have been identified as indicators of subtle physiological changes when identified in body fluids and that circulating miRNAs are very stable (Pratt and Calcatera, 2017).

As an example, circulating miRNA of undoubted origin from testicular tissue (miR-371a-3p) has been established as molecular liquid biopsy biomarker (also in relation to the amount of germ cells) for diagnosis and follow-up of patients with malignant TGCT. However, the aim of a recent study by Boellaard was to demonstrate that miR-371a-3p is also reliable in SF, as a product of germ-cells, and so in direct connection with seminal pathways (Boellaard et al., 2019a).

Most of the studies on SF are generally conducted for male infertility research and many biomarkers are investigated with this purpose. Micro RNAs released in the form of free RNA or associated to exosomes or microvesicles (Tian et al., 2018) (cell-free seminal RNAs - cfsRNA) are promising molecules and their use could find applications also for other diseases and forensic investigations because of their high concentration in seminal plasma with respect to other body fluids (average 1.75 mg/l) (Huang et al., 2009). Table 3 gives a summary of reported potential biomarkers from SF to be used as diagnostic strategies for non-invasive cancer detection.

Table 3
Biomarkers from SF and methods of detection.

Type of cancer	Type of biomarker	Method of detection/Targeted Biomarkers/Ref.
Prostate	Nucleic acid	• Fluorometric quantification and the Electrophoretic analysis - cfDNA (Ponti et al., 2018a) • Fluorimetry - cfDNA & Seminal cell-free DNA (scfDNA) (Ponti et al., 2018c) • Qubit ssDNA Assay Kit and Electrophoresis - cfDNA & ctDNA; scfDNA fragments longer than 1000 base-pairs (Ponti et al., 2019) • Fluorimetric assay and Spectrophotometric quantification - scfDNA molecular profile (Ponti et al., 2018b) • Quantitative real-time PCR (RT-qPCR) and microcapillary electrophoresis – 5′ region of the ACTB (actin, beta) transcript; full-length ACTB and DDX4 [DEAD (Asp-Glu-Ala-Asp) box polypeptide 4] transcripts, (Huang et al., 2009)
	Protein expression	• Quantitative PCR, Western blotting and confocal laser scanning microscopy - chymase (CMA1), matrix metalloproteinases (MMP3, MMP7), and upregulation of MMP14 and tissue inhibitors (TIMP1 and TIMP2) (Neuhaus et al., 2017)
	Metabolites	• Liquid chromatography–Tandem mass spectrometry - NAD & kynurenine (McDunn et al., 2013) • Nuclear magnetic resonance spectroscopy - Citrate & prostatic secretions (Kline et al., 2006)
Testicular germ cell tumor	Nucleic acid	• Quantitative real-time PCR (RT-qPCR) - miR-317a-3p (Boellaard et al., 2019b)
	Cells	• Immunocytological staining - AP-2γ on Germ cells & gonocyte (Hoei-Hansen et al., 2007)

2.3.1. SF handling and storage stability

Despite some drawbacks to the use of SF for analysis coming from erectile dysfunctions or other religious or ethical reasons, the increasing use of this body fluid is forcing many healthcare structures to be equipped to manage and store this kind of specimen. The semen sample collection is already usually performed at home or in dedicated urological laboratories, by masturbation into sterile cups. Patients are invited to return their samples to the laboratory within 2 h for processing (Roberts et al., 2015), 1 h if the aim of the sampling is to investigate cell motility/viability or for sperm banking purposes (https://www.rogelcancercenter.org/fertility-preservation/for-male-patients/sperm-banking-procedures). Also, seminal plasma could be of interest for semen analysis and it is recovered after 1-h sample fluidification at 37 °C, centrifugation at 13000 rpm and supernatant collection at -80 °C. The more widespread use of seminal fluid storage is the cryopreservation (where samples can remain for tens of years) of spermatozoa for further use in Assisted Reproductive Technologies (ART). In these cases, the preferred storage method is liquid nitrogen, in which temperature variations are prevented and the temperature kept at a constant -196 °C in order to allow sperm motility preservation, DNA integrity and to guarantee fertilizing ability of cells (Mestres et al., 2020). A review of the effects on biological molecules and of the available strategies to preserve integrity of DNA and chromatin during cryopreservation is reported in the work of Kopeika and co-workers (Kopeika et al., 2015). Among the protective agent are listed: ascorbic acid, resveratrol, dimethylsulphoxide, antioxidants (β-mercaptoethanol) and glycerol supplementation of culture media. For use of SF as a biomarker source beyond that for reproductive purposes then in addition to cell viability being preserved, there is a need to contain the proteins, metabolites and nucleic acid. One of the works concerning the stability of enzyme activity, electrolytes and mineral concentrations was recently published, establishing that cell-free seminal plasma storage for 7 days at -18 °C does not affect electrolyte and mineral concentrations. AST, ALP and LDH activities are instead significantly reduced by around 20%, 50%, and 65%, respectively, indicating that for these kinds of assays, immediate handling is preferred over delayed analyses (Umbach et al., 2019).

The investigation of some important biological objects in SF, indeed, has been limited in comparison with other biofluids, because of variations induced by enzymatic processes. Some of these events include, in example, the amino acid and small peptide modifications caused by seminogelin proteolysis catalysed by PSA (Roberts et al., 2016) and the variability in concentration of TGM4 (protein-glutamine gamma-glutamyl transferase 4) and KLK3 of 24% and 14% respectively, already after 5 weeks at -20 °C (Drabovich et al., 2019). Protocols allowing metabolic assays include the snap freezing and thawing cycles at 4 and 37 °C, but in this way other components, such as RNA, could be irreversibly damaged. An attempt to overcome this issue has been performed by Roberts and co-workers who developed a strategy to preserve the activity of enzymes involved in the metabolism of choline like Prostatic Acid Phosphatase (a PCa biomarker itself), which has a rapid effect in hydrolysing phosphorylcholine. The inhibition of this enzyme through the addition of tartrate 10 mM to SF samples, immediately before storing them at 4 °C has led to improving the stability of choline and phosphorylcholine levels. This carries the double advantage that tartrate itself resulted in an inner concentration standard for NMR analysis to calculate the absolute molar concentrations of other metabolites of interest, without affecting the stability of mRNA molecules (Roberts et al., 2016).

The integrity of RNA molecules in stored SF has the potential to improve the plethora of cancer biomarkers from a relatively easy-accessible fluid and is very specific for pathologies of male reproductive tract. Xiong and co-workers combined the analysis of microRNA (cfs-miRNA) in order to prove that their presence is quite stable and can be used as biomarker for diagnostic and forensic applications. Semen samples liquefy within 30 min at 37 °C and a pair of samples are centrifuged twice: after the first centrifugation (1600 g for 10 min at

4 °C) the supernatant was collected and centrifuged again (16,000 g) to remove cells and debris. The final supernatant was carefully collected for subsequent assays. Genetic material investigated in this study included some male reproductive organ-specific transcripts (whole process spermatogenesis markers; post-meiotic spermatogenic gene; Epididymis-specific genes; Seminal vesicle-specific genes and Prostate-specific genes); miRNAs existing widely in epithelial cells of male reproductive organs (miR-34a, miR-141), testis-specific miRNA (miR-202, miR-449a), and two piRNAs (piR-013423, piR-023386). The stability of cfs-miRNA was evaluated by a time-course (0, 2, 4, 8, and 24 h, at room temperature) analysis. All these cfs-miRNAs were stable for at least 24 h. Only a very small amount of piRNAs (around 12% for both molecules analysed) remained after 2 min incubation at room temperature, confirming the presence of the RNase activity in semen. Interestingly, the authors observed that the amounts of cfs-mRNA in seminal plasma were almost the same as in the Seminal Microvesicles (SMVs), determined in the supernatant recovery after ultracentrifugation and filtration above 0.22 μm. Instead, if the samples were treated with Triton and proteases, the cfsRNA decreased dramatically. This demonstrates that the most of cfs-RNA is associated to SMVs with a diameter below 200 nm or to other protein complexes, which act as a protective enclave for RNA and allow for its detection (Li et al., 2012).

2.3.2. On chip analysis of SF

The on-chip manipulation of seminal fluid is mainly dedicated to the sorting and selection of cells suitable for in vitro fertilization procedures (Marzano et al., 2019). Indeed, a remarkable number of devices, including passive and active microfluidics have been demonstrated to be able to select high motility from low motility sperm cells (Cho et al., 2003), to sort chromosome X- or Y-loaded spermatozoa (Koh and Marcos, 2015) or to count them (Han et al., 2017). With respect to on-chip diagnosis of diseases, not directly related to sperm cells, none or very few examples are reported in literature. Chip technologies are sufficiently mature to provide tools for SF manipulation, without the need of laboratory instruments, and incorporate as part of protocols for handling SF for key proteome and metabolic biomarkers that have been identified by the standard methods such as NMR (Kumar et al., 2014), to provide a disease fingerprint (Roopa and Mark, 2014). This, considering that thermal stress causes the decay of specific RNAs, changes in translation and degradation rates of transcripts leading to a very high vulnerability of sperm after ejaculation (Paasch et al., 2003), is a possibility which is still unexplored but it would be a new frontier for non-invasive and automatic analysis, helping in fast and self-diagnosis of urology tract-associated diseases.

2.4. Sweat

There has been increasing interest in the use of sweat as a biofluid for POC health monitoring (Chung et al., 2019; McCaul et al., 2017). It can carry a range of biomolecules from small ions and molecules to large proteins and antibodies. Historically, sweat has been used for applications such as medical diagnosis of cystic fibrosis (CF) (Hammond et al., 1994) as well as fluid and electrolyte balance in athletes. These methods have traditionally used off-body measurements and in specialized settings by experts. The emergence of flexible and hybrid electronics allows the development of wearable sweat sensors for timely diagnosis and personalised treatment. A number of promising studies have been reported for various metabolites and neuroimmune biomarkers in sweat (Bandodkar et al. 2019, 2020; Emaminejad et al., 2017; Gao et al., 2016; Hong et al., 2018b; Nyein et al., 2019; Rose et al., 2015).

The transport pathways of biomarkers from blood into the tissue interstitial fluid (TIF) and sweat (Fig. 4) play an important role in sweat sensing and there is a need for more complete understanding of sweat dynamics (Heikenfeld et al., 2019). TIF will be in-depth discussed as a source for liquid biopsy in paragraph 3.2.

Biomarkers in blood enter the TIF through continuous capillaries and are separated from TIF and sweat by cell-based barriers. TIF is present in most of the dermis and surrounds the sweat glands. The secretory coil of the sweat gland is highly vascularised so that the lag time of a biomarker concentration in sweat compared with circulating blood can be relatively short (2–5mins). There is a strong correlation between the sweat and blood plasma concentrations of small hydrophobic (lipophilic) molecules - which can include steroid hormones and drugs – since these are transported transcellularly through the lipophilic cell membranes. Larger hydrophilic (lipophobic) molecules are typically transported through a paracellular route which although are dimensionally large (>10 nm between cells) have tight junctions - formed by different proteins - which leads to a high degree of filtration, e.g. sweat glucose is ~1% of the glucose concentration in the surrounding TIF. The biomarker concentrations can be confounded by other local processes, e.g. sweat lactate is confounded because it is also locally produced during the sweat generation by glycogen breakdown in the cells lining of the secretory coil. Those proteins that are too large to pass through filtering are thought to enter by a tight-junction remodelling effect. This could

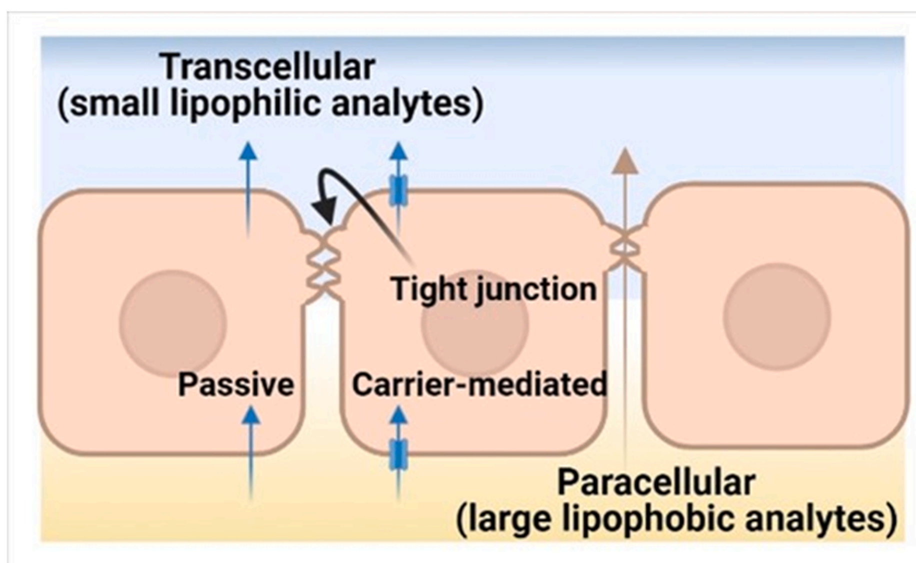

Fig. 4. Partitioning of analytes from blood and ISF to sweat through lipophilic cell membranes via transcellular and paracellular routes.

explain why proteins such as cytokines along with even larger viral antibodies, although very dilute, are found in sweat. The dilution of large analytes has the advantage that proteases and enzymes are also highly diluted which would slow the break down or metabolism of biomarker analytes. Most sweat sensing studies have focussed on endogenous biomarkers (e.g. metabolites and electrolytes), in comparison exogenous biomarkers, such as drugs, and biomarkers for biopsy have been little investigated. Given that most drug molecules have low molecular weight and have high lipophilicity, then they can be expected to partition in sweat in correlation with blood.

Sweat analysis could therefore be used as a proxy for drug concentration in the blood and used for new approaches in therapy management, drug-drug interactions as well as personalised dosing (Lin, 2020; Tai et al., 2018).

Biomarkers from sweat include mostly electrolytes, amino acids, carbohydrates, lactate, urea, volatile organic compounds (VOC) and sebum components excreted by the organism and which could be easily correlated to the general physiological monitoring, both during normal activities and physical exercise (Bariya et al., 2020). Recently, two review papers have addressed “skin metabolomic” (Elpa et al., 2021) and “volatilome” (Opitz and Herbarth, 2018), to encompass the possibilities of using sweat as a source of biomarkers for cancer diagnosis. Examples include Nonanedioic or azelaic acid for lung cancer (Calderón-Santiago et al., 2015); n-aldehydes and 2-ethyl-1-hexanol ubiquitous in sweat of cancer patients and absent among healthy individuals (Monedeiro et al., 2020b). Table 4 provides examples of biomarkers within sweat and the associated cancer types.

2.4.1. Sweat collection and use

Sweat has the simplest matrix in comparison with blood, urine, saliva and seminal fluid and has the advantage that it can be analysed directly on the skin surface where it is secreted; on the other hand, the amount of the sample could be a challenging point without sweat stimulation (in the order of microliters if not induced by stress, warming or exercise). Collection of samples can be achieved by occlusive wrapping, gloves or patches with adsorbent cotton pads placed between a porous layer in contact with the skin and a waterproof external cover or by wiping the skin with a cotton pad moistened with alcohol (Kidwell et al., 1998).

As an example, Human Kallikrein 9 (KLK9), a biomarker associated with higher grade gliomas has been found in sweat after strenuous exercise. The stability of native KLK9 in sweat was established in a 7-day experiment during which sample aliquots were stored at −20 °C, 4 °C and RT. The authors found that in all cases, KLK9 concentration in sweat was stable after 24 h. At RT, on day seven, the concentration decreased by about 40%. KLK9 spiked in serum, instead, decreased by about 60% after 24 h incubation and this was attributed to the formation of heterocomplexes with endogenous serine protease inhibitors (Filippou et al., 2017).

Most standard investigations on sweat are based on mass spectrometry and gas chromatography, and sample manipulation and preparation follows the protocols related to that method including thermal based extraction, centrifugations and addition of buffers (Jiang et al., 2019; Monedeiro et al., 2020b).

Table 4
Biomarkers from sweat and methods of detection.

Type of cancer	Type of biomarker	Method of detection/Targeted Biomarkers/Ref.
Ovarian	Amino acids	• Immunocapture/parallel reaction monitoring mass spectrometry - mat-KLK9 (Filippou et al., 2017)
Lung	Metabolites	• Qualitative tandem liquid chromatography quadrupole time of flight mass spectrometry - Suberic acid, tetrahexose, trihexose, nonanedioic acid & monoglyceride (Calderón-Santiago et al., 2015)
Lung, Gastric, Prostate, Head, Kidney, pancreas	Chiral alcohol	• Gas chromatography with mass spectrometry - 2-ethyl-1-hexanol, hexanal, octanal & 2,6 dimethyl-7-octen-2-ol (Monedeiro et al., 2020a)
Head and Neck	Metabolites	• Headspace solid-phase micro extraction (SPME) gas chromatography (GC) mass spectrometry (MS) – 81 VOMs as potential tumor markers (Opitz and Herbarth, 2018)
Prostate	Amino acids	• Mass spectrometry – KLK4 (Karakosta et al., 2016)
Various	Nucleic acids	• Real-time RT-PCR - DCD (Sakurada et al., 2010)

2.4.2. Wearable technologies for sweat analysis

Recent technological advances in wearable tools for sweat monitoring require a special attention (see Fig. 4). Electrochemical sensors, in example, including electrolyte monitoring of sweat chloride and pH have been fabricated at the inner of nitrile gloves to target high sweat rate sites (Bariya et al., 2020) (Fig. 5A and B). Also, skin-interfaced microfluidic platforms have been realized for the continuous collection and analysis of sweat. Rogers and co-workers fabricated a multi-layered structure, including a thin polyester film, a polydimethylsiloxane (PDMS) microfluidic network obtained by soft lithography in direct contact with skin through an inlet for sweat. All the structures were incorporated into a medical-grade acrylic adhesive layer. Sweat enters through the inlet as a consequence of secretory pressures of eccrine glands and progressively fills the microchannels. Microfluidic module is in direct contact with the sensing module made by flexible polyimide (PI)/copper (Cu) clad sheet hosting an array of interdigitated electrodes, able to perform capacitive measurements to monitor the flow rate of sweat into microchannels (Hourlier-Fargette et al., 2020). Zhang and co-workers in a recent study, developed an intrinsically stretchable sensing patch able to conformally attach to the skin for collection of sweat. Patches, realized in thermoplastic polyurethane nanofiber textiles decorated with silica nanoparticles assembled over a styrene–ethylene–butylene–styrene-based superhydrophobic substrate allowed for the formation of a large wettability contrast resulting in a very efficient sweat concentration. The device they obtained, supports multiplexed colorimetric analysis of sweat for pH and ion concentration measurements, with image acquisition through smartphones (Fig. 5C). There have been interesting developments in technological patches as wearable devices for sweat analysis that have relied on innovative polymeric biocompatible materials. A non-invasive bandage able to collect sweat samples was realized with agarose hydrogel micropatches embedded within a sheet of polytetrafluoroethylene (PTFE) (Dutkiewicz et al., 2014). More recently, Oh et al. fabricated an ELISA assay directly on a PDMS-based hydrogel that was able to perform sampling and rapid chemical profiling of skin metabolites excreted from sweat with a thumbnail-sized circular adhesive patch incorporating an array of microdisks acting as immunospots to quantify the level of several cytokines (IL-1 alpha, IL1RA, IL-17A, IFN-g, and TNF-alpha) of the stimulated skin sites (Oh et al., 2018) (Fig. 7D).

2.5. Stool

Faecal samples analysis is currently performed as a usual test to detect occult blood, associated with chronic inflammatory disease like Crohn’s disease or ulcerative colitis and colorectal cancer (CRC). CRC is one of the most widespread and lethal malignancies globally and it is thought that it takes around 10 years for adenomas, the precancerous lesions, to develop into ascertained carcinoma (Engel et al., 2018). However, clinical diagnosis of inflammatory bowel diseases (IBD) and gastrointestinal cancer is achieved through coloscopy/endoscopy which are well-known for their invasiveness, lack of specificity and intrinsic risks. Investigating the biomarker from stool (molecules, cells, extracellular vesicles and microbiota), in a completely non-invasive way, has the potential to overcome these limitations, and a number of established

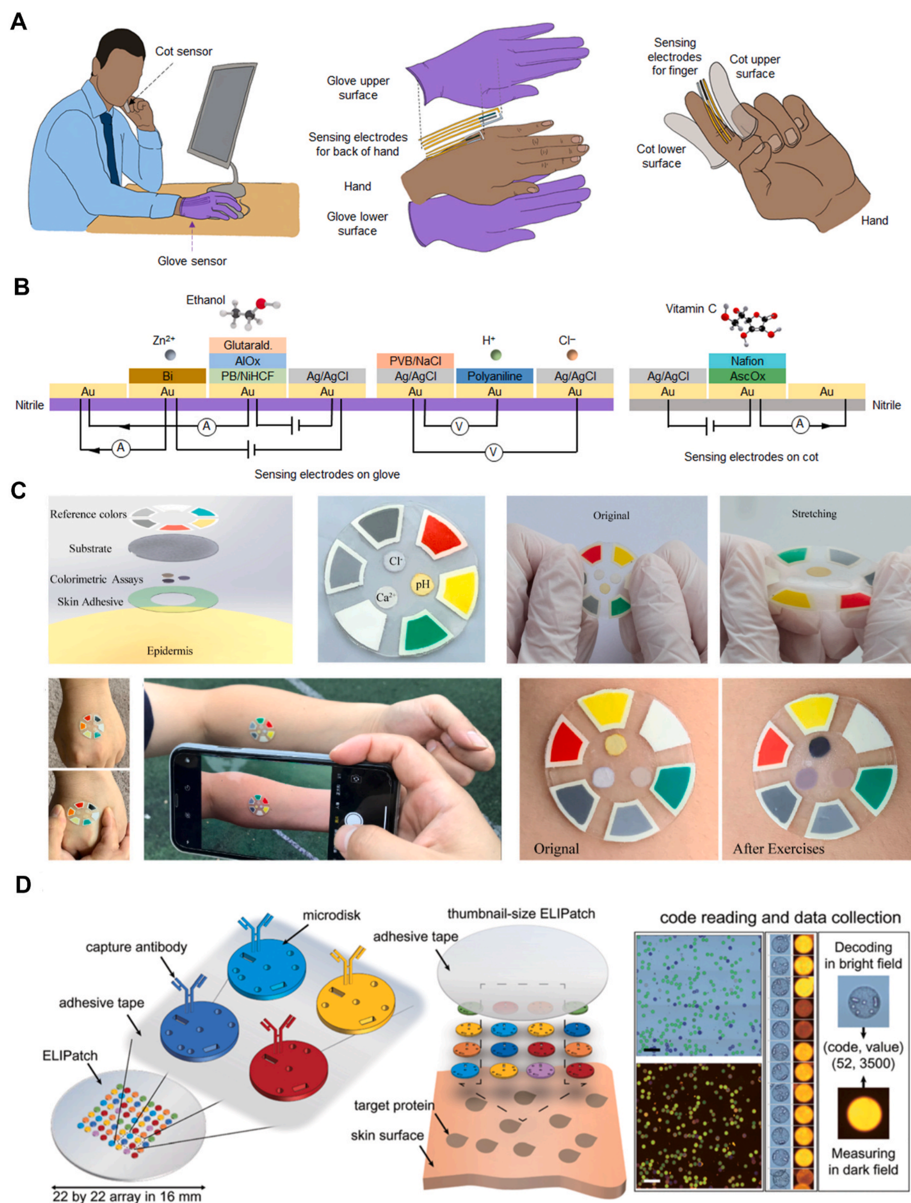

Fig. 5. A. Glove integrated sensing platform to analyse sweat on the fingertips, palm and the back of hand. **B.** Functionalized sensors onto the inner surface of nitrile gloves and measurement schemes for zinc, ethanol, PH, chloride and vitamin C. **C.** Schematic illustration of the wearable and stretchable sweat sensing patch realized by Zhang and co-workers and optical images of the epidermal sensing patch before and after physical exercise, inducing colour modifications of the sensing areas, captured by smartphone interface. **D.** ELIPatch platform including around 500 microdisks encoded by an antibody embedded into adhesive tape to be placed on human skin surface. The data acquisition process is performed by fluorescence. Scale bars, 1.5 cm. Panel of Fig. 5 has been designed adapting figures with Copyright permissions from references (Bariya et al., 2020; Oh et al., 2018; Zhang et al., 2021). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

assays have been proposed over the years. The majority of these, concern the detection of markers related to gastrointestinal tract pathologies (Mima et al., 2016; Pous-Serrano et al., 2017), including hepatic (Boursier et al., 2016; Zhang et al., 2019a) and pancreatic (Frost et al., 2019; Giuliani et al., 2020) disorders.

Moreover, faecal matter comes from the direct contact with the intestinal lumen so cells and extracellular vesicles are exfoliated directly from malignant colonocytes, and it is speculated that the earliest detectable molecular changes caused by CRC are present in stool before they appear in the blood.

Chassaing and co-workers associated the detection of up-regulated faecal lipocalin 2 (Lcn-2) to the presence of intestinal inflammation using a murine model of dextran sulphate sodium (DSS)-induced colitis. Faecal calprotectin, a calcium-binding protein mainly derived from neutrophils and from monocytes and reactive macrophages. This is another emerging biomarker that is significantly associated with the degree of histologic inflammation in the surgical specimen, showing better correlation with endoscopic activity than classic serum markers such as the non-specific C-Reactive Protein. Its presence in faeces, is directly proportional to neutrophil migration toward the intestinal tract and is the result of an inflammatory process which could be triggered in neoplasia, infections, and polyps (Pous-Serrano et al., 2017). During COVID-19 pandemics, the faecal calprotectin levels were found elevated in a significant proportion of patients affected by SARS-CoV-2. The increased calprotectin level could be related to gastrointestinal inflammation as a consequence of the general inflammatory process activated by the infection and could give information about the viral injury to the gastrointestinal epithelium. This implies that patients affected by IBD should also be tested for COVID-19 in the case of flare and diarrhoea (Jena et al., 2021). Moreover, SARS-CoV-2 has also been found in the stool of infected patients, and the association with high faecal calprotectin levels in COVID-19 is the proof of gut tropism (Udeh et al., 2021).

Also, in the case of stool, although the matrix is very complex in comparison to blood or other fluids, miRNAs have been demonstrated to be a very promising biomarker as they remain intact and stable for detection because of the exosomes packaging. For instance, Yu et al. observed that miR-135b could be used to differentiate between different stages of tumor growth. This miRNA can discriminate among adenomas, advanced adenomas, and CRC with 62%, 73%, and 78% sensitivities respectively. In addition, they found miR-18a and miR-221 to be

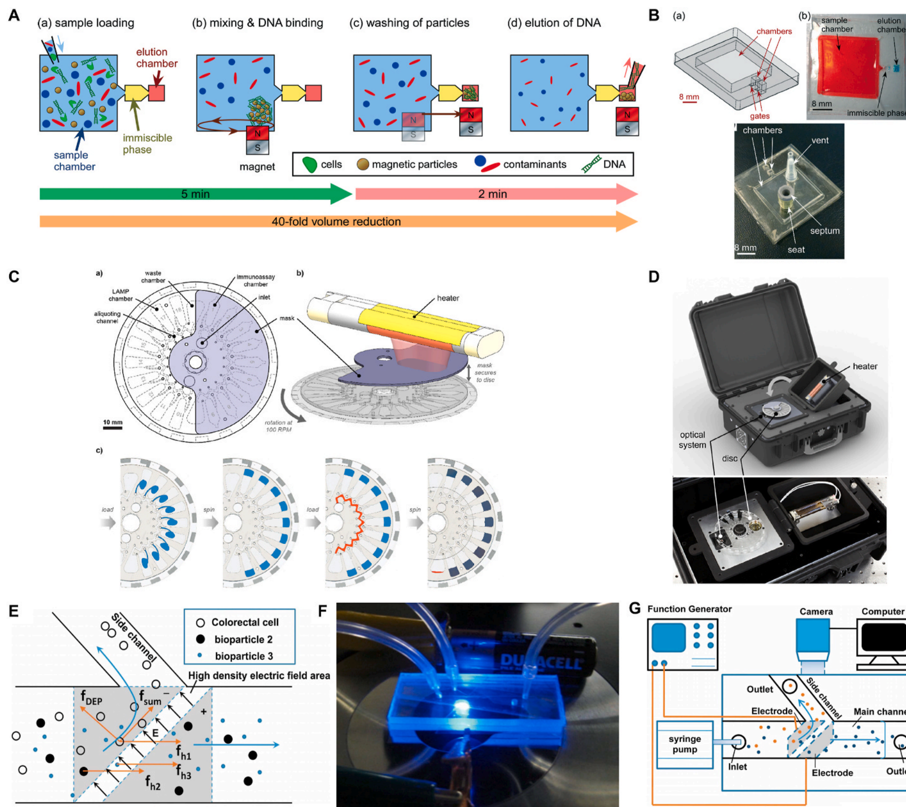

Fig. 6. **A.** Scheme of the DNA extraction process from stool samples based on the magnetic mixing of sample and DNA binding. Magnetic particles allow the movement and washing of the retained DNA molecules through the immiscible phase chamber, washing chamber and elution chamber. **B.** Assembled device with chambers and real device picture. Pictures A and B arranged with Copyright permission permission from (Mosley et al., 2016) **C.** On-disk platform for LAMP and immunoassay detection of intestinal pathogens causing diarrhoea, at the point-of-care. The centrifugal device pushes the sample from the inlet to the different spot of the disk where reactions occur. **D.** The instrument, in its compact assembly, puts the heater over the microfluidic disk mounted in connection with the motor and optical system. Scheme C and picture D adapted with Copyright permission from (Phaneuf et al., 2018). **E.** Geometry of the DEP platform for sorting colorectal cells from bioparticles by using electrodes positioned on the bottom surface. **F.** Optical image of the separation chip, placed under the microscope and connected with the syringe pump. **G.** Schematic illustration of the experimental setup, consisting of an inverted epifluorescent microscope, a function generator, a 10x objective lens, a CCD camera and a syringe pump. Pictures E, F, G adapted with Copyright permissions from reference (Yang et al., 2010).

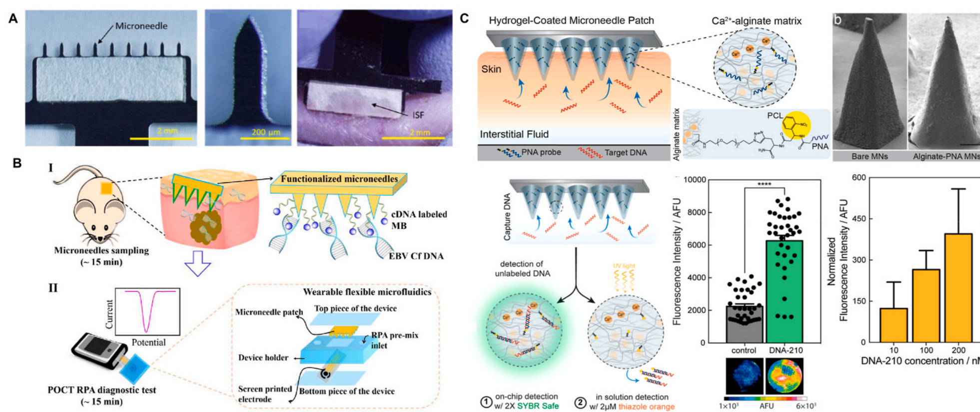

Fig. 7. **A.** Optical image of a patch containing 9 microneedles, each measuring 50 μm × 150 μm in cross section and 650 μm in length and paper reservoirs, before use and during use on hairless rat skin for interstitial fluid collection. **B.** Scheme of the use of microneedle patches for *in vivo* capturing of biomarkers thanks to the needle functionalization (I). Electrochemical microfluidic system for the cell-free DNA of Epstein−Barr virus integrated into flexible substrates and measured by a portable platform. **C.** Graphic illustration of the function of hydrogel-coated microneedle patch, functionalized with peptide nucleic acid probes allowing a doble mechanism of detection of unlabelled nucleic acids. On the left (top), a comparison between bare and alginate-PNA hydrogel coated microneedle by using scanning electron microscopy and (bottom) bar charts considering the efficiency capture probes to detect biorecognition events between PNA and MiRNAs. Figures in the panel have been reproduced and adapted with Copyright permissions from references (Al Sulaiman et al., 2019; Kolluru et al., 2019; Yang et al., 2019).

significantly upregulated in patients with CRC (with sensitivities of 62% and 61% respectively) (Ren et al., 2015). Ahmed et al. presented a work in which 12 miRNAs (miR-7, miR-17, miR-20a, miR-21 and miR106a (Link et al., 2010), miR-92a, miR-96, miR-134, miR-183, miR-196a, miR-199a-3p and miR214) were upregulated and 8 miRNAs (miR-9, miR-29b, miR-127-5p, miR-138, miR-143, miR-146a, miR-222 and miR-938) were downregulated in the stool specimens of CRC patients. The panel of these 20 miRNAs allowed the authors to differentiate not only CRC incidences from healthy controls but also different stages of disease progression (Ahmed et al., 2013).

Other kinds of biomarkers identifiable from the stool are epigenetic modifications of DNA, like aberrant methylation of DNA. Cologuard, the first stool-based CRC screening test approved by the US FDA, show a high degree of sensitivity and specificity (https://www.cologuard.com/en). It includes the detection of hemoglobin, multiple genetic mutations, and BMP3 and NDRG4 methylation sites as biomarkers. The kit is ready to use but it has to be returned to the laboratory within one day from the collection. Cao et al. investigated the clinical performance of stool-based methylated *C9orf50* and methylated KCNQ5 tests for the early CRC detection, claiming that the two methylated sequences, found singularly or together, could reach significantly higher sensitivity than those of Cologuard in the case of identification of precancerous lesions and early stage CRC (Cao et al., 2021).

A high number of biomarkers from stool are linked to the search for altered gut microbiota to intestinal disease, reflecting the importance of the intestinal microbiome (a collection of microorganisms, their genes

and genomes, living in association with the human gut) in complex multifactorial diseases like CRC and IBD. Among these, we can list the Gram-anaerobe *Fusobacterium nucleatum*, as causative agents for CRC, as has already been ascertained for *Helycobacter pylori* in gastric cancer. *F. nucleatum* is under investigation for promoting CRC growth since it induces mucin secretion and inflammatory cytokine tumour necrosis factor (TNF)-α expression, inhibits T cell-mediated immune responses against colorectal tumours and suppresses the activities of natural killer cells, thereby predisposing the host to adenomas or cancer development (Datorre et al., 2021). Microbiota composition identified from stool samples amplifying the V3–V4 region of the 16S rDNA gene from DNA extracts (through Illumina HiSeq 2500 sequencing) could serve even as a marker of inflammation position: the flora in the left-sided colon samples, includes *Clostridium perfringens* and Fusobacterium nucleatum, and may be associated with VEGF expression and colon cancer through DNA damage, methylation, and histone modifications. The microbiota distribution in the right-sided colon samples is less invasive and harmful and particularly rich in *Bifidobacterium dentium* (Zhong et al., 2020).

Table 5 summarizes a list of biomarkers from faeces.

2.5.1. Faeces sampling

The analysis of stool, such as the search of occult blood in faeces, requires sterile boxes for collection into a bar-coded container, chilled to between 0 and 4 °C, and then with specimens being shipped directly to the laboratory within 72 h after collection. The minimal quantity should be 30g and stored at –80 °C until analysis (Imperiale et al., 2004).

There exist several kits for blood identification, based on the colorimetric detection on guaiac paper (including vegetal guaiac resin), of a blue reaction which appears within seconds by applying hydrogen peroxide in the presence of blood. The techniques used for miRNA isolation from stool, typically require the use of commercial kits based on acid guanidinium thiocyanate and phenol-chloroform extraction.

2.5.2. Devices for stool handling and analysis

There are several examples of alternative assays for diagnostics in the field through the analysis of stool samples by microfluidics. Mosley and co-workers developed a rapid (7 min) DNA extraction procedure that allows direct on-chip processing of crude stool samples for the detection of *Helicobacter pylori*. The device is based on the principle of IFAST (immiscible filtration assisted by surface tension) and adapted to include a large volume sample chamber with a septum-based interface for stool sample introduction. The dissolution is helped by solid chaotropic salt and dry superparamagnetic particles (PMPs) which are directly stored on-chip and can be reconstituted upon sample addition, thus facilitating the release of DNA from *H. pylori* cells and its binding to the PMPs. Finally, the PMPs are magnetically moved through a washing chamber in which there is an immiscible oil solution and subsequently to the elution chamber where the DNA is released into aqueous media for subsequent analysis (Fig. 6 A and B) (Mosley et al., 2016).

Phaneuf et al. have realized a miniaturized platform, which is both portable and has high sensitivity for biological detection, using a novel sedimentation-based immunoassay on a centrifugal microfluidic device. The whole system was realized for the detection by LAMP and immunoassay of three enterotoxins (cholera toxin, Staphylococcal enterotoxin B, and Shiga-like toxin 1) and three enteric bacteria (C. jejuni, *E. coli*, and *S. typhimurium*) handling complex sample matrix such as stool. The platform was composed of three subsystems: (i) rotary control with a brushless DC motor paired with an optical switch to allow for both precise velocity control of the microfluidic disc, at a range of speeds from 5 to 5000 RPM for sample loading, centrifugation, and position control; (ii) optical detection system configured for high sensitivity laser induced fluorescence (LIF) measurement (a laser diode, a photomultiplier tube and a filter set); (iii) heating system mounted in an enclosure that swivels in proximity to the microfluidic disc and aligns the heating element to a radial strip on one side of the disk. When closed, a gasket positioned along the perimeter of the enclosure is compressed to form a tight seal, minimizing the loss of heat. Disk rotation at 100 RPM is sufficiently fast to ensure a uniform heating of samples for methods requiring elevated temperatures. Pathogens were detected simultaneously on a single disc at clinically relevant sample concentrations in less than an hour. Assays were performed independently and shown to be highly sensitive (limit of detection = 1.35–5.50 ng/mL for immunoassays and 1–30 cells for isothermal amplification) (Fig. 6 C and D) (Phaneuf et al., 2018). Microfluidic approaches have been described for the isolation of cancer cells from complex matrices, the applicability of these systems for real samples has yet to be fully demonstrated. A DEP-based cell sorter capable of continuous colon cancer cell isolation from other cells takes advantage of the use of microfluidic channels and was developed using plastic lamination-based microfabrication with the electrodes for DEP obtained in ITO (Yang et al., 2010) (Fig. 6E–G). A device with on-chip droplet generator and digital PCR was instead realized by Taly et al. for the quantitative and sensitive detection of KRAS oncogene mutations among wild-type sequences. The developed microfluidic system allowed single target DNA molecules to be confined in microdroplets together with fluorogenic TaqMan probes specific for mutated and wild-type KRAS. Droplets in which the amplification of mutant DNA took place, gave a green-fluorescent signal while the amplification of wild-type DNA resulted in red-fluorescent droplets (Pekin et al., 2011).

Table 5
Stool biomarkers and methods of detection.

Type of Cancer	Type of biomarker	Method of Detection/Targeted Biomarkers/Ref.
Colorectal	Nucleic Acid	• PCR - Stool DNA test 1 and 2 (SDT-1 and STD-2) (Ahlquist et al., 2008) • Fecal immunochemical test (FIT) and quantitative molecular assays - KRAS, NDRG4, BMP3 methylation & β-actin (Imperiale et al., 2014) • Fecal Immunochemical Tests (FIT) and Quantitative RT- PCR – Haemoglobin detection and colonoscopy (van Turenhout et al., 2010) • RT-qPCR - Multitarget stool DNA (mt-sDNA) (Malik, 2016) • Quantitative PCR - CIMP, KRAS, BRAF, PIK3CA & LINE-1 (Mima et al., 2016)
	Cells	• Dielectrophoresis – Colorectal cancer cells (Yang et al., 2010)
Colon	Nuclei Acid	• Fecal immunochemical testing (FIT) and RT-qPCR – mt-sDNA (Berger et al., 2016) • Illumina HiSeq 2500 sequencing - V3– V4 region of 16S rDNA gene (Zhong et al., 2020) • Methylation specific PCR - SFRP2, TFPI2, NDRG4 & BMP3 (Park et al., 2017)
Pancreatic	Metabolites	• Liquid chromatography with Quadrupole time-of-flight mass spectrometry - Leukotriene E4, vitamins B2, B5, B6 & taurocholate (Girlanda et al., 2012)

3. Other easily accessible body fluids

Bodily fluids described in the previous sections (saliva, urine, seminal fluid and sweat) are easy to be collected without the need for specialized personnel. Moreover, their storage and manipulation can be integrated into POC devices for automated analysis. In the following section, other body fluids that need minimal intervention from medical staff for collection or processing are described with associated non-invasive diagnostic assays.

3.1. Exhaled breath

The detection of volatile organic compounds (VOCs) from breath at trace concentrations is one of the most promising analysis methods to be integrated into POC devices with an acceptable accuracy and precision.

This specificity derives from natural physiological activity of alveolar tissues which are the direct interface between circulating blood and substances contained and air exchange. Measuring compounds contained in exhaled breath in the form of VOCs may provide non-invasive, painless, easily repeatable and real-time diagnosis of a number of pathologies. Several biomarkers from breath have been identified, at variable concentrations which strongly depends on breath collection method (Krilaviciute et al., 2015) and usually fall in the range of *ppm* to *ppb* (Bayrakli, 2017). Just to list a few, organic compounds like exhaled acetaldehyde and formaldehyde have been strongly associated with lung cancer (Fuchs et al., 2010; Wehinger et al., 2007); ammonia to the kidneys and esophago-gastric cancers (Kumar et al., 2013); hexanal, heptanal and octanal to breast cancer (Li et al., 2014).

Collecting samples for breath analysis is a non-invasive practice which could be very compliant for patients, but it could be discriminating for the expected concentration of VOC during analysis. Respiratory droplets are generally collected has exhaled breath condensate by asking the patient to exhale steadily and slowly at a normal breathing rate for 5 min into a dedicated device. This could be represented by a gas sampling bag, usually made of polypropylene or PTFE or Teflon or devices holding an adsorbent pad able to fix VOC molecules (Harshman et al., 2020). This method could be a double-edged sword in times of pandemics, as most of them are disposable objects, but some others which contain only a disposable portion should be carefully sterilized, as they come in contact with aerosols which can be the vectors for Sars-CoV 2 particles.

Analysis of exhaled breath is typically conducted by mass spectrometry, but new methods allowing integration into portable systems, based on laser spectroscopy methods or electronic nose, which allow the real time detection of examined compounds are on the rise. One of these, developed by Spagnolo and co-workers considers the QEPAS method. It is based on a 2f wavelength modulation-based Quartz Enhanced Photoacoustic Spectroscopy, which allows the fast gas exchange inside a compact gas cell with a volume <5 mm^3, very effective for real time breath measurements (Tittel et al., 2012). Other methods include the sensor arrays of polymeric materials like polyaniline nanocomposites (Le Maout et al., 2018) or semiconductor-based, miniaturized transductors for acetone, NO_2, NH_3, H_2S sensing, providing new opportunities for the design of highly integrated breath-analysis systems easily integrable into Internet Of Thing (IOT) systems (Yoon and Lee, 2017).

3.2. Interstitial fluid

Tissue interstitial fluid (TIF) is the fluid that can be found at the interface between circulating body fluids and intracellular fluids (Sun et al., 2010). Interstitial fluid originates from the blood and then leaks out of capillaries bringing nutrients to cells in the surrounding tissues. Because interstitial fluid is in direct communication with the cells, alterations could be reflected in TIF, making it an interesting source of disease biomarkers. If compared with plasma, TIF has the advantage of containing proteins in high concentrations in the medium surrounding cells and they are not muddled with unrelated proteins from other organs and tissues. Proteomics of TIF could take great advantage from this aspect, since low-abundance proteins are enriched in comparison to other biological fluids. In the case of tumor tissues, TIF proteomics could provide a new and promising avenue for detecting and discovering tumor biomarkers. TIF, indeed, contains many secreted proteins derived from tumor or tumor-associated cells released in the microenvironment, which can act as messenger factors. Furthermore, unlike in the serum and urine, in TIF the interference of highly abundant proteins is relatively low, making a comparison of the expression of TIF proteins between tumor tissues and adjacent healthy tissues easier. It has been estimated that the concentration in tumor TIF of a potential biomarker might be approximately 1000–1500-fold higher if compared to its concentration in serum (Wang et al., 2016).

From TIF proteomic assays, Celis and co-workers demonstrated that TIF contained some major serum proteins as expected, but its 2D PAGE protein profile was notably different in comparison to serum. Other abundant serum proteins (albumin, haptoglobins 1 and 2, ferritin and immunoglobulin) were detected in a comparable manner, while a few classical serum proteins like apolipoproteins C-III and J were absent (Celis et al., 2004). In the discovery path of biomarkers, proteins contained in tumoral TIF have been differentially identified for several tumor diseases. Just as few examples, Wang et al. identified the abundances of tubulin beta-5 chain (TUBB5), leucine-rich alpha-2-glycoprotein 1 (LRG1) and immunoglobulin J chain (IGJ) in a significantly higher concentration in the interstitial fluid of colorectal cancer mice than in control animals (Wang et al., 2016). Two ECM-associated proteins, SPARC and thrombospondin-2 (THBS2), were selected for further validation as biomarkers for hepatocellular carcinoma (HCC) in the case of low serum AFP levels and were associated with patients with poor prognosis by bioinformatic methods (Zhang et al., 2017a).

Halvorsen et al. in 2017 identified also miRNAs in the interstitial fluid of breast cancer tissues, as key factors in intercellular communication responsible for the cross-talk among cells in the tumor microenvironment. In particular, they found a total of 266 miRNAs at higher levels in the TIF of tumor samples if compared to normal tissue. Of these, 61 were detected in $>75\%$ of the serum samples. 7 of the 61 miRNAs were associated with poor patient survival and 23 with the colonization of immune cells and adipocytes (Halvorsen et al., 2017). More recently, the role of non-coding exosomal-associated Circular RNAs has been identified in the interstitial space by all types of cells as well as in the tumor microenvironment, opening the way to novel cancer-associated biomarkers (Tang et al., 2021).

Due to a number of technical difficulties associated with harvesting interstitial fluid from native tissues, TIF protein profiling may be rather problematic and in literature, there are no methods by which interstitial fluid can be isolated entirely free from any contamination. Even though interstitial fluid doesn't clot like blood, one of the issues related to recover TIF is to avoid a mixing with blood and peculiar practical concerns need to be taken into consideration. Among these, factors which can affect the quality of specimen are: the presence low-concentrated proteins *in situ* or diluted during the process of fluid recovering; the handling process could damage cells causing a release and contamination by structural or other non-secreted proteins into the extracellular space; an uncontrolled proteolytic activity may occur during isolation procedure. In a detailed review paper, Gromov and co-workers describe methods to obtain TIF from biopsies and they focus their attention on tissue centrifugation at low G-forces and passive elution from fresh tumor specimen (Celis et al., 2004; Gromov et al., 2013).

In vivo sampling of interstitial fluid without recurring to surgical biopsy could represent a very invasive method, above all if the tissue under investigation is not easily accessible. Due to this drawback, some methods now obsolete, consider animal models. Among these we can list *in vivo* insertion of a glass capillary (Sylven and Bois, 1960) and the implantation of small chamber with a porous membrane inserted into a growing tumor. TIF is drained into the chamber and it is collected for a certain time, allowing sampling at various time points (Gullino et al., 1964). Large pores microdialysis along with open flow microperfusion is another method which offers great promise in determining interstitial tissue concentrations of biomarkers, including the monitoring of therapeutic proteins such as monoclonal antibodies and also helping in understanding pharmacokinetic-pharmacodynamic relationship in tumor microenvironment. The most commonly used membrane probes for microdialysis are used for high molecular weight substances such as cytokines and proteins. Permeable membranes of an implanted microdialysis probe are typically made of nanoporous materials (polycarbonate, polyethersulfone, regenerated cellulose or polysulfone). In addition, the membranes have to be biocompatible to maximize host compliance and exert high flexibility. Usually, the materials used in microdialysis probes are not biodegradable. Microdialysis membranes made of biodegradable or reabsorbable materials may have the

advantages of long-term sampling and don't need additional surgical removal. On the other hand, the insertion of the probe carrying the membrane is untargeted, so the fluid could also hold intracellular content from lysed cells. Moreover, the injection may induce tissue trauma and alteration, causing local tissue inflammation and leading itself to alteration of cytokines levels, thus confounding results (Jadhav et al., 2016).

To overcome these limitations, the road to minimally invasive investigation of TIF primarily led to the development of tiny needles with an average length of 500 μm, that could potentially provide a new clinical monitoring and diagnostic testing tool. The microneedles, embedded into patch devices, are able to collect or directly analyse TIF through dermal interstitial fluid (Kolluru et al., 2019) (Fig. 7A).

The group of Samant et al. used a patch containing five solid microneedles to create an array of micropores in the skin coupled with mild suction. By pressing the patch at an angle into the skin of 21 human subjects, they reached only the outer layer of skin containing TIF. They were able to monitor glucose and caffeine pharmacodynamics without recurring to permanent subcutaneous sensors (Samant et al., 2020). A further step toward the integration of sensors on the surface of microneedles was made by Wang and co-workers, who functionalized microneedles with biorecognition elements. The tools penetrate the stratum corneum (or periosteum) and were able to selectively capture protein biomarkers from local TIF. Then, the microneedle patch was peeled off from the skin and captured protein biomarkers were quantified by an ultrasensitive fluoroimmunoassay (Wang et al., 2021). Yang et al. combined microneedles functionalized with hydrogel coated with capture DNA, to selectively capture Cell-free DNA Epstein-Barr Virus *in vivo*. Then, they achieved quantitative detection of the biomarker by incorporating a recombinase polymerase amplification electrochemical platform into flexible wearable technology (Yang et al., 2019) (Fig. 7B).

Recently, the group of Sulaiman was able to obtain the *in situ* detection of the biodetection from TIF through the development of a hydrogel-coated microneedle array able to sample up to 6.5 μL of fluid in 2 min and detect cell-free nucleic acid biomarkers. Microneedles in this case are covered with alginate polymers functionalized with peptide nucleic acid (PNA) capture probes, thanks to a specifically developed covalent chemistry, in order to allow a sequence-specific immobilization of miRNA of interest (Fig. 7C). Detection and quantification of the biorecognition event were possible by two ways: a direct visualization of the isolated biomarker directly on the microneedle patch or an alternative detection in solution of PNA/miRNAs by a more complex process involving light-triggered release from the hydrogel (Al Sulaiman et al., 2019).

3.3. Tears

The tear system of the eye, including nasolacrimal duct and tear glands are responsible for the production of tears, composed of water, salts, lipids and proteins (varying from 6 to 10 mg/ml) mainly including lysozyme, lipocalin, lactoferrin, lipophilin and albumin. When the eyelid blinks, tears are spread across the eye and it keeps wet for comfort. As the ocular mucous membrane (or conjunctiva) is a barrier to the external environment, the tear film also represents a barrier that contains high IgA levels (McKay et al., 2020). This is only one of the multiple functions of tear film, which include lubricating eyelids, conjunctiva, and cornea, removing foreign materials, supplying nutrients to eye tissues, neutralize bacterial infections thanks to high concentrations of lysozyme and lactoferrin. Proteomic analysis of tear fluid, identified mainly by HPLC, MS and SDS-PAGE, has provided information about the pathogenesis of diseases and led to new diagnostic possibilities (von Thun und Hohenstein-Blaul et al., 2013). Altered tear cytokine levels of interleukins (IL)-1α, IL-1β, IL-6, IL-8, TNF-α have been associated with the autoimmune ocular disease Sjogren's syndrome and in glaucoma patients (Chong et al., 2010). Transmembrane mucins such as MUC1 (Ca 15.3) and MUC16 (Ca 125) constituting Glycocalyx proteins are commonly detected in blood as cancer progression biomarkers and are present in tears at significant concentrations (Argüeso, 2020). Bohm et al. identified more than 20 proteins upregulated or downregulated in the tear fluid of control and breast cancer groups (Böhm et al., 2012) and more recently, also concerning metastatic breast cancer, oncogenic miRNAs miR-21 and miR-200c have been isolated in the exosomes from tears of patients (Inubushi et al., 2020).

A method for collection of tears makes use of Schirmer's strips, placed at lower conjunctival sacs of both eyes for 5 min. Acting by capillarity, they allow the water in tears to travel along the length of a paper test strip. The strips can then be immediately dried at 70 °C for 1 min and stored properly in plastic bags and used for proteomic assays (after protein extraction) (Qin W., Zhao C. (2019) Tears: Potential Window for Monitoring Systemic Conditions. In: Gao Y. (eds) Urine. Springer, Singapore.). This method has drawbacks including being time-consuming, high risk of contamination as well as analyte dilution and consequently compositional variations of tears which arises from tear overflow induced by irritation (Moreddu et al., 2019).

There have been recent innovations for the investigation of tear composition through direct contact with the eye surface using engineered contact lenses that incorporate detection tools as wearable technologies. The most appealing application in the development of such devices, was the continuous monitoring of sugar level of the body fluid, considered a red flag for diabetes and diabetes-associated diseases (Bruen et al., 2017). Parviz et al. developed a glucose-sensitive contact lens with embedded microfabricated amperometric sensor with the electrodes fabricated using multi-layered metal structures (Ti/Pd/Pt). Glucose oxidase is immobilized in a titania sol–gel layer, allowing a sensitivity enhancement to detect the low-level detection of glucose in tear fluid down to 0.01–0.07 mM (Yao et al., 2011). The configuration proposed, however, suffered from the visual obstruction and the offline measurements of the tears, as connection to the electrodes were required to obtain the results (Fig. 8A). In a recent work, Park and co-workers demonstrated the realization of transparent (>91%) and stretchable sensors on wearable soft contact lenses, able to detect glucose and intraocular pressure wirelessly. Key components of the devices are graphene and its hybrid with metal nanowires, ensuring comfort and unobstructed vision when the soft contact lens is worn by users. Wireless operations were achieved by sensor coupling to an external antenna via a magnetic field able to detect resistivity changes of the sensor (Fig. 8B) (Kim et al., 2017). A different approach for tears biosensing was explored by Yetisen et al. who developed a paper-based assay fully integrated into laser-ablated surface of contact lenses and embedded into a poly-HEMA lab-made contact lens, chemically bonded to seal the device. An inlet allows tear fluid to reach the sensing areas to detect tear pH (variations in the range of 6.0–8.0), glucose (from 2 to 10 mM), proteins (BSA concentrations from 0 to 8.0 mg mL^{-1}), L-ascorbic acid (LOD of 59 mg L^{-1}), and nitrites (0–160 μmol L^{-1}) in the physiological range. A smartphone-based readout of the colorimetric assay classifies the devices as a POC tool for self-monitoring of health parameters (Moreddu et al., 2020).

3.4. Earwax

Cerumen or earwax, is a substance produced by the external auditory canal apocrine sebaceous glands, including debris from exfoliated keratinocytes of stratum corneum. Keratin indeed is the main component (around 60%), other constituents are long-chain hydrocarbons, diterpenoids, alcohol and steroids (cholesterol and squalen). The earwax functions are related to the lubrication and moistening of ear channel, the removal of dust and water as a consequence of its lipid composition (Feig et al., 2013). The most direct application of cerumen analysis is the diagnostic application of local pathologies of the ear, as in example otomycosis or types of benign or malignant/inflammatory (otitis) pathologies of the outer ear. Moreover, as for hair analysis, cerumen is only a snapshot of a momentary condition, as in the case of liquid biopsy from

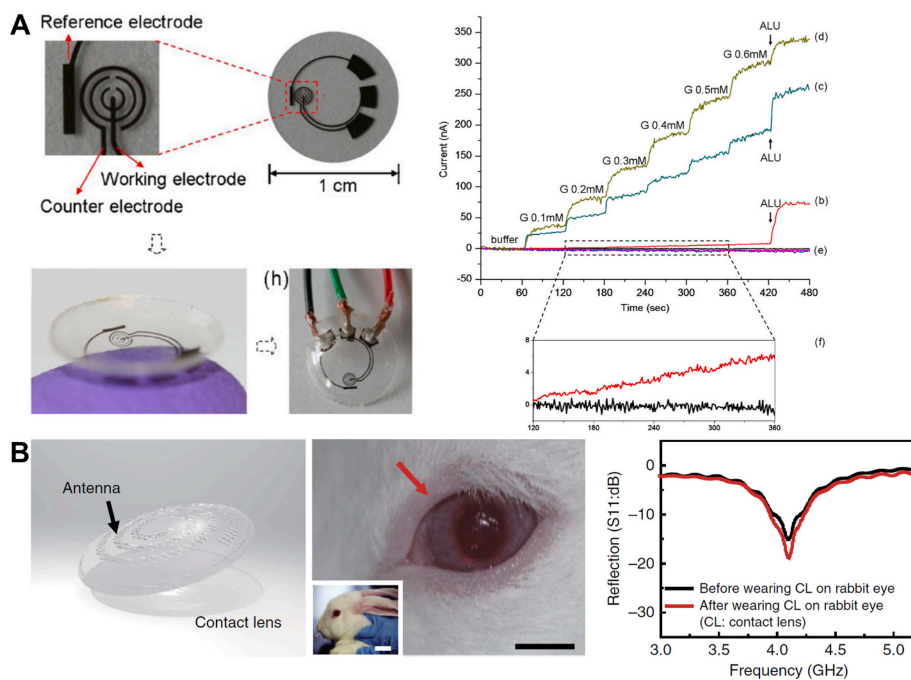

Fig. 8. Working principle of two contact lens devices with integrated glucose sensor. **A.** Scheme and optical image of a complete sensor for monitoring tear glucose level, before and after connected for testing. Amperometric measurements of the sensor after each step of functionalization. **B.** The wireless monitoring of glucose concentration from tears, achieved using magnetic field antennas, has been performed on the eyes of a rabbit, avoiding view obstruction problems. Black and white scale bars in the living experiment, are 1 cm and 5 cm, respectively. Picture arranged with Copyright permissions from references (Kim et al., 2017; Yao et al., 2011).

blood or the other fluids described in this review, but it can be the result of an accumulation of secretions over time captured in a single sample. To validate the applicability of cerumen as a novel biofluid used in clinical diagnostics for systemic diseases and stratification stage, there is a need to unravel earwax composition which has been a topic of increasing recent research interest.

Heales and co-workers have correlated earwax composition with some metabolic pathologies related to enzymatic deficiency or protein metabolism such mitochondrial fatty acid oxidation defects, phenylketonuria or organic acid defects and they associated the abnormal presence of some molecules in cerumen (acylcarnitine and derivatives, creatinine, amino acids not normally present like alloisoleucine, argininosuccinate, higher level of serine or phenylalanine) to each of these defects (Krywawych et al., 2020).

More recently, the analysis of earwax has been exploited for the detection of cancer biomarkers. In the work of Gonçalves Barbosa et al. the cerumen of around 100 volunteers divided between healthy and cancer patients (lymphoma, carcinoma or leukaemia) were analysed for the presence of Volatile Organic Metabolites (VOMs). 158 VOMs were identified by HS/GC-MS including organooxygen, organosulfur, carboxylic acids, organonitrogen and hydrocarbons. Of these, 27 VOMs selected by a genetic algorithm were able to successfully discriminate from cancer and a control group on the basis of a discrimination pattern (Barbosa et al., 2019).

Altered miRNAs were also investigated as inflammatory modulators of the host immune response in case of infective or inflammatory disorders. The study was conducted on healthy and otitis affected dogs and miRNAs expression was profiled by Next Generation Sequencing and validated by RT-qPCR (Lecchi et al., 2020), thus demonstrating that they can represent a biomarker identifiable in cerumen with analytical significance also for other species and other pathologies.

Sampling cerumen can be a home-made practice, or it can require the intervention of specialized personnel. Cerumen can be in the form of wet or dry, depending on ethnicity. The phenotype of cerumen is indeed determined by two alleles at a single gene termed as ABCC11 gene which results in the dry type typically prevalent in East Asians (95%) but rare in Europeans and Africans (3%). A mixed rate of dry and wet types is present in Native North Americans, Central Asia, the Pacific Islands, Turkey and Asian ancestry.

Sample collection of cerumen requires some hygiene practice in order to avoid contamination if cerumen will be employed for forensic testing or genetic material research. Sampling cerumen can be obtained using sterile plastic swabs gently rotated in the outer section of the ear canal. Also, plastic curettes, metal scoops or wooden spatulas can be used for dry earwax, while the so-called Jobson-Horne probe can be used for both wet and dry sampling. In these cases, a health professional intervention is required to avoid damages of the ear and infections. In the case of VOM analysis, there is a need to avoid the use of fragranced bath shower soaps to prevent assay interferent. Assay interferences are avoided by removing the first cerumen and disposing as waste, the ear is then irrigated with water and the remaining cerumen is collected 24 or 48h later using a moistened cotton swab soaked with an alcohol-ether mixture (Shokry and Antoniosi, 2017). Cerumen collection and storage was achieved by immediately storing samples at -80 °C until they are ready for use. The cerumen can be resuspended in Urea and Thiourea until full dissolution, filtered and centrifuged. Alternatively cerumen can be extracted from the cotton bud in 500 μl of methanol and stored at -20 °C for analysis (Krywawych et al., 2020).

To the best of our knowledge, no LOC devices have been realized for earwax processing and analysis.

4. Conclusions

The ongoing pandemic of COVID-19 has pointed out the necessity of distanced interactions in our social contexts and, unfortunately, this is also in the case of healthcare settings. The possibility of using liquid biopsy has the potential to strongly impact on the management, epidemiology and large-scale screening of disease and above all in cancer. This is particularly the case for the current pandemic, in which prevention and follow-up checks are neglected to avoid infection of already immunocompromised subjects. Mainly cancer has counted on an outstanding reduction in newly diagnosed cases and dangerous delays in surgical therapies and periodic follow-ups. The use of body fluids biomarkers and related technologies allows an innovative vision for traditional liquid biopsy, which has mainly been associated with blood analysis and significantly revolutionizes the standard approach for diseases. Recently, research dealing with innovative methods has seen a large expansion looking for a near-future strategy for overcoming the

limitations of standard examinations which often suffer from non-specific outcomes, low patients' compliance and sometimes the false positive response. Moreover, such an approach could be of great importance because of its feasibility of translation into POC applications and, in some cases, the technological development is already mature enough to allow the leap into the market with a relatively small prototyping effort. Indeed, while LOC technology devices may have high commercial potential, only a few devices have achieved commercial success. Many aspects need to be taken into account to add value: standardization of technologies and materials, scale economies for the mass market, having the right application and, perhaps more importantly, integration of the sample preparation process. Validation rules and regulatory authorities should be also taken into account.

The recent years have seen tremendous progress in LOC device development but, despite the high performance achieved in terms of sensitivity/specificity, they require additional equipment to run and they frequently use off-chip pre-processed samples to analyse. In many cases, samples must be handled by qualified professionals before being tested *on-chip* and the results of the analysis must be elaborated by expert scientists. On the other hand, LOC developed for sample preparation often don't allow the analysis to be performed on the same platform. In all of these cases, it is more realistic to call these devices not *lab-on-a-chip* but *chips-in-a-lab*.

For commercial applications, the "*chip in a lab*" bottleneck must be sadly overlooked in favour of a more simplistic yet crucial technology that prioritizes usability and smooth component integration. Moreover, the choice of materials, user-friendly design and *plug-n-play* connections (Zoupanou et al., 2021a) are basic components that are necessary for ensuring market penetration and exploiting the added value that the technology offers. In many cases, indeed, the proof-of-concept devices use methods and materials which allow a high customization of architectures but are not suitable for the rapid shift to industrial context. Leakage problems, instability of connections, rapid degradation of chemically modified surfaces are undesirable for commercial manufacturing (Berthier et al., 2012; Nguyen et al., 2020). One example is the use of materials like glass, SU8, PDMS in combination with optical or soft lithography, which are very common in research laboratories, but require further modification, expensive post-processing procedures and standardization before reaching the industrial scenario. Their success requires a drastic reduction in the costs of technology implementation and single device prices is a crucial aspect for mass production. Parameters of response time and portability are strictly related to the

Table 6
Technology readiness grade for liquid biopsy samples analysis.

Sample	Test	Market readiness: H (high); M (medium); L (Low)	Offline Sample prep required	Trained personnel required	Response time	Notes
Saliva	Chromatographic strip kit	**H**: disposable cartridge, reagent kits. Low-costs	No	No	Less than 15 min	Needed other confirmatory tests https://www.screenitalia.it/test-rapido-covid-19-saliva/
	Microfluidic polymer platform and fluorescent integrated reader	**H**: disposable plastic LOC cartridge (low-cost), portable fluorescence reader	No	No	20 min for fluid flow	T. U et al. (2020)
	Magnetic immunoassay	**M**: automatic reaction available, materials used not suitable for industrialization (PDMS) and microplate reader required for measurements	No	Yes	60 min	Lin et al. (2018).LOD 4 ng/ml
	Electrowetting	**L**: materials used not directly prone to industrial exploitation, but suitable to miniaturization and automation.	Yes	Yes	N/A	Abdelgawad (2020); Srinivasan et al. (2004)
Urine	Colorimetric, paper-based microfluidics. Naked eye detection.	**H**: already available on the market for pregnancy, ovulation and others	No	No	Less than 15 min	Abarghoei et al. (2019)
	Microfluidics	**M**: sample purification device, measurements off-chip. Materials used not suitable for industrial exploitation.	No	Yes	N/A	(Liang et al. (2017); Rzhevskiy et al. (2020)
Seminal fluid	Microfluidics for sample preparation	**L**: material used not suitable for industrial exploitation, off chip analysis	Yes	Yes	N/A	Huang et al. (2020)
Sweat	Wearable devices	**H**: sensor-integrated skin patches.	No	No	Real-time detection	Dutkiewicz et al. (2014); Hourlier-Fargette et al. (2020)
Stool	Colorimetric detection	**H**: Blood identification kits already on the market (low-costs).	Yes	No	Few seconds	https://www.mayoclinic.org/tests-procedures/fecal-occult-blood-test/multimedia/fecal-occult-blood-test-kit/img-20007541
	Microfluidics, (IFAST - immiscible filtration assisted by surface tension)	**H**: PMMA and magnetic-beads based device (low-costs), sample analysis on-chip.	No	Yes	7 min	Mosley et al. (2016)
	centrifugal microfluidic device	**H**: automatic rotary motor paired with an optical switch, followed by isothermal amplification of pathogen DNA.	No	Yes	<1 h	Phaneuf et al. (2018)
Exhaled breath	laser spectroscopy methods or electronic nose	**M**: sensor arrays with high degree of miniaturization and multiplexing	No	No	N/A	(Le Maout et al. (2018); Tittel et al. (2012)
Interstitial Fluid	Microfluidic sampling	**H**: collection and analysis integrated into skin patch- microneedles, low-costs	No	No	Real-time	Al Sulaiman et al. (2019); Kolluru et al. (2019)
Tears	Wearable metal nanowires sensors	**H**: contact-lens integration of sensors and wireless detection	No	No	Real-time	Kim et al. (2017)
	Paper-based microfluidics	**H**: contact-lens integration of sensors and smartphone based colorimetric detection	No	No	N/A	Moreddu et al. (2020)
Earwax	VOM analysis	**L**: chemical contaminants could affect measurements.	Yes	Yes	N/A	Shokry and Antoniosi (2017)

advancement of technology and materials used to contribute to the suitability to user-friendly devices spreading.

Considering the whole aspects of market readiness, following summary table (Table 6) reports technologies investigated in this review paper, together with the assignment of High (H) Medium (M) and Low (L) classification.

Furthermore, in some cases, once features of robustness and industrial grade are achieved, the gap to overwhelm is the validation of the assay by international regulatory authority in compliance with standard performances in terms of sensitivity, specificity and reliability, and this is not an aspect of secondary importance.

In conclusion, issues raised in this review can't be approached by a single point of view and we are convinced that a multidisciplinary vision can help to overcome the gap between basic research and applied technologies. The right consideration of biological fluid is fundamental as well as the possibility of sample collection without pain and inconvenience for patients. Secondly, the self-collection of specimens is related to the stability of the sample itself, or to the possibility of including the sample into a conservative medium, able to preserve the integrity of the components to be analysed. There are several biomarkers from biological fluids indeed, which are understated despite their high diagnostic value. One of the problems is the difficulty in sample management and processing, or the lack of experience in storage and stability preservation of biomarkers contained. On the other hand, advances in technologies for Point-Of-Care analysis and rapid sample treatment have made remarkable progress, which allow for rapid detection and prompt diagnosis. With the COVID-19 pandemic emergency, the needs of these new technologies accelerate. Thus, we would like to highlight that it's time to push mature non-invasive and Point-Of-Care technologies toward a prompt market exploitation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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