Paper-based biosensors for cancer diagnostics

Claudia Pereira,[1,2,3,¥] Claudio Parolo,[1,4,¥] Andrea Idili,[1,5] Roger R. Gomis,[6,7] Lígia Rodrigues [3,8], Goreti Sales[2], Arben Merkoçi[1,7]*

[1] Nanobioelectronics & Biosensors Group, Institut Català de Nanociència i Nanotecnologia (ICN2), Campus UAB, Barcelona, Spain.

[2] Biomark, Sensor Research, Superior Institute of Engineering of Coimbra, Rua Silvio Lima 3030-790, Coimbra, Portugal

[3] CEB - Centre of Biological Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal

[4] ISGlobal, Barcelona Institute for Global Health, Carrer del Rosselló 132, 08036, Barcelona, Spain

[5] Department of Chemistry, University of Rome Tor Vergata, Via della Ricerca Scientifica, 00133 Rome, Italy

[6] Institute for Research in Biomedicine (IRB Barcelona), The Barcelona Institute of Science and Technology, 08028 Barcelona, Spain.

[7] ICREA, Institució Catalana de Recerca i Estudis Avançats, Barcelona, Spain.

[8] LABBELS – Associate Laboratory, Braga, Guimarães, Portugal

*Correspondence: arben.merkoci@icn2.cat (Arben Merkoçi)

¥ the authors contributed equally

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Abstract

The implementation of a wide diagnostic campaign to early diagnose cancer could save millions of lives and billions of dollars every year. Unfortunately, cancer diagnostic is extremely complicated and current approaches rely on the use of expensive equipment and specialized personnel, which hamper their deployment in low- and middle-income settings. Here, we analyze the technical challenges that must be overcome to achieve precise cancer diagnostics and we describe how such hurdles have limited the development of point-of-care (PoC) sensors. Then, we explain why we believe recent

achievements in the field of paper-based sensors could allow their use as widely-available sensing platforms for cancer detection. Finally, we present our vision of what should be done in order to make paper-based sensors widely used diagnostics platforms for cancer.

Global impact of cancer

From a global perspective, cancer is considered a disease of epidemic proportions, accounting for 1 in every 6 deaths in 2018 and an estimated 9.6 million deaths, according to World Health Organization (WHO) [1-3]. The introduction of new treatments has allowed an improvement in the overall outcome for patients, greatly decreasing death rates and increasing their quality of life [4,5]. However, this is not the real picture if we observe homogeneously worldwide (Figure 1). The clinical improvement in diagnostic techniques and treatments comes with higher costs and this could threat the stability of healthcare systems worldwide in the next future[6]. In fact, the financial burden has reached such proportions that, in some countries, physicians are forced to balance out the course of testing and treatment per patient in order to minimize costs, leading to ineffective diagnosis and inadequate treatments [7]. Hence, the question is: should we be more focused on how to improve treatments or diagnosis? One widely accepted idea, regardless the type of cancer, that has been constantly discussed, is that early diagnosis holds the best chances to decrease the financial impact of cancer [8,9]. This relates with the fact that patients in more advanced stages of cancer require costlier treatments, such as chemotherapy or targeted therapy [10,11]

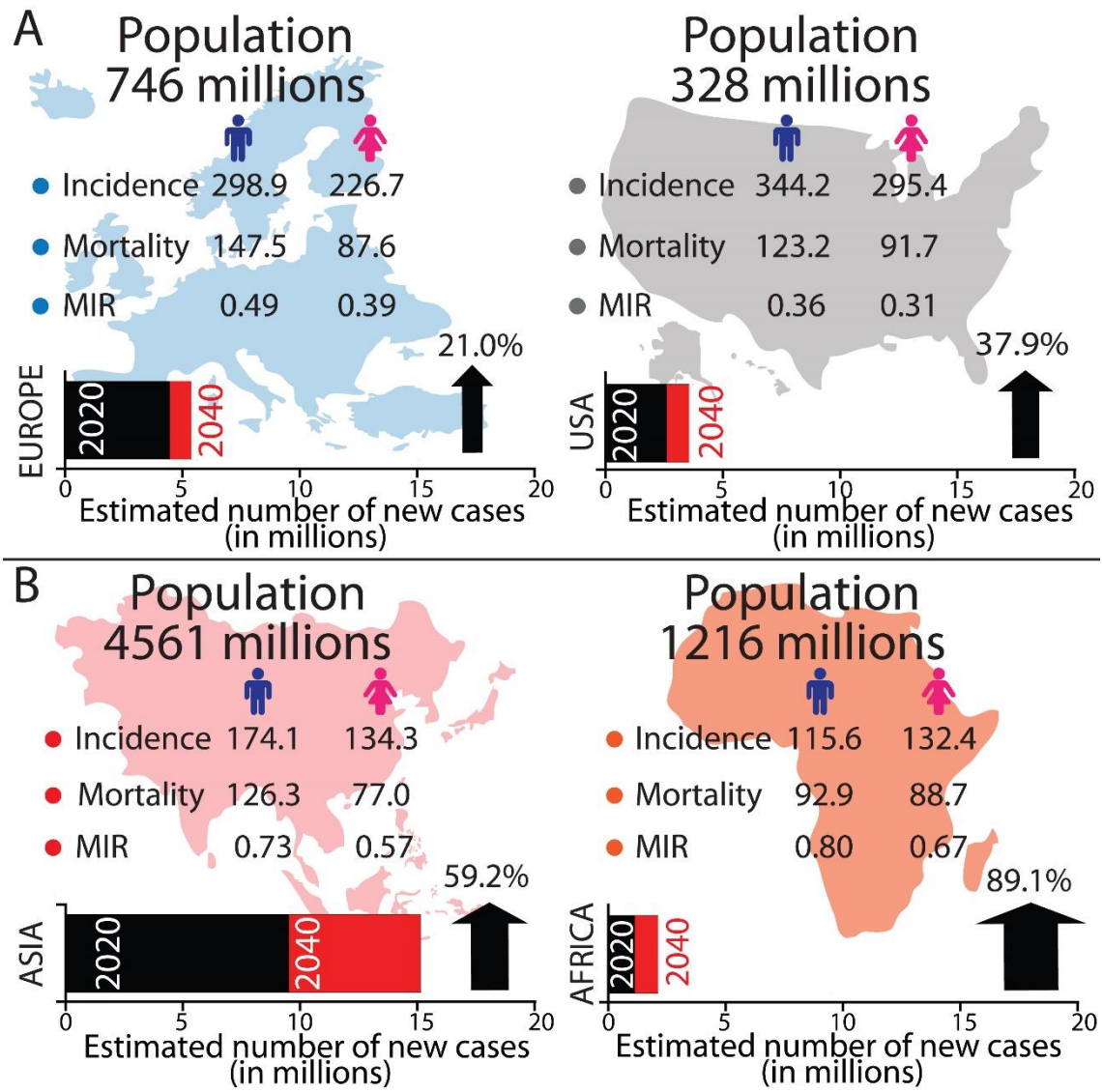

Figure 1: The impact of cancer on healthcare worldwide. Despite the global increase in the direct costs associated to cancer, the incidence and mortality have not homogenously improved, showing a great disparity between high-income and middle-/low-income countries. Specifically, Asia and Africa display a lower overall cancer incidence and mortality rates compared to higher-income regions such as USA and Europe, and these trends are observed in both sexes. However, this pattern is the reverse if we consider the Mortality to Incidence Ratio (MIR), which is found to be higher in Asia and Africa compared to USA and Europe in both sexes, with men having a higher MIR compared to women across all regions [12]. Besides the high cost of new treatments, this disparity is also due to the need for lower-income countries to deal with other epidemics related to infectious diseases, which undeniably take a good portion of the healthcare budget [9,13]. Worrisomely, a projection for 2040 shows how cancer cases will increase

more in Asia and Africa than in USA and Europe. Therefore, unless this increment is compensated by a MIR decrease, these areas will face an increase mortality as well.

Cancer diagnosis limitations

The accurate diagnosis of cancer requires the identification of three main characteristics: the tumor primary site, the type of cells, and the tumor stage (Figure 2) [14]. Indeed, knowing where the cancer initially developed and the type of tissue involved is essential to understand how it will behave and progress [15]. Specifically, relying on the primary site alone is not the most accurate strategy due to tumors' and patients' heterogeneity (Box 1). That is, even tumor cells from the same organ can show distinct morphological and phenotypic profiles that lead to a different disease progression [16,17]. For this reason, pathologists routinely examine multiple sites of the same tumor in order to identify the prevalent type of cells and, therefore, select the best treatment [18]. During this complex process, medical doctors often rely on the use of diagnostics exams that can help them to better classify the tumor.

Looking into diagnostics, imaging techniques and laboratory-based tests (e.g., Immunohistochemistry, ELISA, PCR) use harvested tissue and blood samples to detect and discriminate the cancer type [19]. Although they are still efficient and used routinely, these techniques will soon become insufficient for a proper diagnosis [20]. In fact, cancer's complexity, entails the need for more accurate screening methods able to identify the type and stage of cancer [20]. One example is breast cancer screening, where false-positive rates in conventional mammography canbe as high as 20% [21]. Another important pitfall of current techniques is their cost. For example, in the US the mean costs for diagnostic mammograms are 493 dollars, for ultrasounds are 134 dollars and for biopsies 2,343 dollars [22]. Additionally, considering that false negatives account for an estimated 2,8 billion dollars annually, the need for more sensitive, accurate, and cost-effective diagnostic tools becomes clear [22].

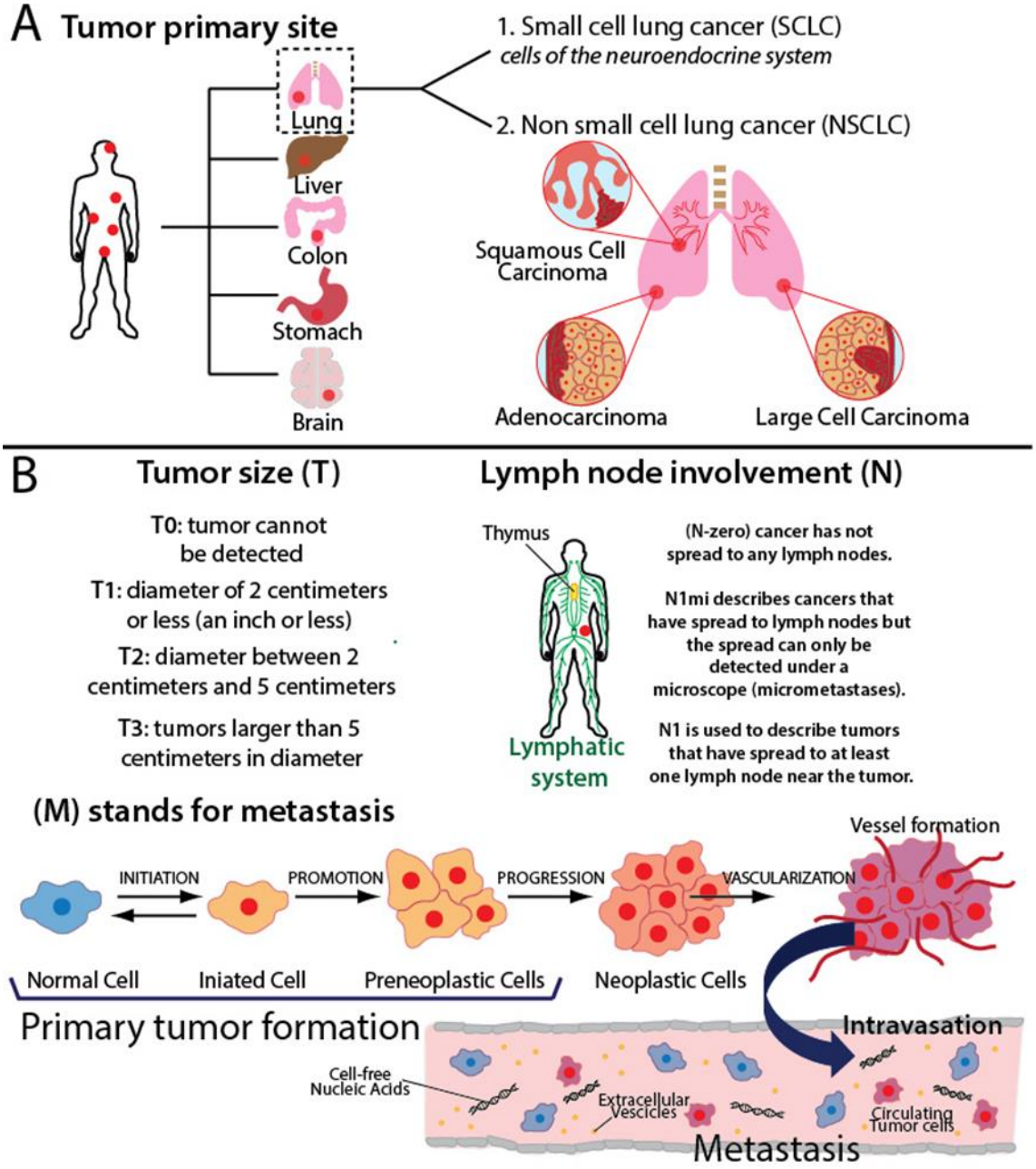

Figure 2: Cancer classification. The TNM is the standard system for cancer malignancy classification and helps understand the aggressiveness of the tumor. This system considers the tumor´s morphological characteristics and translates into the classification of stages from 0 to 4, where 0 is related to a tumor that has not spread, while 4 relates to metastasis and high malignancy. [23]. A) The tumor's primary site is the starting point for classification. B) The system allows to assess the tumor size (T), invasion to adjacent tissue/nodes (N) and possible metastasis (M) when malignant cells' migration through blood vessels to another site is implied [23].

Multiplexability

Due to their heterogeneity, many types of cancer display abnormal levels of specific biomarkers that not only are not exclusive to this disease but also present levels that may vary from stage to stage [24]. For this reason, the detection of multiple cancer biomarkers could help not only to determine the presence of a tumor, but also to discriminate its typology and stage in a cost-effective manner [25]. Some studies have been performed to validate this multiplexed approach [26,27]. For example, in 2008 a study compared the accuracy of diagnosis when monitoring levels of the noncoding RNA transcript PCA3 in urine alone over the monitoring of six putative prostate cancer biomarkers. [26]. The authors concluded that the multiplex approach provides higher accuracy when compared to the analysis of either PCA3 or prostate-specific antigen (PSA), the standard biomarker for prostate cancer screening.

Sensitivity

If we search the literature for cancer biomarkers, we will find thousands of hits. However, out of these thousands, only a small group of 20 biomarkers are FDA approved for their use in diagnostic tests [28]. Indeed, finding new clinically-relevant biomarkers for cancer detection is not an easy task. Looking at a molecular level, cancer status often implies countless of overexpressed genes that not always correlate with over-expressed proteins [29]. Moreover, since most biomarker candidates belong to pathways common to both normal and oncologic tissues (e.g., angiogenesis, proliferation, and differentiation), even when there is a higher expression of a given protein biomarker it may be difficult to discriminate it from other physiological conditions [29]. Even when we consider a gold standard technique like histology, for example, the lack of sensitivity imposes a problem for early detection. For instance, although more than 15 years may pass from cancer initiation to patient's death, such time window drops to just 4 years using histology as diagnostic method [29]. In fact, considering that average size of a metastatic tumor is 9 mm in diameter, in order to reduce mortality by 50% (e.g., in ovarian cancer) the tumor should be detected when its size is only 5 mm [29]. At date, only a few expensive and laboratory-based techniques (e.g. biopsies, mammography, ultrasounds) are able to detect tumors of such dimensions [19].

Paper-based diagnostics

Up until a decade ago, the required sensitivity and multiplexability for cancer diagnostic were only possible by employing laboratory-based sensing platforms, such as polymerase chain reaction (PCR) for genetic screening or enzyme-linked immunosorbent assay (ELISA) for antigenic testing [30]. However, these technologies are not suitable for a widespread use. In 2016, the WHO estimated that 1 billion people were deprived of healthcare services worldwide [31]. This led several nations to reformulate public health policies taking into account cost effectiveness and new sustainability goals. These changes included the extensive use of PoC biosensors that adhere to the REASSURED (Real-time connectivity, Ease of specimen collection, Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end-users) criteria (Figure 3) [31,32]. In this context, in the last few years, paper-based devices made huge steps towards bringing multiplexability and ultra-sensitivity out of the laboratory. This is also reflected by their market size, with projections estimating that, for the USA alone, it will reach 10.5 billion dollars by 2025 [71].

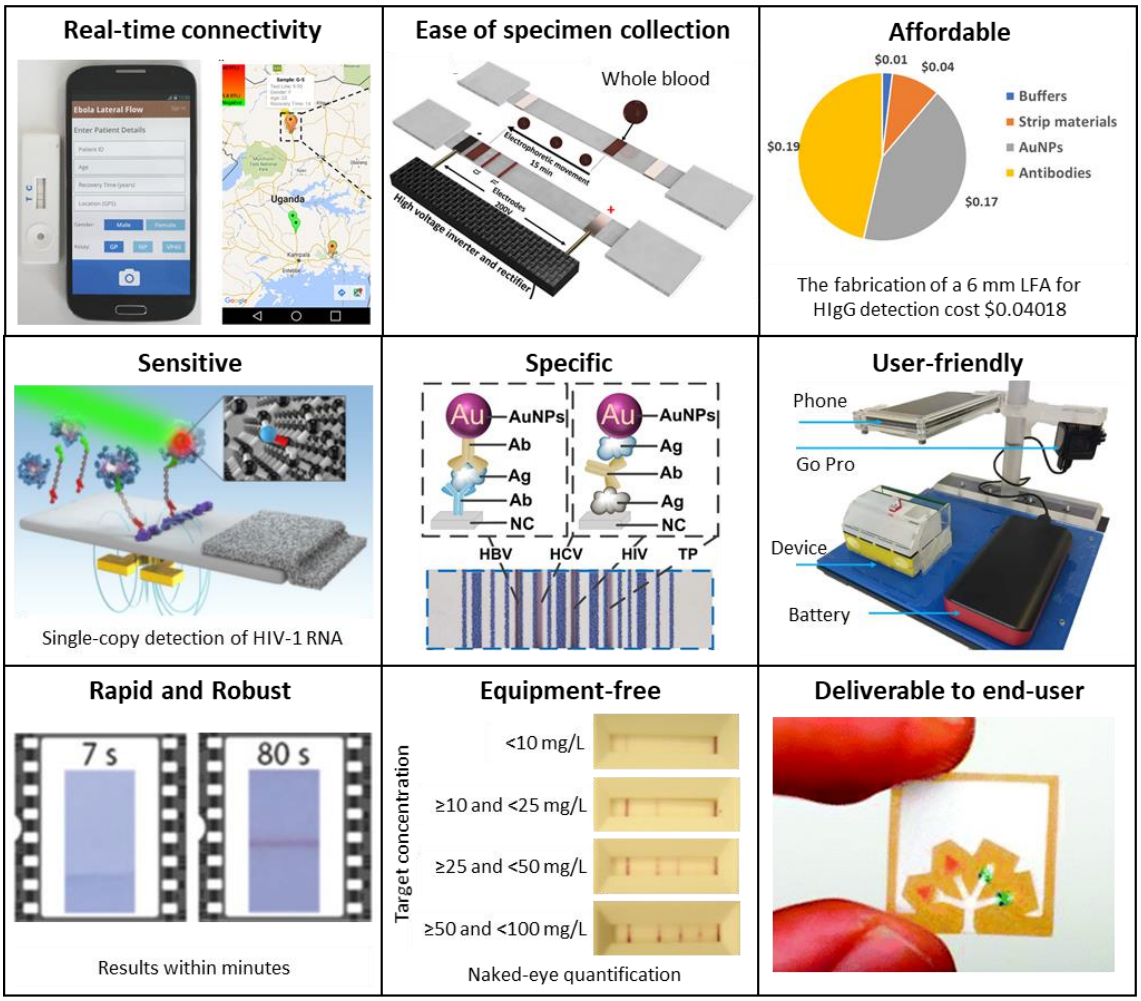

Figure 3: Paper-based sensors as REASSURED devices. Although the REASSURED criteria was originally formulated to define PoC sensors for infectious diseases [32] (which require a prompt (ideally sub-hour) diagnosis), it can now be extended to cancer diagnostics as well. During the last two decades, advances in the development of paper-based diagnostic devices have allowed to achieve analytical and usability performances that matches those required by the REASSURED criteria [32]. That is they have/are: real-time connectivity [33], ease of specimen collection [34], affordable [35], sensitive [36], specific [37], user-friendly [38], rapid and robust [39], equipment-free [40] and deliverable to the end user [41,42]. Besides accuracy and usability, paper-based sensors also provide great economic and environmental sustainability, supporting their use in mass-scale diagnostics campaigns.

Paper-based devices for cancer biomarkers detection

After conducting an extensive literature research in the major peer-reviewed article databases, such as PubMed, Scopus, Web of Science, and Google Scholar, we found a total of 57 (Table 1 in Support Information) research studies that correspond to the search in the title/abstract field of the following group of keywords: “paper-based” and “biosensor” and “cancer”; “paper-based” and “sensor” and “cancer”; “biomarker” and “biosensor” and “cancer”; “paper-based” and “biosensor” and “tumor”; “paper-based” and “device” and “cancer”. We then excluded all the reviews and studies without disclosed recognition elements or indirect cancer biomarkers. Considering that using only the key words “biosensor” and “cancer” gave over 1900 hits, we consider 57 studies to be a substantially low number. However, as we already mentioned, until now PoC sensors have mostly been employed for the detection of infectious diseases biomarkers. Out of the 57 manuscripts, only 23 tested the biosensor in human serum (Table S1, in blue), 14 of which are immunosensors. Moreover, none of these biosensors are considered to be ready for clinical use, since they not fully adhere to the REASSURED criteria [32]. In the following section, we analyze the major advances achieved to date and how they can represent the foundation for a new era in cancer diagnostic.

Paper-based tests for multiplex biomarker detection

The simultaneous analysis and quantitative detection of multiple biomarkers at the point-of-care is essential to achieve the required clinical sensitivity and specificity for cancer diagnostics [25,27,43]. Historically, multiplexed PoC devices have suffered many drawbacks, such as poor reproducibility, difficult and long optimizations, high cost and potentially complicated signal read-out (especially by naked eye) [35]. However, recent technological innovations in the field of paper-based sensors have overcome most of the aforementioned limitations.

The majority of advances aiming at the development of multiplexed paper-based sensors have been focused on the detection of biomarkers for infectious diseases or metabolic disorders [37,44-46]. For example, Yang *et al.* developed a multiplexed barcoded paper-based device using inkjet printing [37]. The authors claim that, by using their method, it is possible to obtain barcodes with 16X higher coding ability that the standard Codabar. As test bed, they detected 8 different biomarkers (for Hepatitis B Virus, Hepatitis C Virus, Human Immunodeficiency Virus type 1, and human Treponema Pallidum) 8 different drugs (penicillin, sulfadimidine, tetracycline, erythromycin, enrofloxacin, spectinomycin, thiamphenicol, and dexamethasone) and several nucleic acid sequences (for Ebola virus, hepatitis A virus Vall7 polyprotein gene, hepatitis B virus surface antigen gene, and human immunodeficiency virus) in human serum samples. Using a different approach, Joung *et al.* used a vertical assemble of functionalized paper layers to perform the rapid (20 min) colorimetric multiplex analysis (13 individual immunoreactions) in patient's serum samples for the detection of Lyme-specific antibodies [45]. Furthermore, the use of smartphone and cloud-based analysis provided the system with real-time connectivity allowing images to be sent to a server for quick analysis and be returned back to the user. On the contrary, Li *et al*. exploited a microfluidic 3D approach to fabricate 3D branched microfluidic channels which allow to temporally resolve chemiluminescence signals. As test bed for their system, they detected with good sensitivity small molecules such as glucose, lactate, cholesterol, and choline [46]. Finally, Guo *et al*. developed a paper-based fluorogenic immune-device that, by using zinc oxide nanowires (ZnO NWs), could enhance fluorescence signals up to 5-folds. They demonstrated its applicability simultaneously detecting 3 cardiac biomarkers (human heart-type fatty acid binding protein, cardiac troponin I and myoglobin) with good sensitivity and selectivity in a 5 minutes test, without compromising portability [44].

In the cancer field, we found 14 studies where the authors developed a multiplex approach, 8 of which tested the biosensor in human serum. Wang *et al.* [47] developed a chemiluminescent sensor for the detection of three cancer biomarkers (α-fetoprotein, CA 125, and CEA) in their clinically-relevant range. The novelty of this work relies on the modification of paper with chitosan in order to achieve the covalently modification of the paper surface with antibodies, therefore, conferring higher stability and robustness to the device. Building up on this work, Ge *et al.* [48] used a fluorescent approach for the detection of the same biomarkers, along with CA 153, improving the detection limits of two-order of magnitude [48]. However, we must note that both works employ an ELISA-like procedure (i.e., including blocking and washing steps) that compromises their use at the point-of-care, being not fully user-friendly nor rapid. Another innovative work based on the use of fluorescence signal is the one by Deng *et al* [49]. In particular, the authors built a paper-fluidic chip device that allowed to detect simultaneously two small RNAs sequences from tumor samples. In this work, the authors combined several innovative solutions to achieve the desired analytical performance keeping a high usability. They used poly(ether sulfone) to extract and purify the RNA, an hairpin probe to exponentially amplify the targets, magnetic sheets to facilitate the assembly of the device and quantum dots to obtain a strong fluorescence signal.

In the electrochemical field, Wang *et al.* [50] used wax- and screen-printing to fabricate paper-based electrochemical aptasensors for the detection of CEA and neuron-specific enolase in clinical serum [50]. Specifically, they obtained excellent analytical properties (with linear ranges covering five orders of magnitude) by modifying the working electrode with a combination of nanomaterials (including amino functional graphene, gold nanoparticles and Prussian blue), while at the same time including features such as automatic sample filtration and auto injection that facilitated the use of the device by non-trained personnel. In a different work, Wang *et al* [51] fabricated a 3D electrochemical immunosensor based on a multi-wall carbon nanotubes assemble. Using screen-printed electrodes and a sandwich detection system, the HRP-O-Phenylenediamine-H_2O_2 allowed to simultaneously detect 2 tumor biomarkers with high sensitivity [51]. Also using a sandwich immunosensor, Ortega *et al* [52] employed a paper-based electrochemical detection system to detect Claudin 7 and CD81 in extracellular vesicles. The channels were fabricated using wax printing and the electrodes were printed with graphene oxide

and silver ink [52]. Remarkably, the authors successfully validated the performance of their device using 80 patient samples.

More recently, Xu *et al* [53] proposed a novel sensing method for the detection of six cancer biomarkers using mass spectrometry and a paper-based substrate. Specifically, they developed a one-step hexaplex device that uses just 10 µL of sample for screening the six biomarkers within 30 min. The paper spray ionization (PSA) was employed in circle-cut papers and, along with a sandwich assemble resembling the ELISA approach, the final device was able to overcome some of the challenges associated with PSA-MS technique [53]. Furthermore, the authors successfully applied their method inserum samples from 12 patients who have been diagnosed with cancers at several sites, including breast tumors (benign and malignant), gastric cancer, and liver cancer.

On the bright side, we have just seen how several works successfully used paper-based sensors for the multiplexing and quantitative detection of clinically-relevant biomarkers. This is a crucial aspect to achieve an early cancer diagnosis as well as a personalized treatment. However, there is still a margin to improve. In fact, while a few works detected more than five analytes, the vast majority still detect just up to three different biomarkers, which is not enough to really impact the way cancer is diagnosed. Similarly, the usability of those approaches should be improved. Undoubtedly, all of them are much easier to use than an ELISA, but they are probably not easy enough to be deployed at the point-of-care. In this respect, we believe that including usability studies in future works will strengthen the awareness that achieving excellent analytical and clinical performance is not enough to have an impact in the real world.

The enhanced sensitivity of recent paper-based tests

The quantitative analysis of biomarkers in the sub-nanomolar range is essential to achieve a precise cancer diagnostic. Looking at the past fifty years, it is clear how paper-based tests have been "relegated" to the detection of highly-concentrated biomarkers. From a technical perspective this is reasonable considering that most of these sensors do not have incubation or washing steps. However, this limits the time window for the bioreceptors to interact with the target biomarker and impeding the active removal of potential interferent molecules [35]. More recently, however, advances in bioengineering and

nanotechnology have provided solutions (Figure 4) enabling to achieve remarkable low detection limits and quantitative results while keeping their ease of use [36,54-59].

As for multiplexing, the majority of the works achieving ultra-sensitivity in paper-based devices are focused on non-cancer-related biomarkers [60]. Probably the most impactful work to date is the work from Miller *et al*., where the authors, using spin-enhanced nanodiamonds, improved the sensitivity of the biotin-avidin model in a lateral flow assay over five orders of magnitude reaching the impressive limit of detection of 8.2×10^{-19} molar concentration [36]. Using the same set up, and following a 10-min isothermal amplification step, the authors also detected a single-copy of HIV-1 RNA using clinical plasma samples. The authors achieved such outstanding limits of detection by harnessing the fluorescence detection of nanodiamonds. Looking at their work from a REASSURED perspective, the only concern we find in the work from Miller *et al.* is its ease of use and therefore applicability. In fact, nowadays it is possible to find commercially available, miniaturized, optical sources and filters at reasonable prices [61], as well as companies selling nanodiamonds of different sizes for a cost similar or lower to the one of AuNPs. However, being able to detect such low target concentrations must be related to the complete removal of the non-specific accumulation of nanodiamonds on the test line. Currently, this is possible just by including extra washing steps that makes the whole process longer and cumbersome.

When it comes to improving sensitivity, the use of amplification techniques is always a convenient solution. In particular, due to lower cost and simplicity, isothermal amplification techniques tend to be easier to integrate into point-of-care diagnostic devices than the PCR. In this context, a remarkable work using the recombinase polymerase amplification method is the one made by Jauset-Rubio *et al.,* where the authors developed a lateral flow assay able to detect 8.67×10^5 copies of DNA using a constant temperature (37° C) in less than 15 min (including both amplification and detection) [54]. Besides the analytical and usability performance, the authors designed the probes in such a way that the recognition event on the paper-based test happen via hybridization rather than using antibodies/hapten couples. This sensing architecture allow to improve the stability of the device and its shipping and storing capabilities. Instead, using the loop-mediated isothermal amplification, Suea-Ngam *et al.* were able to detect single copies of methicillin-resistant *Staphylococcus aureus* (MRSA) using silver nanoparticles as labels in a lateral flow assay [59]. The authors managed to integrate the

entire assay into the paper surface and used a smartphone camera for quantitatively analyze the results, making the whole device portable and extremely easy to use. Finally, Chen *et al* designed a testing platform for the detection of miR-31 using the CRISPR/Cas12a system on a paper-based strip [57]. This platform combined the CRISPR/Cas12a system with the "invading stacking primer" (IS-primer) amplification reaction in a one-pot reaction at a constant temperature. Using their system the authors could detect the target in diluted saliva without extraction procedures in 90 min at attomolar levels [57]. A common criticism to the use of isothermal amplification technique is the requirement of hardware that maintain a constant temperature for the whole duration of the assay. Fortunately, recent advancement (such as the use of paper-based batteries [62,63] allows to achieve such control at a low cost and in an integrated form, making possible the use of isothermal amplification techniques also at the point-of-care.

Besides optical readouts, paper-based electrochemical sensors have also achieved excellent limits of detection [63] For example, Yao *et al* developed an ultrasensitive wireless aptasensor for kanamycin detection (down to 30 fg/mL) which is based on flexible freestanding graphene paper [56]. In order to achieve such low limit of detection, the authors integrated in the sensor a nuclease-assisted amplification strategy, where the nuclease (DNase I) allowed the catalytic recycling reaction of target. This is an important aspect since, while the use of enzymes allows a dramatic amplification of the signal, when employing them at the point-of-care it is always essential to verify their activity after prolonged storage and shipping. In another work, , Sun *et al*. used molecular imprinted polymers obtaining a limit of detection of 0.87 pg/mL for glycoprotein ovalbumin [55]. Combining the use of nanomaterials (i.e., Au nanorods, nanoceria) and the hybridization chain reaction, the authors obtained very low limit of detection However, the synthesis and fabrication of several nanomaterials, as well as the use of enzymatic amplification, may limit the deployment of the strategy at the point-of-care. Finally, Cinti *et al.* showed the possibility to detect breast cancer mutations employing the signal ON and OFF approach in a paper-based electrode [58]. Particularly relevant from this work is the extremely ease of use of the device, requiring just the addition of the sample (in this case whole blood) directly on the working electrode to obtain a quantitative readout. However,

the sensitivity of the device does not allow to achieve the detection of the biomarkers in their clinical relevant range.

Overall, the strategies discussed to increase the sensitivity of paper-based sensors rely on some external or integrated hardware which either control the temperature to achieve the ideal conditions of isothermal amplifications or is required to carry out the fluorescent/electrochemical measurements. However, in most cases, the hardware employed has a low cost and can be easily miniaturized, making the use of this amplification strategies potentially suitable for point-of-care applications, as long as they maintain an adequate ease of use (as it has been the case of glucometers). In our opinion, as we discussed for the work of Miller *et al.* [36], the main challenge will be minimizing/eliminating non-specific adsorptions. However, we believe that the implementation of flow control strategies such as the work of Sena et al. [34]may allow to achieve ultra-sensitivity without sacrificing the ease of use.

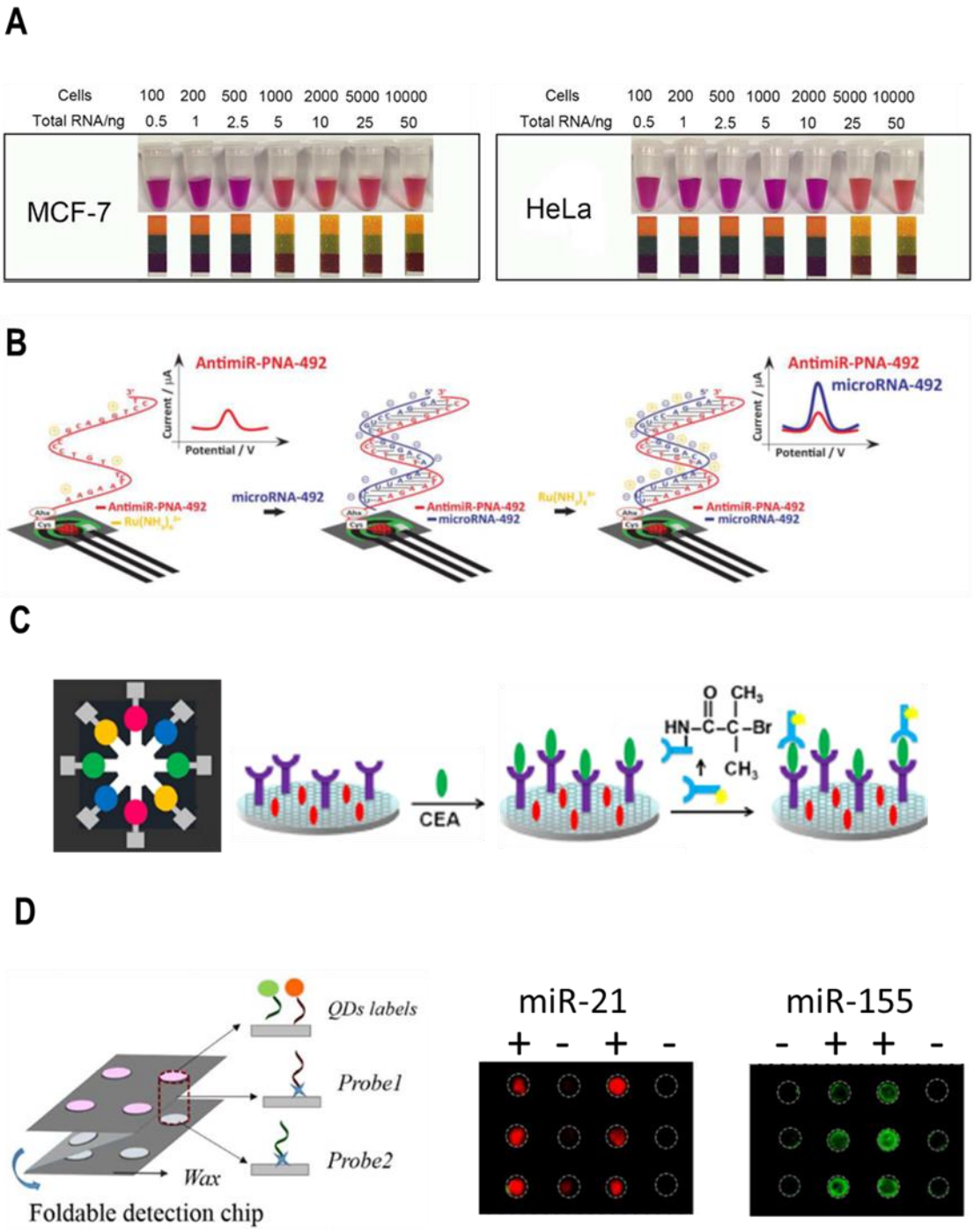

Figure 4: Paper-based sensors for cancer biomarker detection. Although the use of paper-based sensors for cancer detection is still at its dawn, several interesting approaches have been reported. In particular efforts have been focused on addressing sensitivity and multiplexing issues in order to make the results of paper-based sensors clinically relevant. Regarding the sensitivity in A) Feng *et al*. proposed a paper-based colorimetric test that supports the naked-eye detection of cancer-related miRNA down to 0.41 amol/ng in HeLa cells [64]. B) In another example Moccia *et al*. detected down to 6 nM of cancer-related

miRNA using a paper-based electrode [65]. Regarding multiplexing C) Wu *et al.* have reported a paper-based microfluidic electrochemical device able to carry out a radical polymerization reaction that allow to achieve the detection of 4 difference cancer biomarkers down to 0.01 ng/mL [66]. D) Finally, Deng *et al*. developed a fully-integrated paper-based device capable to both extract, purify and fluorescently detect down to 3×10^6 copies of multiple miRNAs [49].

Translation of the technology into clinical practice

The current scenario in the field of paper-based sensors is quite promising. Novel sensing platforms with improved analytical performance are coming out on a monthly basis. Despite this huge productivity, the development of bioanalytical devices is still mostly based on a trial and error approach during the whole process of the fabrication and optimization. While this approach has been fruitful so far, we should also note how the improvements are generally not very significant or very rarely are successfully transferred to the market. In this context, we believe that developers could take advantage of the use of a more computerized approach that may allow a faster and optimized analytical performance of their device [67-70]. This in turn will generate a substantial decrease of the developing costs and a higher chance of getting the product to the market. Similarly, the implementation of artificial intelligence models during the data analysis may result in a convenient tool to improve the sensitivity and specificity of the sensor, without the need to further modify the biological or hardware components [67,71].

Besides the challenges inherent to technology development and the lack of a universal model of diagnosis, there are socio-economic aspects to consider in order to achieve a massive cancer diagnostic campaign. For example, even a high-sensitive test able to detect multiple cancer biomarkers may not be able to provide a clear positive or negative diagnosis per se, since variations of patient's biomarkers may be associated to other medical conditions. Therefore, the test results and other patient's symptoms should always be analyzed by a trained oncologist. In the perspective of a mass-diagnostic campaign, this implies the formation and deployment of numerous healthcare professionals and potentially some adaptations of current infrastructures (i.e., storage and disposal of samples and tests).

Another challenge is managing the impact that such diagnosis can have in a patient's life [72]. For example, even just the idea of being tested for cancer creates such an emotional stress that patients may avoid the testing altogether. At the same time, patients diagnosed as positive for cancer may require psychological support that should be implemented immediately after the test result. This creates the need for other new professional figures that accompany the patients during the whole screening process [72]. Besides the psychological burden of the testing, the patient must be guaranteed that third parties cannot access the results. In fact, a breach in the data security could imply a dramatic increase in the cost of health and life insurance, as well as discrimination during job applications. Therefore, it seems clear that to succeed in implement a massive cancer-diagnostic campaign it is essential to build around it an effective socio-economic framework.

CONCLUDING REMARKS

Our fight against cancer cannot afford to remain stacked in current laboratory-based detection methods, but must aim to implement a widespread use of point-of-care diagnostic devices that adhere to the REASSURED criteria. In this respect, paper-based sensors appear to be the most viable solution since they combine excellent analytical performance with economic and environmental affordability. Specifically, we showed how recent advances in the field of nanotechnology allowed the development of multiplexed and ultra-sensitive paper-based devices that have the potential to provide the required clinical sensitivity and specificity for cancer diagnostic. Accompanied with the right ethical and care management, this could decrease the economic burden of healthcare systems and improve the outcomes of cancer patients.

BOX 1 – Diagnostics for cancer personalized medicine

In addition to the difficulty to early identify the type and stage of cancer, the idea that one treatment fits all does not take into consideration the biological uniqueness of each patient. In chemotherapy, for example, it is crucial to control the drug dosage in order to maximize its effectiveness and minimize secondary effects[73]. Indeed, the metabolic and physiological differences of individuals affect differently the pharmacokinetics of drugs and they are often not considered in the posology [73]. This leads to unnecessary side effects due to the under- and over-dosing of the therapeutic drugs, which increase direct, informal and indirect costs [7]. To overcome this, medical care has been driven towards personalized medicine with the idea to customize the standard procedures to a patient's specific needs [74]. Nowadays, roughly half of the oncological drugs have an associated diagnostic kit to assure the drug is administrated only to patients that can benefit from it (i.e., using genetic tests)[75]. As healthcare switches from fee-for-service (a fee per service rendered) to value-based (payment based on patients' health outcome) medicine, proper analytics will be key to improve treatments' success that is translated into a more cost-effective health system [76,77].

In cancer, current personalized medicine relies tremendously on genetic and antigenic testing [78,79]. The former provides high levels of specificity and sensitivity; however, it requires long experimental protocols, the need of specialized personnel, and expensive equipment which hampers its use on a wide scale. Contrarily, the latter can potentially be used in point-of-care (PoC) sensors providing rapid and quantitative results within minutes, and without the need of specialized personnel; however, their low accuracy and sensitivity has often prevented their use for the detection of biomarkers in the low and sub-picomolar range (concentrations often required to achieve early diagnosis) [80]. It appears clear that a technology capable to provide enough sensitivity and specificity at the point-of-care could revolutionize the way how clinicians can perform cancer diagnosis. However, to achieve such accuracy, it is paramount to find the right combination of biomarkers in order to provide the desired specificity and a sensing technology able to quantify their concentrations in their clinically-relevant ranges.

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Table S1: Summary of the paper-based biosensors developed for cancer biomarkers detection. In blue are highlighted the studies conducted in real samples.

Type of paper	Signal detection	Recognition element nature	Biomarker	LOD / Range	Approach	Ref
Chitosan	Chemi-luminescence ELISA	Antibodies	α-fetoprotein CA 125 CEA	0.1 - 35.0 $ngmL^{-1}$ 0.5 - 80.0 UmL^{-1} 0.1 - 70.0 $ngmL^{-1}$	Covalently immobilize antibodies on µPADs; Sandwich CL-ELISA on µPADs;	[47]
Chitosan	Amperimetric	Antibodies	α-fetoprotein CA 125 CA 153 CEA	$5x10^{-4}$-100 $ngmL^{-1}$ $1x10^{-4}$-100 UmL^{-1} $1x10^{-4}$-100 UmL^{-1} $5x10^{-4}$-100 $ngmL^{-1}$	Two polyethylene terephthalate substrates, with one paper layer with the sensing sites and the other layer with counter electrode and reference electrode.	[81]
Whatman chromatography paper 1#	Electrochemical	Antibodies	CA125 CEA	0.001 – 75.0 UmL^{-1} 0.05- 50 $ngmL^{-1}$	Microfluidic paper-based wax-patterned device based on multi-walled carbon in a sandwich mode nanotubes	[51]
Whatman chromatography paper 1#	fluorescence	Antibodies	α-fetoprotein CA 125 CA 153 CEA	0.001 - 100 $ngmL^{-1}$ 0.0002 - 100 UmL^{-1} 0.0002 - 100 UmL^{-1} 0.005 - 200 $ngmL^{-1}$	Covalently immobilization of capture antibodies; CuO NPs-labeled secondary antibody in a sandwich-type immunoreaction;	[48]
Whatman chromatography paper #1	Electrochemical	Antibodies	α-fetoprotein CA 125 CA 153 CEA	0.001 -100 $ngmL^{-1}$ 0.001 -100 $ngmL^{-1}$ 0.005 -100 $ngmL^{-1}$ 0.005 -100 $ngmL^{-1}$	SiO2 nanoparticles in which antibody and HRP were co-immobilized were used as the signal reporter	[82]
Whatman chromatography paper 1#	Electrochemical	Antibodies	α-fetoprotein CA 125 CA 153 CEA	0.01 -100 $ngmL^{-1}$ 0.05 -100 $ngmL^{-1}$ 0.05 -100 $ngmL^{-1}$ 0.01 -100 $ngmL^{-1}$	Use of graphene to modify the immunodevice surface to accelerate the electron transfer;	[66]
Whatman chromatography paper 1#	Electrochemical	Antibodies	PSA	0.0012 $ngmL^{-1}$; 0.005 - 100 $ngmL^{-1}$	Sequential growing gold nanoparticles and manganese oxide nanowires networks on a freestanding three dimensional origami flexible device;	[83]
Whatman chromatography paper #114	Electrochemical	Aptamers	HL-60 cells Anti-cancer drugs	4 cells/10 µL; 500 – $7.5x10^{7}$ $cellsmL^{-1}$	Au-paper electrode fabricated and employed as the working electrode; Sequential in-electrode 3D cell culture	[84]

Whatman nitrocellulose	Electrochemical	Antibodies	PSA	6 $pgmL^{-1}$	Pre-loads capture antibodies onto a paper disk serving as a small electrochemical cell.	[85]
Not disclosed	Electro-chemiluminescence	Antibodies	PSA	1.0$pgmL^{-1}$; 1.0$pgmL^{-1}$ - 100 $ngmL^{-1}$	Two hydrophilic cells patterned connected by a carbon ink, with enhanced response through modification of the bipolar electrode cathode with multi-walled carbon nanotubes	[86]
Whatman chromatography paper#114	Electro-chemiluminescence	Aptamers	MCF-7 cells	250 $cellsmL^{-1}$; 450 - 10^7 $cellsmL^{-1}$	Microfluidic paper-based electro-chemiluminescence by origami construction with aptamers functionalized with 3D macroporous Au-PCE	[87]
Whatman filter paper #1	Impedance	nanocomposite	K562 cells	500 $cellsmL^{-1}$; $7.5x10^2$ – $3.9x10^6$ $cellsmL^{-1}$	Gold nanorods modified ITO electrodes	[88]
Whatman filter paper #1	Electrochemical	Antibody	CEA	25.8 $\mu Ang^{-1}mLcm^{-2}$; 2-8 $ngmL^{-1}$	Composite of poly(3,4-ethylenedioxythiophene):poly(styrenesulfonate) and reduced graphene oxide	[89]
Nitrocellulose paper (Whatman AE99 and mid CLW-040)	Infrared temperature gun	Antibody	MCF-7 cell	600 cells;	Graphene oxide-gold nanoparticles-anti-EpCAM composite added into nitrocelulose with immobilized target cells	[90]
Filter paper	Fluorescence	Antibody	CEA	0,89 $ngmL^{-1}$; 0 – 100 $ngmL^{-1}$	Lanthanide-doped upconversion nanoparticles tagged with specific antibodies printed to the test on the test paper	[91]
Whatman chromatography paper 1#	Electrochemical	Antibody	CEA	10 ng mL^{-1}; 50 $pgmL^{-1}$ - 500 $ngmL^{-1}$	Device with microfluidic channel built through wax printing and electrodes through screenprinting; Working electrodes modified with NH2-G/Thi/AuNPs nanocomposites	[92]
pH strips	Colorimetric	NA	miR-21	9.3 fM; 20 fM - 20 nM	Isothermal amplification through a netlike rolling circle amplification technique; quantification through correlation between hydrogen ions production and miR-21 concentration	[64]
Whatman chromatography paper, grade 2	Fluorescence	DNA probes	miR-21 miR-155	$3x10^5$ copies 3x105 to $3×10^8$ copies	Extraction and purification of small RNA through polyethersulfone paper chip; downstream detection was performed with Quantum dots as signal labels.	[49]
Whatman #1 filter paper	Electrochemical Smartphone readout	Antibody	Neuron-specific enolase	10 pg mL^{-1}; 1 - 500 ng mL^{-1}	NH_2-G/Thi/AuNPs-modified µPADs, combined with electrochemical detector and Android's smartphone,	[93]

Filter paper	Naked eye	Boronic acid modified N, S co-doped carbon quantum dots	Glucosamine in cancer cell HepG2	1.3 µM at 340 nm	Boronic acid and carbonyl groups structurally rigid N-S doped carbon quantum dots	[94]
Not disclosed	Fluorescence	Aptamer	EpCAM	250 pM 1 – 100 nM	Immobilized aptamer linked quantum dots and Cy3 labeled complementary DNA	[95]
Whatman #1 chromatography paper	Electro-chemiluminescence	Aptamer	MCF-7 cells	40 cellsmL^{-1}; 100 - 10^6 cellsmL^{-1}	Carbon ink-based closed bipolar electrode with wax printed reaction zone and use of AuPd nanoparticles serving as both as a capture aptamer carrier and as catalyst for the reaction of luminol and H_2O_2.	[96]
Whatman chromatography paper #1	Electrochemical	Antibodies peptide	CEA PSA	0.005 -100 ngmL^{-1} 0.001 – 40 ngmL^{-1}	Paper working electrodes modified with gold nanoparticles and cyclodextrin functionalized gold nanoparticles	[97]
Not disclosed	photoelectrochemical	DNA probe	CEA in MCF-7 cells	0.001 - 200 ng mL^{-1}	A rotatable paper-photocontrollable switch that allows light source to selectively activate desired working zones of the cyto-sensor	[98]
Filter Nitrocellulose	Naked eye	Antibody	$p16^{INK4a}$	Positive/Negative result	AuNPs functionalized with specific antibody conjugated with HRP for increased signal	[99]
Whatman paper	Colorimetric	Antibody	CEA	0.51 ngmL^{-1} 0.002 - 75.0 ngmL^{-1}	Use of ionic liquid to minimize unspecific binding and secondary antibodies stacked on the surface of the carboxylated $CO_2(OH)_2CO_3$-CeO_2 nanocomposite with intrinsic peroxidase-like activity	[100]
Nitrocellulose	Fluorescence	DNA probe	BRCA-1 gene	0.4 µM for a 250 dilution factor	DNA hybridization coupled with a smartphone based readout system	[101]
Whatman chromatography paper	Colorimetric	Antibody	PSA	3 pgmL^{-1}	Gold nanoparticles labelled with biotinylated ssDNA sequences and streptavidin-HRP for enzymatic signal enhancement.	[102]
Not disclosed	Electrochemical and colorimetric	Aptamer	MCF-7 cells	20 cellsmL^{-1} 50 - 10^6 cellsmL^{-1}	Polyhedral AuPd alloy nanoparticles and three-dimentional reduced graphene oxide	[103]
Micro-pore filter paper	Electrochemical	Antibody	PSA	1.18 ngmL^{-1} 0.002 - 75.0 ngmL^{-1}	Sheet-type biosensor based on a multi wall carbon nanotubes activated with a PSA antibody	[104]

Not disclosed	Colorimetric	Antibodies	PSA EphA2	89 $pgmL^{-1}$ 400 $pgmL^{-1}$	Erbium ion (Er^{3+})-doped and thulium ion (Tm^{3+})-doped upconversion nanoparticles as two independent reporters on two-color lateral flow strips	[105]
Whatman chromatograp hypaper#1	Electrochemical	Aptamer	PSA	10 $pgmL^{-1}$; 0.05 - 200 $ngmL^{-1}$;	Gold nanoparticles/reduced graphene oxide/thionine nano composites to coat working electrodes for aptamer immobilization	[106]
Whatman filter #1	Colorimetric	Modified aminoacid	Citrate	0.4 µM; 1.0 µM -10 mM	Cysteine-capped gold nanoclusters on Y shaped paper-based microfluidic device	[107]
Whatman filter paper #1	Electrochemical	Antibody	CEA	10.2 $µAng^{-1}mLcm^{-2}$ 4 - 25 $ngmL^{-1}$	paper modified with nFe2O3@PEDOT:PSS/WP	[108]
Filter paper	Electrochemical	Aptamer	miR-21 miR-141	0.1 fM	Biosensor composed by a first layer of silver nanowire and a second layer MoS2/AuNPs.	[109]
HF180 nitrocellulose membrane	Naked eye	Enzyme	TP53 gene missmatch	11 nM	Inactivated uriase printed onto paper microzones. DNA containing C−C mismatches allows reactivation of urease activity, hydrolysis of urea and alteration of pH	[110]
Pure cellulose	Electrochemical	Antibody	CA125	0.01 UmL^{-1} 0.1 - 200 UmL^{-1}	Nanocomposites of reduced graphene oxide/thionine/gold nanoparticles coated onto working electrodes	[111]
Filter paper	Plasmonic	Antibody	urine perilipin2	- 50 to $5x10^3$ $pgmL^{-1}$	Gold nanorattles coated with cetyltrimethylammonium chloride functionalized with monoclonal antibody by means of a bifunctional polyethylene glycol	[112]
Not disclosed	Electrochemical	Aptamers	CEA NSE	0.01-500 $ngmL^{-1}$ 0.05-500 $ngmL^{-1}$	Composites of graphene-thionin-AuNPs and of Prussian-blue-PEDOT-AuNPs were synthesized in order to promote electron transfer and the targets' aptamers immobilization	[50]
Not disclosed	Electrochemical	Antibody	CA-125	0.78 U/mL 0.78 – 400 U/mL	A silver nanoparticles/reduced graphene oxide nano-ink on flexible paper substrate	[113]
Filter paper	Electrochemical	DNA probe	H1047R	Presence or absence of missense mutation	Signal ON and OFF in a filter paper-based waxed strip that uses gold nanoparticles to anchor the recognition probe	[58]
Whatman filter paper #3	Electrochemical	Aptamer	CEA	1.06 $ngmL^{-1}$ 0.77–14 $ngmL^{-1}$	Paper modified with graphene and poly (3,4-ethylenedioxythiophene):poly(styrenesulfonate)	[114]

Office paper sheet	Electrochemical	Peptide	miRNA-492	6 nM 10 - 100 nM	A customized PNA probe on a paper surface with a two layer wax print and a nanostructured working electrode surface modified with gold nanoparticles.	[65]
Filter paper	SERS	Aptamer	Cytochrome c	1.79 $pgmL^{-1}$ 0 $pgmL^{-1}$ –10 μgmL^{-1}	(SERS)-based biosensor composed of gold nanourchins, an aptamer and a complementary DNA labelled with Cy5	[115]
Nitrocellulose Glass cellulose Filter paper	Fluorescence	Antibody	NMP22 BTA	Positive or negative	Chip manufactured with paper and wax; Dual-channel µPAD with colloidal gold immune labeling	[116]
Filter paper	Fluorescent	Aptamers	miRNA 21 miRNA 31	3.07×10^{15} M 3.28×10^{15} M in A549 cell lysates	Microfluidic paper-based laser-induced sensor that uses duplex-specific nuclease for fluorescent signal amplification	[117]
Chromatographic paper	Colorimetric	Primers	PCA3	0.34 $fgmL^{-1}$	A sponge-like polyvinyl alcohol pad and Calcine-preloaded dry filter paper were placed in the amplification and detection zones of a 3D-printed chip	[118]
Nitrocellulose	Colorimetric	Antibodies	α-fetoprotein MUC 16	1.054 $ngmL^{-1}$ 0.1 – 100 $ngmL^{-1}$ 0.413 $ngmL^{-1}$ 0.1 – 10 $ngmL^{-1}$	AuNPs conjugated with cysteamine immobilized on a nitrocellulose membrane	[119]
Nitrocellulose membrane	Colorimetric	Aptamer	Osteopontin	0.1 $ngmL^{-1}$ 10 - 500 $ngmL^{-1}$	Biotinylated aptamer for OPN pre-capture, immobilized antibody on the test line for second specific target identification and streptavidin-modified gold nanoparticles on the conjugation pad for color detection.	[120]
Not disclosed	Electro chemiluminescence	CRISPR RNA	miRNA 17	10^{-15} M	Use of *trans* cleavage activity of CRISPR/Cas13a to mediate subsequent exponential amplification and a "light-switch" molecule $[Ru(phen)_2dppz]^{2+}$ to avoid electrode modification and washes	[121]
Standard copier paper	Fluorescence	Antibodies	uPA	100 pM	graphene-gold nanoparticle platform with quantum dots	[122]
Not disclosed	Colorimetric	Antibodies	PEAK1	1 ng mL^{-1} 10^{-9} - 10^{-6} gmL^{-1}	Use of the properties of gold nanoparticle in color dye degradation	[123]
Whatman filter #1	Colorimetric	DNA probe	miRNA-21	10 – 10^3 pM	A DNA-templated Ag/Pt nanoclusters with peroxidase mimetic activity.	[124]

Chromatography Paper (Whatman #1)	Electrochemical	Antibody	VEGF-C	10 pg mL^{-1} 0.01 – 100 $ngmL^{-1}$	Surface of working electrodes modified amino-functional single-walled carbon nanotubes and gold nanoparticles	[125]
Not disclosed	Mass spectrometry	Antibodies	CA 15-3 CA 19-9 CEA CA 125 HE4 α-fetoprotein	25 UmL^{-1} 40 $mUmL^{-1}$ 5 $ngmL^{-1}$ 35 UmL^{-1} 140 $pmolmL^{-1}$ 10 $ngmL^{-1}$	Gold nanoparticles functionalized with antibodies for a imuuno sandwich assay with readout through online mass spectrometry	[53]
Whatman paper #1	Electrochemical	Antibodies	Claudin 7 CD81	0.4 $pgmL^{-1}$ 2 – 10^3 $pgmL^{-1}$ 3 $pgmL^{-1}$ 0.01 – 10 $ngmL^{-1}$	A dual sandwich-type immunosensor that employ reverse phase proteomic array	[52]

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