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Microfluidic Platforms for Biomarker Analysis

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ABSTRACT

Biomarkers have been described as characteristics, most often molecular, that provide information about biological states, whether normal, pathological, or therapeutically modified. They hold great potential to assist diagnosis and prognosis, monitor disease, and assess therapeutic effectiveness. While a few biomarkers are routinely utilised clinically, these only reflect a very small percentage of all biomarkers discovered. Numerous factors contribute to the slow uptake of these new biomarkers, with challenges faced throughout the biomarker development pipeline. Microfluidics offers two important opportunities to the field of biomarkers: firstly, it can address some of these developmental obstacles, and secondly, it can provide the precise and complex platform required to bridge the gap between biomarker research and the biomarker-based analytical device market. Indeed, adoption of microfluidics has provided a new avenue for advancement, promoting clinical utilisation of both biomarkers and their analytical platforms. This review will discuss biomarkers and outline microfluidic platforms developed for biomarker analysis.

1. INTRODUCTION

Biomarkers (biological markers) have been hailed central to the future of medicine, which is progressing towards a greater focus on prevention and personalisation [1]. Accordingly, there has been substantial academic and corporate investment in this field over recent times. Biomarkers are characteristics, most often molecular, that 'mark' or provide indication of biological states [2]. They supply information about what is occurring in the body, whether normal, pathological, or therapeutically modified. This information is highly valuable, from assisting diagnosis and prognosis, to monitoring disease, and assessing therapeutic response. Such clinical guidance is linked to significant benefits including greater capacity for disease prevention (through identifying predisposition and risk), better patient health outcomes, and reduced healthcare costs [3, 4]. While there are certain biomarkers used clinically, translation from discovery to clinical implementation has been slow and challenging, with many not being successful [5]. Indeed, of the thousands of candidate biomarkers identified, only about 100 have been implemented into clinical use [3]. A number of issues have been suggested as contributing factors to this [6]. These include potential challenges posed at every stage of the biomarker development pipeline, in addition to those posed by academic research characteristics, task complexity, costs, regulatory requirements, and stakeholder miscommunication [5, 7], as discussed more comprehensively in the paper. Utilisation of microfluidics in the biomarker field may provide an opportunity to address or overcome some of these obstacles.

Microfluidics is the science and technology of manipulating small amounts of fluids within micro-scale channels [8]. Microfluidics offers the advantage of very small sample volumes, but also offers highly precise control over these fluids due to the micro-scale fluid behavior, often displaying laminar flow characteristics. With origins in the early 1950s, microfluidics is still a fairly young field [9]. However, due to undergoing rapid development, microfluidics has become firmly established in the academic and industrial sectors as a multi-purpose tool [10]. There are several advantages of microfluidic platforms, many deriving from the small scale [10, 11]. These include faster processing and response times, enhanced efficiency, sensitivity, and portability, reduced requirement of samples and reagents, and lower costs. In addition, the capacity for parallelisation, multiplexing, and automation add further benefits to this approach [12].

Although some biomarkers are already having a clinical impact, current biomarker research and development is being hampered by various challenges; there also remains much room

for further growth and advancement in biomarker development and biomarker-based analytical device development and usage. Given the benefits offered by microfluidics, there has been an increasing interest in the utilisation of microfluidics for biomarker development and clinical analysis, in order to help overcome some of the obstacles faced and address untapped opportunities. This review will firstly discuss prominent aspects concerning biomarkers, with reference to those biomarkers already used clinically. Next, a brief overview of some of the microfluidic platforms that have been developed for biomarker analysis will be presented. Finally, some comments on outlook and concluding remarks will be offered.

2. BIOMARKERS

2.1 Origins, Classifications, & Utility

The term 'biomarker' first appeared in the medical literature in 1977 [13, 14], although unaccompanied by a definition or explanation. A standardised definition was produced by the National Institutes of Health (NIH) Biomarkers Definitions Working Group in 2001. According to this, a biomarker is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".

One system of classifying biomarkers is according to the purpose for which they are used, with this approach comprising of five classes [15]. The first is 'antecedent biomarkers', which are used to evaluate the risk of developing particular diseases. The second contains 'screening biomarkers', which enable identification of disease at the subclinical stage. 'Diagnostic biomarkers' make up the third class, these are used in diagnosing overt disease. The fourth class contains 'staging biomarkers', which assess the stage and severity of the disease. Finally, 'prognostic biomarkers' constitute the fifth class, which are used to predict the course a disease will take, with consideration of the therapeutic response and efficacy, and likelihood of recurrence (**Figure 1**).

Biomarkers can be molecular, physiological, or physical characteristics [2]. Although the latter two groups were previously the main types, it is currently accepted that the term 'biomarker' usually refers to traits of a molecular nature [5, 16]. Molecular biomarkers can be organised into categories, which include protein, genomic (DNA and RNA), lipid, carbohydrate, and metabolites. These can be further classified into subcategories, that in turn contain specific molecular entities [2]. Molecular biomarkers are sourced from various bio-samples, including blood, saliva, urine, faeces, cerebrospinal fluid, amniotic fluid, tissue/cells, among others [2, 15] (**Figure 1**).

The substantial interest in biomarkers has been driven by their great utility and potential benefits. As shown by the classifications according to purpose, there are multiple ways biomarkers can be used that grant the capacity for improved health outcomes. Accordingly, diseases may be prevented through detecting predisposition and risk, or reduced in severity through early detection by screening [4]. Diagnosis and prognosis can also be more informed with improved accuracy. The therapeutic strategy may be tailored to the individual, reducing risk of adverse events and increasing the likelihood of positive responses. In

addition, ongoing biomarker monitoring can track the course of the disease allowing for updated management as necessary [17]. Biomarkers can be of great value for informing medical decision-making because they provide clinically-relevant information in a fast and accessible manner when analysed using point-of-care (POC) testing [18]. Biomarker research can also enhance understanding of disease mechanisms, which has far-reaching benefits. Moreover, pharmaceutical and biotechnology research and development may be assisted by biomarkers [19]. The potential for reducing medical costs is another major benefit of biomarkers, which may occur for example, through using expensive therapies only on patients identified likely to benefit [3, 19].

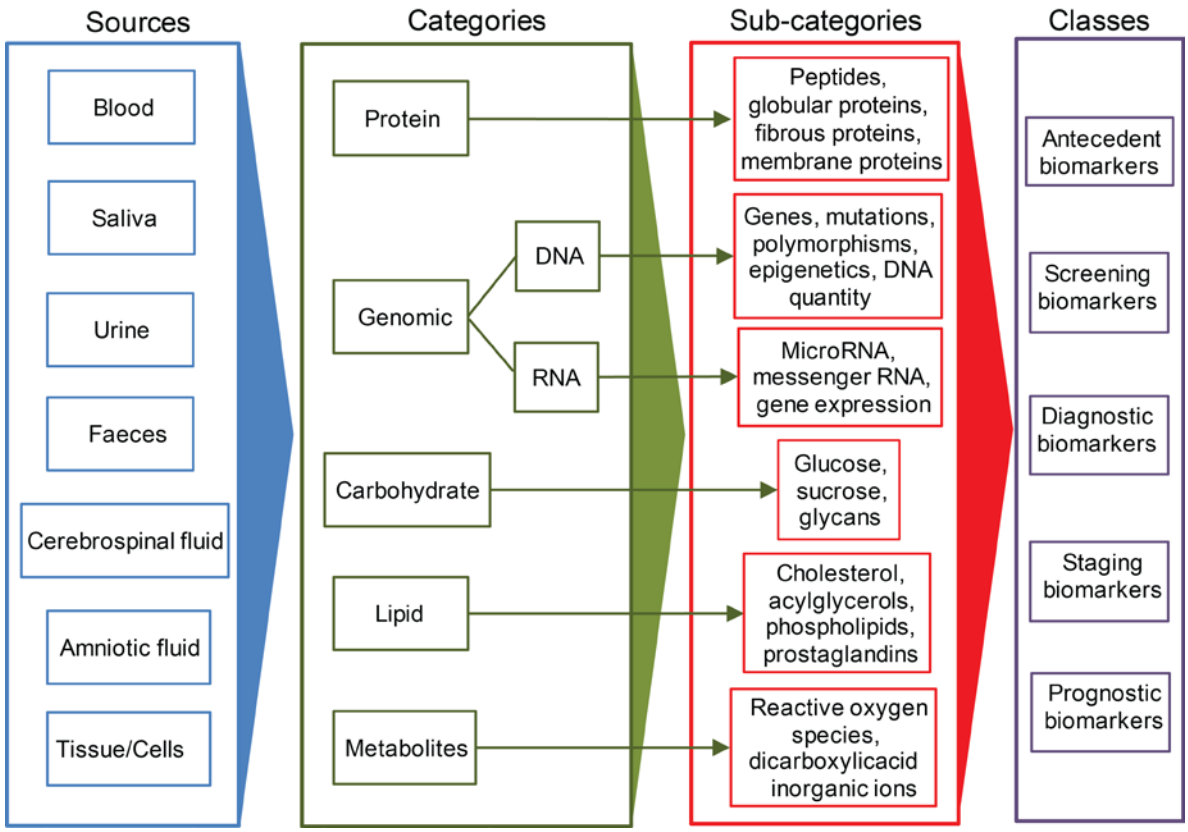

Figure 1. Overview of biomarkers; including the major sources, categories, sub-categories, and classes (according to purpose).

2.2 Biomarker development, market, & developmental challenges

There are multiple steps in the pathway from biomarker discovery to use in clinical practice [5, 16]. This development pipeline includes biomarker discovery, prioritisation, qualification, verification, validation, and implementation. Given the potential economic rewards, in addition to the health and societal benefits, there has been substantial investment by the academic and private sectors in order to see through these development steps [1, 20]. Accordingly, during the period from 1986 to 2009, close to 29,000 biomarker research grants (containing the term biomarker) were awarded by the NIH [20]. Those awarded in 2008 and 2009 alone totaled over US$2.5 billion. While more recently, more than 14,000 biomarker grants were awarded by the NIH between 2009 and 2011 [21].

Furthermore, BCC Research [22] findings estimated the total global biomarker market in 2010 at US$13.5 billion, with projections to grow to almost US$33.3 billion by the end of 2015. Genomics was found to be the largest biomarker area, which was predicted to continue as the largest and also the fastest-growing element pushing for biomarker technology advancements. Currently, oncology commands the highest revenue, although cardiology biomarker revenue is forecast to experience a higher growth rate in the upcoming years [23].

Despite the explosive growth in the biomarker research field and market, only a small number of the thousands of proposed biomarkers have been adopted into clinical practice [5]. Indeed, Poste [3] estimates that there have been 150,000 published papers reporting thousands of claimed biomarkers, while only approximately 100 biomarkers are routinely used clinically. Various elements contribute to this translational inefficacy, with the extensive process of biomarker development fraught with numerous potential challenges [16]. Each component of the development pipeline is associated with obstacles [5]. This includes insufficient strategies for biomarker characterisation and validation, and failure of biomarkers to meet sensitivity and specificity requirements [6]. Moreover, lack of sufficient scientific rigor is a prominent issue [7]. Concerns surrounding study design and experimental execution are present, such as insufficient sampling, inadequate description of experimental parameters (e.g. data generation), hidden multiple hypotheses, and inadequate standardisation of procedural conduct [3, 6, 7, 24]. Lack of statistical validation, in addition to inadvertent bias and use of cherry-picked data for biomarker identification are also problems [7, 25]. Further, the lack of coherence of the development pipeline has also been

raised [16]. Together these issues can contribute to inefficient and/or scientifically unsound, and ultimately unsuccessful development.

Furthermore, academic research organisation and culture has also been identified as a hindrance, with lack of large-scale collaborative networks across institutions and publication bias potentially holding back future research [3, 24]. The complexity of the task required of biomarkers likewise presents a challenge. Biomarkers should ideally detect diseases early, in people from a range of backgrounds (e.g. age, sex, ethnicity, etc.), from complicated biological matrices at low concentrations [6]. This requires more resources and strategies to achieve such goals. Additionally, the costs involved, in terms of finance, resource, and time, can be quite substantial [6, 26]. The considerable expense and length of the process can be a deterrent to investments in the field. Furthermore, the need to meet regulatory requirements can be both difficult and costly [16]. Miscommunication between stakeholders, such as industry and regulators can also lead to inefficiencies and lost opportunities [7]. These and other challenges faced contribute to the slow progress and failure of many biomarkers; further strategies to address or overcome these are needed [27].

2.3 Prominent molecular biomarkers, commercial analytical devices, conventional methods of analysis & comparison of analytical methods

Even with the challenges associated with translation of biomarkers into clinical use, there are those that have been successful and are having an impact. For example, human epidermal growth factor receptor 2 (HER2) is a biomarker for breast cancer with multiple potential clinical uses [28]. Detection of HER2 overexpression and amplification in breast cancer patients (approximately 15-30% of cases) provides prognostic information, as it is associated with enhanced aggressiveness and a greater relapse risk. It is also used for therapeutic benefit prediction, such as to select patients for an anti-HER2 drug, Herceptin (trastuzumab monoclonal antibody). Other biomarkers recommended for breast cancer clinical use by the American Society of Clinical Oncology in their evidence-based clinical practice guidelines include carbohydrate antigen 15-3 (CA 15-3), cancer antigen 27.29 (CA 27.29), oestrogen receptor, and carcinoembryonic antigen, among others. **Table 1** and **Table 2** provide a summary of biomarkers for diseases identified as leading causes of death in high-income countries and low- &/or middle- income countries, respectively [29].

181 **Table 1.** Summary of biomarkers for diseases identified as leading causes of death (in
182 order) in high-income countries, according to the World Health Organisation [29].

DISEASE	BIOMARKER CLASS	BIOMARKER DETAILS	IN CLINICAL USE?	REF
Cardiovascular Disease	Proteins- blood, saliva	B-type natriuretic peptide (BNP) & N-terminal pro-B type natriuretic peptide (NT-proBNP)	Yes	30, 31
		C-reactive protein (CRP)	Yes	30 32
		Cardiac troponin (cTn) I and T	Yes	30, 31
		Myoglobin (MYO)	Yes	31, 32
		Soluble CD40 ligand (sCD-40L)	Yes	33 32
		Soluble intracellular adhesion molecule (sICAM-1)	No	15
		Heart-type fatty acid binding protein (H-FABP)	Yes	32
		D-dimer	Yes	15 33
		Ischemia modified albumin (IMA)	Yes	32
		Fibrinogen	No	30 33
		Pregnancy-associated plasma protein A (PAPP-A)	No	32
		Creatine kinase (CK-MB)	Yes	31
		Myeloperoxidase (MPO)	Yes	15
		Lipoprotein-associated phospholipase A2 (Lp-PLA2)	Yes	15
	Protein & lipid	Lipoprotein a (Lp(a))	Yes	15
		Apolipoproteins	Yes	15 30
	MircoRNAs (miR)- blood	Specific signature for each cardiovascular condition. E.g. Ischemic heart disease: miR-1, miR-30c, miR-133, miR-145, miR-208a, miR-208b, miR-499, miR-663b, miR-1291, are elevated, while miR-126 miR-197 miR-223 are decreased.	No	34
	Lipids	Cholesterol	Yes	33
		Triacylglycerols (triglycerides)	Yes	30
Lung cancer	Proteins- tissue	Epidermal growth factor receptor (EGFR)	Yes	35
		Anaplastic lymphoma kinase (ALK)	Yes	35

	Proteins- blood	Cytokeratin 19 fragment (CYFRA 21-1)	No	[36, 37]
		Carcinoembryonic antigen (CEA)	No	[37]
		Neuron-specific enolase (NSE)	No	[36, 38]
		Progastrin-releasing peptide (ProGRP)	No	[39, 38]
		Tumour M2-pyruvate kinase	No	[39]
	DNA	Mutations in K-ras & p53	No	[39, 38]
Dementia	Proteins- cerebrospinal fluid	Amyloid-β (Aβ)	Yes	[40, 41, 42]
		Tau and Phosphorylated tau	Yes	[40, 43, 44]
	DNA	Apolipoprotein-E (apoE) allele	No	[42, 41]
		Mutations in Presenilin 1 or 2 (PSEN1, PSEN2) or Amyloid beta (A4) precursor protein (APP) genes	Yes	[42]
Colorectal cancer	Proteins- blood	Trefoil factor 3 (TFF3)	No	[45]
		Growth/differentiation factor 15 (GDF15)	No	[45]
		Carcinoembryonic antigen (CEA)	Yes	[46, 47]
	Protein- stool	Haemoglobin	Yes	[46]
	Carbohydrate-blood	Carbohydrate antigen 19.9 (CA 19.9)	Yes	[46, 47]
	Genomic	K-ras gene mutation	Yes	[48, 49]
		Microsatellite instability	No	[48, 49]
		Guanylyl cyclase C (GCC) messenger RNA expression	Yes	[48]
Diabetes	Carbohydrate	Glucose	Yes	[50, 51]
	Glycated proteins-blood	Haemoglobin (Hb) A1c	Yes	[52, 53]
		Fructosamines e.g. glycated albumin	Yes	[50]
Breast cancer	Proteins- blood	Human epidermal growth factor receptor 2 (HER2)	Yes	[28, 54, 55]
		Carbohydrate antigen 15-3 (CA 15-3)	Yes	[28, 56]
		Carcinoembryonic antigen (CEA)	Yes	[28, 56]
		Cancer antigen 27.29 (CA 27.29)	Yes	[28, 57]
		Tissue polypeptide antigen (TPA)	Yes	[56]
		Tissue polypeptide specific antigen (TPS)	Yes	[56]
	Proteins- tissue	Urokinase-type	Yes	[28]

		plasminogen activator (uPA)		
		Plasminogen activator type 1 inhibitor (PAI-1)	Yes	[28, 55]
	Genomic	Oestrogen receptor	Yes	[28, 55]
		Progesterone receptor (PR)	Yes	[28, 54]
		Topoisomerase (DNA) II α (TOP2A)	Yes	[54, 55]
		Breast cancer 1, early onset (BRCA1*)* and Breast cancer 2, early onset (BRCA1*)* mutations	Yes	[54, 58]
Prostate cancer	Protein- blood, urine	Prostate specific antigen (PSA)	Yes	[59, 60, 61]
		B7-homolog 3 (B7-H3)	No	[60, 62]
		Alpha-methylacyl-CoA racemase (AMACR)	No	[60, 63]
	Genomic	Phosphatase and tensin homolog (PTEN)	No	[61, 64]
		Prostate cancer antigen 3 (PCA3)	No	[60, 63, 64]

Table 2. Summary of biomarkers for diseases identified as leading causes of death in low- &/or middle- income countries (those not already listed in table 1), according to the World Health Organisation.

DISEASE	BIOMARKER CLASS	BIOMARKER DETAILS	IN CLINICAL USE?	REF
Lower respiratory infections	Proteins- blood, bronchoalveolar lavage	C-reactive protein (CRP)	Yes	[65, 66]
		Procalcitonin (PCT)	Yes	[66, 67]
		Pro-adrenomedullin	No	[67, 68]
		Mid regional pro-atrial natriuretic peptide (MR-proANP)	No	[67]
		Pro-vasopressin (CT-proAVP)	No	[67]
		Proadrenomedullin (proADM)	No	[67]
	Protein- oral fluid	Neuraminidase	Yes	[69]
	Protein- nasopharyngeal aspirate	Influenza nucleoproteins	Yes	[69]
	Carbohydrate- urine	C-polysaccharide	Yes	[69]

Human immunodeficiency virus/ Acquired immunodeficiency syndrome (HIV/AIDS)	Nucleic acid	HIV-1 & HIV-2 viral load	Yes	70
	Proteins- blood, oral fluid, urine	HIV-specific antibodies	Yes	71, 72
	Protein- blood	HIV p24 antigen	Yes	73 74
Tuberculosis	Nucleic acid- sputum	*Mycobacterium tuberculosis* DNA	Yes	75, 76
	Proteins- blood	Interferon (IFN) γ	No	77, 78
		Neopterin	No	76-78
		Antibodies specific for 38kDa antigen and 6 kDa early secretory antigenic target (ESAT6)	No	78, 79
	Protein- urine	Lipoarabinomannan	No	76, 80
Malaria	Proteins- blood	*Plasmodium falciparum* histidine-rich-protein2 (PfHRP2)		81-83
		Plasmodium lactate dehydrogenase (pLDH)	Yes	81-83
		Plasmodium aldolase	Yes	81-83
	Proteins- blood	Antibodies specific for: gSG6-P1; *Plasmodium falciparum* reticulocyte binding-like homologue protein 2 & 4 (PfRh2 & PfRh4); Dihydrofolate reductase (DHFR)	No	83-85

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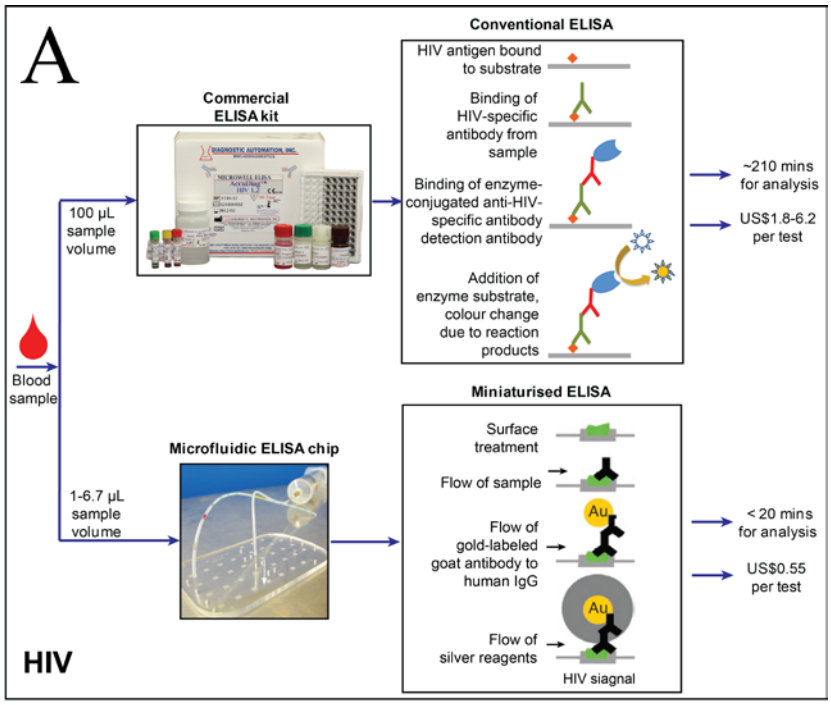
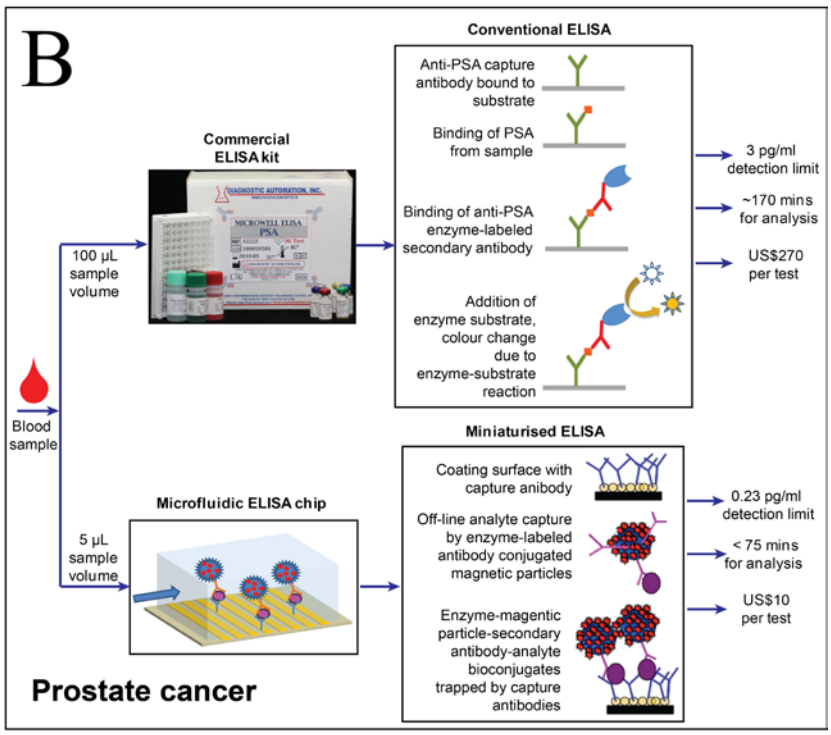
Similar to the case with biomarker development, there has been rapid expansion in the investment and market of biomarker-based analytical devices [86]. According to Drucker and Krapfenbauer [6], there are currently 153 approved molecular diagnostic tests commercially available, manufactured by more than 15 different companies. This includes POC devices, which increase biomarker analysis accessibility and reduce the time from sample collection to analytical answer [86]. Hence, the informative power of biomarkers is made available at the earliest time possible. Two widely used biomarker-based analytical devices that are not limited to the clinic but are patient/consumer-operated in their own homes, include those for pregnancy detection and glucose monitoring. Pregnancy tests, which operate according to immunocapillary principles (lateral flow tests), were first made available "over-the counter" in the late 1970s [10, 87]. Unlike the initial multi-step home pregnancy tests, current tests involve only a single step. The first electrochemical glucose biosensor was produced in 1962 [88]. Considerable development since then has seen over 40 different personal blood glucose monitors become commercially available, most of which use disposable enzyme electrode test strips.

There are also a range of devices for use in the clinic [86]. One of the oldest POC devices available is the Abbott iSTAT, which launched in 1992 [89]. This device has the capacity to detect a range of analytes from blood samples including glucose, coagulation markers, dissolved gases, and cardiac markers (e.g. troponins). Leading the POC market in the U.S., the iSTAT is now used in a third of U.S. hospitals. Interestingly, the iSTAT is microfluidics-based, which could be a key factor of its success. Other companies with commercial POC instruments include Roche Diagnostics (blood analysis [90]), Siemens (blood and urinalysis [91]), and Samsung (blood analytes, including cholesterol and creatinine [92]).

Conventional methods of biomarker analysis rely on established technologies, as summarised in the **Supplementary Table (S1)**. Although in widespread use, these techniques have various limitations. Such drawbacks include being time-consuming, labour-intensive, and expensive, requiring large sample and reagent volumes, and trained operators, while also having limited- or no- portability, among others. [86, 93]. **Figures 2A & 2B** provide direct comparisons of conventional and microfluidic methods of analysis using the model biomarker cases of HIV and PSA. Further, as can be seen from the comparison provided by **Figure 2C**, compared to conventional methods, microfluidics can offer lower costs, reduced sample volumes, and faster analytical times. In addition, microfluidics can

overcome some of the other drawbacks of conventional techniques such as having greater portability and greater capacity for automation and multiplexed analysis. It is also reported that microfluidics provides opportunity for higher sensitivity [10] and greater POC capacity [94]. However, commercial availability and standardisation of results are areas in which conventional methods are currently superior.





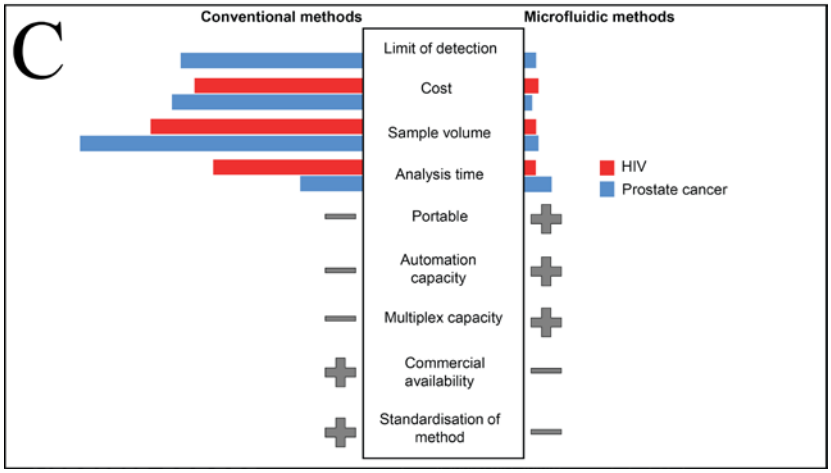


Figure 2. Comparison of biomarker-based analytical devices using conventional and microfluidic methods. **(A)** Comparison of methods for HIV-specific antibodies analysis (HIV biomarker). The microfluidic chip detection method involves the reduction of silver ions onto to the gold nanoparticles conjugated to antibodies. The signals produced can be examined using low-cost optics (enabling quantification) or the human eye. Images and data from Chin et al. [95] and Diagnostic Automation / Cortez Diagnostics, Inc.[96] **(B)** Comparison of methods for PSA analysis (prostate cancer biomarker). For the microfluidic device, amperometric detection was used whereby the enzyme labels (attached to the magnetic particle-secondary antibody-analyte bioconjucates) were activated by hydrogen peroxide, and then reduced by the electron transfer between the electrode and enzyme labels via an applied mediator. Images and data from Chikkaveeraiah et al. [97] and Diagnostic Automation / Cortez Diagnostics, Inc. [98] **(C)** Comparison between conventional and microfluidic methods, using the model cases of HIV and PSA analysis (with data from (A) & (B)).

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2.4 Development of biomarker-based analytical devices, stakeholders, & the need for microfluidics

The development and translation of diagnostic devices into clinical practice is a significant and complex process, with a number of stakeholders involved [99]. Over recent years the rate of diagnostics reaching the market has declined; this is perhaps contrary to anticipations given the important scientific accomplishments and investments made over the period. This 'pipeline problem' is multifaceted, with the actions of stakeholders playing a role [99]. Stakeholders include researchers, industry, clinicians, healthcare payers, government policy makers, and patients, which each face their own set of risks and rewards. Researchers face numerous challenges, prominent among them are to develop a device that operates safely and effectively, while ensuring there is demand for their product and securing investment for its translation. Industry (e.g. diagnostic and biotechnology companies) and venture capital firms are sources of this investment. Although ultimately driven by return, investment always carries risks and inadequate investment return is a possibility [99]. Competition exists for industry attention amongst different research groups developing similar technologies, but also within the diagnostic market from revival technologies [86]. Healthcare payers, for example national healthcare systems and private healthcare insurers, need to ensure the legitimacy of the testing and assess the economic impact it will have, for example whether test expense would overcome the potential savings of personalised care [86]. Healthcare providers are direct customers of diagnostic devices, with the hesitance of clinicians and medical institutions to adopt new diagnostic devices affecting diagnostic device uptake [86]. Governments have a responsibility to ensure safety, but also to facilitate healthcare developments. Regulatory requirements imposed by government agencies (e.g. US FDA) can also present hurdles to bringing diagnostics to market [86]. Patients are the ultimate beneficiaries of benefits new diagnostics offer, although their access to them depends on the decisions made by the other stakeholders.

There thus remains substantial room for optimisation of current systems to improve performance and flexibility, and moreover, development of new analytical devices for those biomarkers for which there is no current commercial analytical platform. This gap can be addressed by microfluidics. After undergoing its own development and establishment as a technology over past years, microfluidics is now being applied to areas in which its advantages will be of most benefit. Biomarker analysis is one such area where incorporation of microfluidics has the potential to transform the field. Moreover,

microfluidics is crucial to biomarker development and clinical utilisation because it can be far more efficient and effective than other methods and in several cases can offer unique capabilities that cannot be achieved by other means. Indeed, the need for microfluidics is illustrated in complex diseases such as cancer. The heterogeneity of cancer can mean multiple biomarkers are required for tumour diagnosis and characterisation, as single marker detection may provide insufficient information [100] [101]. Molecular characterisation of cancers can identify disease subtypes of individual patients, facilitating personalised medical care [102] [101]. By informing prognosis, in addition to assisting prediction and assessment of treatment benefits, tumour characterisation can enable targeted therapies [26].

Accordingly, clinical usage of cancer biomarkers necessitates the capacity to analyse biomarkers rapidly with high sensitivity and selectivity, and importantly, to do so multiplexed. While conventional techniques are unable to deliver this, microfluidics-based approaches have shown their capability to do so. Zheng and colleagues' silicon-nanowire sensor [100] is a notable demonstration of achieving such requirements. The versatility of microfluidic channel layouts, such as numerous channel manifolds organised on a single device, enable microfluidic systems to deliver the high throughput needed for clinical applications [103]. Microfluidic devices can offer detection of multiple analytes from a single sample simultaneously in one channel, and/or screening of numerous samples in parallel for one target on a single chip [104]. Further, microfluidics is suited to achieving accurate quantitative analysis of biomarkers, due to principle requirements of biomarker analysis (e.g. highly precise metering and handling of liquids) being strengths of microfluidics [10].

Determination of biomarker profiles correlated with different cancer pathogenesis stages may also assist early detection, as well as guide tailored treatment [100]. Multiplex biomarker chips have been suggested as the appropriate device for detecting these biomarker 'codes' [102]. Hence microfluidics is critical to the adoption of clinical biomarker usage in complex diseases, an area that greatly contributes to the disease burden and has much to benefit from the clinical guidance biomarkers provide. Similarly, disease diagnosis in resource-limited settings including developing countries, demands portable, simple, rapid, and low-cost analysis, while also having sufficient sensitivity and specificity. Microfluidics is ideal to achieve such requirements, as shown by the 'mChip' ('mobile microfluidic chip for immunoassay on protein markers') which diagnosed HIV and syphilis in Rwanda with sensitivity and specificity comparable to conventional laboratory assays [95]. It further boasts of needing only a needle-prick volume blood sample, the capability of using whole blood,

sera or plasma, and no requirements of user signal interpretation or external infrastructure/electricity. Such devices have a substantial public health impact, overcoming resource barriers to enable early detection of disease in a cost-effective manner. Thus, there is a real need for microfluidics in biomarker-based analytical devices, a selection of those already developed will be discussed in the next section.

3. MICROFLUIDIC BIOMARKER-BASED ANALYTICAL PLATFORMS

Devices employing microfluidics do so via performing fluidic unit operations. These operations include transport, mixing, metering, and control of fluids, as well as concentration, separation, and detection of molecules or particles, among other actions [9]. Here, a selection of microfluidic platforms that have been developed for biomarker analysis will be presented. Analysis in this case refers to investigative procedures, such as pre-concentration, detection, quantification, characterisation, etc. Almost all of the following platforms are non-commercialised devices.

3.1 Protein biomarkers

Proteins have particular characteristics that make them valuable biomarkers [105-107]. Firstly, proteins are more dynamic and diverse than other molecular groups due to factors such as post-translational modifications and alternative splicing, conferring the capacity to carry more information [105]. They also provide a greater reflection of cell physiology due to the nature of their involvement within it. Furthermore, given the vast number of proteins and their highly specific biological roles, it is likely that proteins are the most ubiquitously affected molecular domain in pathological processes, responses to disease and therapeutics, and recovery [16]. Accordingly, great focus has been placed on proteins in biomarker research. Protein biomarkers include peptides, globular proteins, fibrous proteins, and membrane proteins (**Figure 1**). Their differential expression/abundance (including absence), and structural or functional alterations (e.g. via post-translational modifications) can provide markers of certain biological states [108].

Blood contains a multitude of proteins, representing the most comprehensive and most sampled human proteome [16, 109]. In addition to the hundreds of classical plasma proteins (corresponding to tens of thousands of molecular forms), there are also other proteins present including receptor ligands (e.g. cytokines and peptide hormones), leaked tissue proteins, and immunoglobulins [109]. Although the blood proteome is easy to sample and very informative as to the physiological/pathological state, the substantial dynamic range of protein abundance spanning more than 10 orders of magnitude enhances the complexity of detection [109].

Immunoaffinity capture has been one of the major mechanisms used to separate proteins of interest from biological samples on microfluidic platforms [110]. Detection and quantification of target proteins is enabled due to the sensitivity and specificity of antibody-antigen

interactions [93]. Employing immunoaffinity, Chikkaveeraiah and colleagues [97] developed a microfluidic electrochemical immunoarray for the simultaneous detection of the cancer biomarkers prostate specific antigen (PSA; prostate cancer), and interleukin-6 (IL-6; lung, oral, colorectal, and prostate cancers). The 8-electrode array immunosensor was based on the 'sandwich' immunoassay detection method, although analyte capture was conducted off-line rather than the conventional on-line strategy. This involved the protein analyte binding to the enzyme-labeled antibody-conjugated magnetic particles prior to delivery into the system, to be subsequently bound by capture antibodies located on the sensor surface. Amperometric detection was used whereby the enzyme labels were activated by hydrogen peroxide, and then reduced by the electron transfer between the electrode and enzyme labels via an applied mediator. Likewise, Malhotra, et al. [111] used off-line capture methods with their electrochemical microfluidic array for the simultaneous detection of oral cancer biomarkers IL-6, IL-8, vascular endothelial growth factor (VEGF), and VEGF-C. To improve detection sensitivity, Ikami and coworkers [112] patterned hydrogel pillars, which contained polystyrene beads functionalised with capture antibodies along the microchannel. The microchip was employed for on-line sandwich immunoassays of C-reactive protein (CRP), α-fetoprotein (AFP), and PSA; biomarkers for inflammation and cardiovascular disease, tumours, and prostate cancer, respectively.

Using a modified enzyme-linked immunosorbent assay (ELISA) technique, Stern et al. [113] captured the cancer biomarkers PSA and carbohydrate antigen 15-3 (CA 15-3; breast cancer) from blood samples (**Figure 3A**). Their two-stage detection approach involved first capturing the biomarkers on the microfluidic purification chip (MPC), followed by a second specific binding event to secondary antibodies at the connected silicon nanoribbon detector. This method increased selectivity while reducing the detector sensitivity required.

Furthermore, Schaff and coworkers' [114] conducted immunoassays on their lab-on-a-disk. In lab-on-a-disk platforms, centrifugal force is the principle propulsion mechanism of fluid through microchannels within a disk. By isolating the capture beads from the reaction mixture by sedimentation generated by the centrifugation, wash steps were reduced compared to conventional ELISA wash procedures. The device quantified the inflammation biomarkers CRP and IL-6 from whole blood samples, demonstrating a detection limit of <100 pM. Similarly, Park et al. [115] presented a lab-on-a-disk for multiplex immunodetection of cardiovascular disease biomarkers (high sensitivity CRP, cTnI, and N-terminal pro-B type natriuretic peptide) from saliva and blood. The disk featured interconnected reaction

chambers enabling communal reaction processes, which then became isolated via the action of microvalves for individual biomarker detection. The assay limits of detection were comparable to those of standard ELISA (**Figure 3B**).

Alternatively, Zhu and colleagues [116] detected the breast cancer biomarker CA 15-3 from serum samples using an opto-fluidic ring resonator (OFRR) sensor. Anti-CA 15-3 antibodies functionalised to the inner surface of the OFRR captured CA 15-3, and the subsequent change in refractive index was detected by the surrounding optical resonator for concentration determination. Likewise, Gohring, et al. [117] used an OFRR sensor to detect human epidermal growth factor receptor 2 (HER2) extracellular domain, also a breast cancer biomarker (**Figure 3C**). Their device enabled detection in serum samples at clinically relevant levels of 13-100 ng/mL.

Microfluidic paper-based analytical devices (μPADs) have provided a novel approach for biomarker analysis. Wang and associates [118] adopted this method for performing electrochemical immunoassays. Their μPAD comprised of a wax-patterned paper layer together with a substrate layer containing screen-printed electrodes. This device was used for the diagnosis of the serum tumour biomarkers cancer antigen 125 (CA125) and carcinoembryonic antigen (CEA). In addition, Yan et al. [119] performed electrochemiluminescence (ECL) immunoassays on a 3D μPAD for CEA quantification from serum samples, a cancer biomarker (breast, esophageal, intestinal, cervical) (**Figure 3D**). The immunoarrays consisted of complexes of capture antibody, CEA analyte, and the signal antibody, immobilised to the screen-printed working paper electrode on board-A. ECL reactions were triggered when TPA (tris-(bipyridine)-ruthenium [$Ru(bpy)_3^{2+}$]-tri-*n*-propylamine) reactant was added to the device, where board-A worked together with the reference- and counter- electrodes on board-B. Good sensitivity (0.008 ng/mL detection limit for human control serum samples) and specificity (negligible effect of presence/absence of other analytes) was demonstrated.

Electrophoresis methods employing different mobilities of ions in an electric field have been widely used for isolation and identification of proteins in microfluidic systems [120]. One such method, capillary electrophoresis, was integrated with immunoaffinity capture by Yang and associates [121]. Their microdevice utilised an affinity column consisting of a reactive polymer lining a microchannel with four specific antibodies bound to it. The antibodies used in this study corresponded to those capturing AFP, CEA, cytochrome C,

and heat shock protein 90 (HSP90) cancer biomarkers. Following antigen capture, capillary electrophoresis was subsequently used to separate and quantify the biomarkers. The device was able to simultaneously quantify the different antigens at normal and pathologically elevated levels. Likewise utilising capillary electrophoresis, Yu et al.'s [122] microfluidic chip enabled on-chip fluorescent labeling and separation of HSP90. In contrast, Bottenus and associates' [123] microfluidic chip employed isotachophoresis (ITP). ITP separates sample components into almost pure zones, according to their electrophoretic mobilities between the leading electrolyte (high mobility ions) and terminating electrolyte (low mobility ions). The separated zones are concentrated up to several orders of magnitude [124]. The chip achieved a 10,000-fold increase in the concentration of the cardiovascular disease biomarker cardiac troponin I (cTnI).

Aptamer-based biosensors (aptasensors) used for clinical applications have recently become incorporated within microfluidic systems [125]. Aptamers are single-stranded oligonucleotides (RNA or DNA) that bind to target molecules with high specificity and affinity. Aptamers are selected via systematic evolution of ligands by exponential enrichment (SELEX) in vitro, from large random-sequence oligonucleotide libraries. Microfluidic aptasensors have been developed for various proteins. Thrombin is an enzyme (serine protease) biomarker for coagulation and atherothrombosis [126]. Tennico and colleagues' [127] developed a microfluidic aptamer-based assay for detection and quantification of thrombin. A sandwich assay method was used whereby aptamer-functionalised magnetic beads located in the microchip reaction chambers captured thrombin, with subsequent binding of a quantum dot-functionalised aptamer to a different thrombin epitope. Florescence microscopy was used for on-chip detection. This device demonstrated a limit of detection of 10 ng/mL. Wang et al. [128] presented a similar device, although using electrochemical detection.

Mitsakakis and colleagues [129] developed an integrated surface acoustic wave (SAW) biosensor system which enabled multiplex protein biomarker detection. The adsorption or binding of target proteins on the sensor surface causes a change in the phase and amplitude of the acoustic wave. The utility of the system was demonstrated by detecting the cardiac biomarkers creatine kinase MB (CK-MB), cardiac reactive protein (CRP), D-dimer, and pregnancy-associated plasma protein (PAPP-A) [130]. Similarly, Lee et al. [131] developed a SAW immunosensor for Hepatitis B surface antibody (HBsAb) detection and measurement.

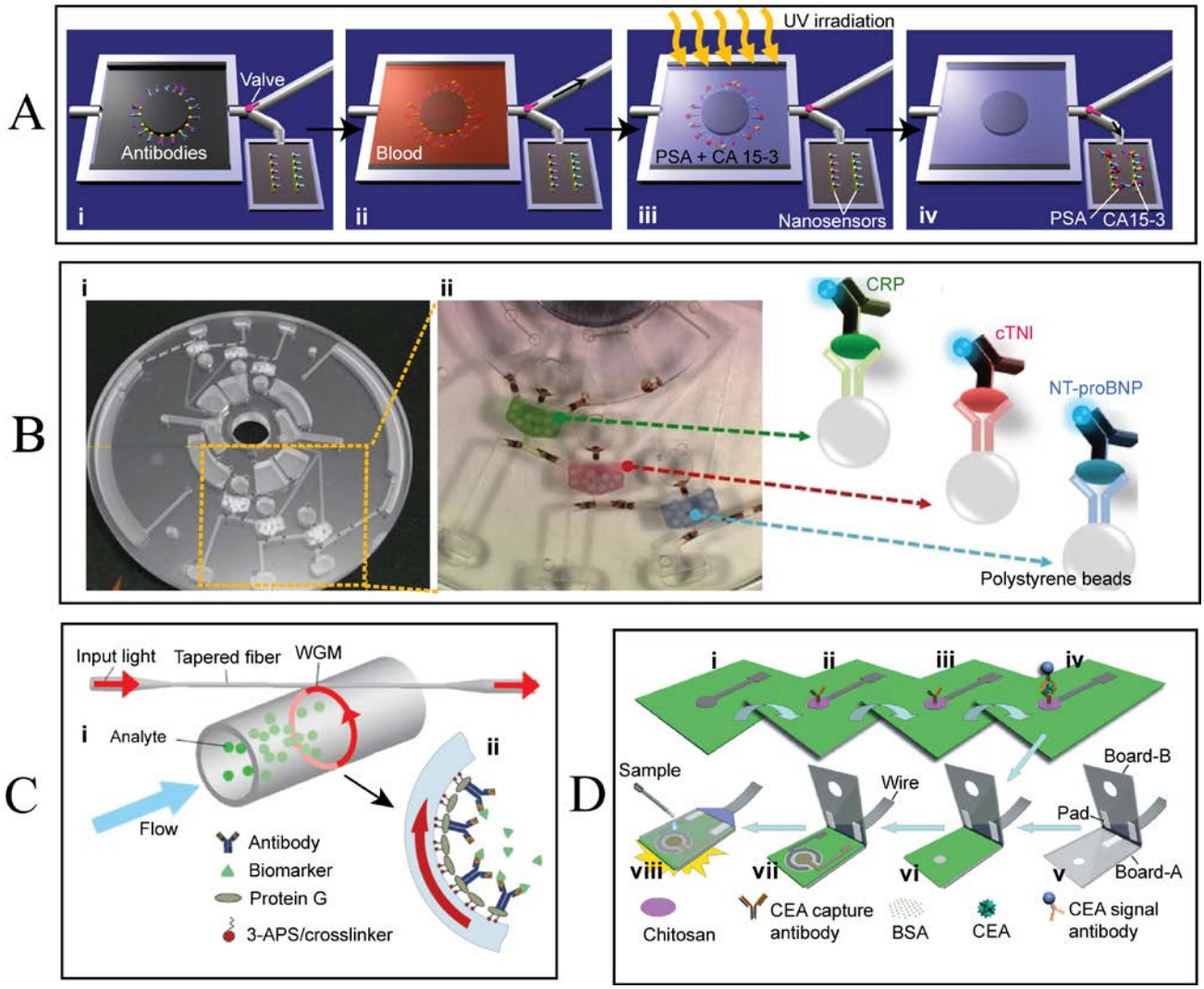

Figure 3. Microfluidic platforms for protein biomarker analysis: **(A)** Schematic illustrating operation of Stern et al.'s [113] microfluidic purification chip (MPC): i) PSA and CA 15-3 antibodies are bound via a photocleavable crosslinker to the MPC, ii) Blood sample is delivered onto the chip; biomarkers present bind to their respective antibodies, iii) Sensing buffer is added for washing purposes. Cleavage of photolabile crosslinker occurs upon UV irradiation, iv) Released antibody-antigen complexes flow (directed by valve) into the nanosensor reservoir for biomarker detection. © Macmillan Publishers Limited 2010. **(B)** Park et al.'s [115] lab-on-a-disk: i) Photograph of the disc. The disc contains one analytical unit for the unknown sample and another for the positive control sample, ii) Image showing the main reaction chambers isolated from each other, and preloaded with ELISA reagents. © American Chemical Society 2012. **(C)** The opto-fluidic ring resonator biosensor [117]: i) Overview of mechanism whereby sample passes through the capillary, which has whispering gallery modes (WGM) circulating in the cross-section resonator, ii) HER2 biomarker present in sample binds to antibodies functionalised on the capillary inner

surface, the subsequent change in refractive index is registered by the resonator enabling biomarker detection. © Elsevier B.V. 2010. **(D)** Schematic demonstrating the fabrication and assay steps involved for the microfluidic paper-based electrochemiluminescent 3D immunodevice developed by Yan et al. (2012): i-iv) Immunoarrays for CEA biomarker generated on the screen-printed working paper electrode (SPWPE), v) Device-holder, vi) Modified SPWPE (from steps v-iv) inserted face-down onto board-A, vii) Screen-printed reference electrode placed face-up onto modified SPWPE, viii) Device-holder secured shut and TPA reactant administered to initiate electrochemiluminescent reaction. © Wiley-VCH Verlag GmbH & Co. 2012.

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3.2 Genomic (Nucleic acids)

Genomics is the largest segment in the biomarker market [22]. The development of nucleic acid biomarkers has been greatly facilitated by advances in genomic analysis technologies and techniques [108]. Moreover, nucleic acid detection is generally easier than protein or metabolite detection; the availability of nucleic acid amplification methods being one contributing reason [132]. Although single gene mutation testing has been used clinically for years, it has been stated that drug discovery and development is the most important current application for genomic biomarkers [133]. Genomic biomarkers include mutations, gene detection and expression, circulating nucleic acid quantity, messenger RNA, micro RNA, epigenetic modifications, and polymorphisms (e.g. single nucleotide polymorphisms), **Figure 1**.

3.2.1 Deoxyribonucleic acid (DNA)

Quantitative detection of mutations has been performed using droplet-based microfluidic devices. Pekin et al.'s [134] platform involved the creation of thousands of droplets, within which genetic analysis took place. Digital PCR DNA amplification and detection within droplets reduced reagent volume and time required for analysis. The system demonstrated highly sensitive and specific detection of the tumour biomarker *KRAS* oncognene mutations, using DNA extracted from cultured cells.

Droplet-based microfluidic platforms have also been adopted for detecting the presence of specific genes identified as biomarkers. Zhang and associates' [135] droplet-based microfluidic device demonstrated successful detection of remodeling and spacing factor-1 (Rsf-1) gene, an ovarian cancer biomarker, in addition to genetic identification of the bacterial pathogen *Escherichia coli* (**Figure 4A**). The single device incorporated sample preparation, droplet-contained genetic analysis, and detection using fluorescence. Silica super-paramagnetic particles were utilised for solid phase extraction of DNA within distinct droplets, as well as for being carriers to transfer DNA via magnetic actuation.

DNA microarrays capture single-stranded target DNA sequences via complementary nucleotide base-pairing with probes immobilized on the solid array surface, which are arranged in an ordered layout [136]. While this technology has become an indispensible tool in genomics, the requirement for multiple array components, additional equipment, and trained operators, has seen the technique limited to advanced laboratories [137]. However, microfluidic systems with integrated microarrays are emerging, leading to many advantages

including faster assay times and enhanced portability, which promote more widespread use [138]. Accordingly, Seefeld et al.'s [139] microfluidic microarray device enabled detection of both DNA biomarkers and proteins using surface plasmon resonance imaging (SPRi). The chip design consisted of four microchambers, each containing three discrete SPRi gold film microarray spots used for detection. Target DNA was detected at a limit of 10 fM when RNase H amplification was used. Utilising fluorescently-labeled reporter oligonucleotide probes, Zhang and Hu [140] demonstrated multiplex detection of HIV-1 and HIV-2 via target DNA detection. The single quantum dot nanosensor was able to simultaneously detect the DNA targets at the single-molecule level, using coincidence detection and fluorescence-resonance-energy-transfer (FRET) detection.

Alterations in DNA quantity can provide important information relating to various physiological states including disease and cell cycle phases [141, 142]. Correspondingly, Liu et al. [143] analysed circulating nucleic acids (CNAs) from serum samples, a marker relevant to cancer and fetal medicine. The size and number of CNAs present was determined by performing microfluidic cylindrical illumination confocal spectroscopy as fluorescently-labeled CNAs passed through the analysis constriction on the chip. This procedure did not require complex preparation steps such as DNA isolation or amplification, only addition of a DNA intercalating dye to the sample was needed. Additionally, MIC has been used to quantify cellular DNA content, an indicator of changes in the cell cycle and cytotoxicity [142].

3.2.2 Ribonucleic acid (RNA)

Messenger RNA (mRNA) is central to gene expression, which in turn plays a critical role in cellular physiology. Accordingly, disease and therapeutic interventions can often involve changes to the expression of certain genes, which can thus be analysed through mRNA detection and quantification [133]. Zhang and associates [144] used droplet-based microfluidics to detect gene expression differences of the cancer biomarker epithelial cell adhesion molecule (EpCAM) amongst distinct cancer cell types (**Figure 4B**). The use of two aqueous inlets for their emulsion droplet-generating device produced uniform agarose droplets with specified amounts of constituent sample and reagents. Further, the system enabled single RNA molecule emulsion reverse transcription polymerase chain reaction (RT-PCR) to be performed within agarose droplets, at high throughput levels. Flow cytometry and fluorescence microscopy were used for analysis of the agarose beads. A similar droplet-based microfluidic platform for gene expression analysis using mRNA

samples was developed by Mary and coworkers [145] to study TATA-binding protein (TBP) gene (cancer biomarker). In contrast, Mousavi et al. [146] developed a system to detect the mRNA lung cancer biomarker heterogeneous nuclear ribonucleoprotein B1 (hnRNP B1) using SPR analysis.

The goal of a "sample-in, answer out" diagnostic platform for mRNA detection in clinical specimens has been advanced by Gulliksen and colleagues [147] (**Figure 4C**). Their microfluidic system integrated sample preparation procedures on a preparation chip (e.g. cell lysis, nucleic acid extraction) and analytical procedures on the nucleic acid sequence-based amplification (NASBA) chip (e.g. amplification, fluorescent detection). Human papillomavirus (HPV) E6/E7 mRNA was detected from cervical cytology specimens, a biomarker of cervical cancer. In addition, a microfluidic platform for neutrophil isolation from blood samples with subsequent on-chip isolation of mRNA (and protein) has been described [148]. Genome-wide RNA microarray examinations revealed discernible temporal changes in transcriptional patterns with clinical injury course.

MicroRNA expression levels in tissues and blood have recently been investigated as potential biomarkers of normal and abnormal (disease) physiology [149]. Moltzahn et al.'s [150] search for potential serum mircroRNA biomarkers for prostate cancer involved combining multiplex quantitative RT-PCR with a microfluidic chip. The biomarker profiles produced enabled distinction between healthy, high-, medium-, and low- risk prostate cancer patient samples. Analysis on the nanolitre scale increased time and cost efficiencies. Similarly, Jang and coworkers [151] performed microfluidics-based real-time quantitative PCR (qPCR) analyses for microRNA lung cancer biomarker discovery. While Garcia-Schwarz and Santiago [152] integrated ITP and DNA-functionalized hydrogels on their microfluidic chip to achieve enhanced microRNA detection sensitivity.

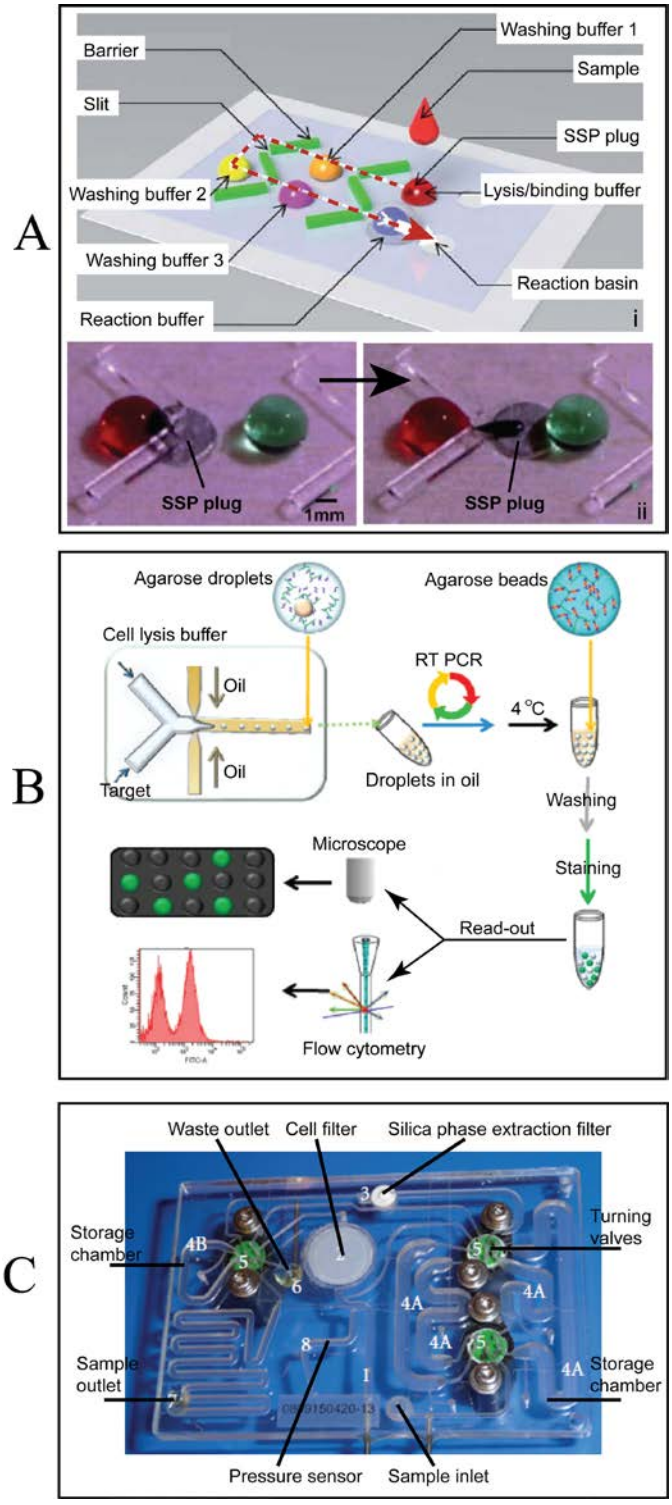

Figure 4. Microfluidic platforms for nucleic acid biomarker analysis: **(A)** Schematic of the microfluidic platform developed by Zhang and associates [135]: i) With sequential movement of droplets through the device (enabled by silica superparamagnetic particle (SPP) magnetic actuation) sample preparation and genetic analysis steps are performed on-chip within individual droplets. V-shaped micro-elevation slits facilitate SPP splitting from droplet, ii) Experimental setup showing the SSP splitting from the red droplet and squeezing through

the microelevation (which blocks the red droplet's movement) via magnetic actuation; the SSP plug then merges with the green droplet. © The Royal Society of Chemistry. **(B)** Procedure employed by Zhang et al. [144] for single-molecule RNA analysis. The two-aqueous-inlet microfluidic droplet generator produces agarose droplets containing reagents and one RNA molecule. RT-PCR is performed within the droplets and subsequent detection via fluorescence microscopy and flow cytometry follows. © American Chemical Society 2011. **(C)** Gulliksen and coworkers' [147] microfluidic platform for point-of-care mRNA analysis: i) Sample preparation chip, which performs cell preconcentration and lysis, and extraction of nucleic acid. © Gulliksen et al. 2012.

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3.3 Carbohydrates

Due to their abundance and the importance of the roles they play in cellular functioning, carbohydrates can be useful biomarkers [153]. Despite this, progress in glycomics has been hindered by the available analytical methodology, as it is quite far behind that of proteomics and genomics [154]. This category includes simple monosaccharides and disaccharides such as glucose and fructose respectively, and more complex oligosaccharides and polysaccharides (**Figure 1**).

The glucose concentration in blood and urine is a biomarker for diabetes diagnosis and monitoring. To address this, Sheng et al. [155] developed a microfluidic device for electrochemical measurement of blood glucose concentration. Their platform consisted of a tunable microreactor, created by localising magnetic nanoparticles functionalised with glucose oxidase within the microchannel by applying an external magnetic field. The device demonstrated good accuracy for human serum samples, with results comparable to conventional testing. In contrast to using glucose-oxidase, Hou et al.'s [156] device employed heated colorimetric reaction and spectrophotometric detection. Lankelma and colleagues [157] also used an alternate approach, measuring urine glucose concentrations with a μPAD. Moreover, multi-target microfluidic chips have also been developed whereby the device can detect glucose in addition to other biomarkers such as uric acid [158], urea and creatinine [159], or tumour proteins [160].

Glycans are oligosaccharide or polysaccharide chains, and may be potential biomarkers for various cancers such as breast and prostate [154]. Investigating this, Cao et al. [161] developed a microfluidic platform for detecting alterations in cell-surface glycan expression, with proposed applications to cancer metastasis. Within the microchannel, glycans selectively bound to the lectins functionalised on the electrodes (**Figure 5A**). Glycan expression was evaluated by simultaneous electrochemical impedance spectroscopy and optical microscopy, which enhanced assay accuracy and sensitivity. Changes in glycan expression in response to drug exposure were also observed.

Furthermore, microfluidic platforms have been used to identify candidate glycan biomarkers. The serum N-linked glycome was profiled using Chu and coworker's [162] microfluidic system. The device separated and identified N-linked oligosaccharides using on-chip nano liquid chromatography, coupled with time-of-flight mass spectrometry. Good sensitivity and repeatability was demonstrated.

3.4 Lipids

The diversity and particular properties of lipids makes their characterisation, detection, and quantification challenging [163]. Despite this, lipids can provide important clinically-relevant information [164]. Cholesterol, for example, has been used clinically as a biomarker for heart disease for more than 50 years [163]. Lipid biomarkers include cholesterol, acylglycerols, phospholipids, and prostaglandins (**Figure 1**).

Cholesterol is a biomarker for cardiovascular conditions such as hypertension and atherosclerosis, which can lead to cardiovascular disease (e.g. myocardial infarction and cerebrovascular accidents) [164]. Utilising the cholesterol-cholesterol oxidase reaction, Ali et al.'s [165] microfluidic cholesterol biosensor employed indium tin oxide (ITO) electrodes coated with a thin film of anatase-titanium dioxide nanoparticles, functionalised with cholesterol oxidase (**Figure 5B**). Cholesterol sensing was achieved using the electrochemical response generated upon exposure to samples. Alternatively, Ruecha and colleagues [166] demonstrated rapid and highly sensitive cholesterol detection using microchip capillary electrophoresis coupled to amperometric measurement.

Triglycerides can indicate the risk of developing conditions such as coronary heart disease and hypertension [164]. Chen et al. [167] analyzed serum triglycerides using a microfluidic bead-based enzymatic carrier chip. Enzymes (lipase, glycerokinase, and glycerol-3-phosphate oxidase) co-immobilised on magnetic nanoparticles reacted with triglycerides in serum samples, with electrochemical detection occurring at a gold nanoband microelectrode. Moreover, the components comprising triglycerides, which are fatty acids and glycerol, have been individually analysed in near real-time from adipocyte secretions using microfluidic devices [168, 169]. In addition, Muck et al. [170] analysed a range of lipids including fatty acids, mono-, di-, and tri- glycerides on their chips using laser desorption ionisation time of flight (LDI-TOF) mass spectrometry.

3. 5 Metabolites

Metabolites are small molecules that are intermediates or products produced in metabolic processes [108]. Detection of metabolites can be challenging due to their very diverse chemical and physical properties, including solubility, instability, and vast concentration ranges [171, 172]. However, they are highly sensitive to gene-environment interactions, and changes in metabolite concentrations can provide information about metabolic processes and in turn, associated diseases and therapeutic responses [171]. This group includes small molecules produced through metabolism, such as reactive oxygen species and dicarboxylic acids (**Figure 1**).

A vast array of metabolites are present within blood due to its important physiological roles, with one being a transport system for secreted and excreted substances [173]. For example, a multi-step quantitative assay for the oxidative stress biomarker hydrogen peroxide (H_2O_2) was performed using the digital microfluidic automaton developed by Jensen and coworkers [174] (**Figure 5C**). This device comprised of a 3-layer glass and polydimethylsiloxane (PDMS) structure, with a 2-dimensional 8×8 rectilinear microvalve array. Microvalves acted as reaction chambers, in addition to controlling fluid flow upon receiving signals from pneumatic channels. Programming different sequences of microvalve actuations enabled various operations such as mixing, rinsing, and dilution of reagents to be conducted. The assay demonstrated high accuracy with a detection limit at the sub-micromolar level, and thus able to detect normal serum H_2O_2 levels.

In addition, the microfluidic platform developed by Lin and associates [159] enabled detection of creatinine and urea (also glucose), biomarkers for kidney disease (**Figure 5D**). The device integrated a field effect transistor into a microfluidic chip. The biomarkers reacted with the appropriate enzyme (urease or creatinine deiminase) located within microbeads, which were immobilised at the sensor surface via a magnetic field and physical obstacle. Production of hydrogen ions from the reaction changed the sensor potential, which was measured to determine the analyte concentration. The targets were analysed sequentially, demonstrating a range of detection of 1-16 mM for urea and 0.01-10 mM for creatinine. Serum zinc concentrations, a biomarker for a range of conditions including dementia, have also been measured using a centrifugal microfluidic device [175].

Other researchers have used μPADs for analysing nitrite ion (NO_2^-) and uric acid (UA), which are biomarkers for renal and lung diseases [176]. In an exciting development, through

developing an accessory and application for a smartphone, Oncescu and colleagues [177] demonstrated a portable and accessible tool to rapidly monitor pH (a measure of the hydrogen ion (H^+) molar concentration) in both saliva and sweat.

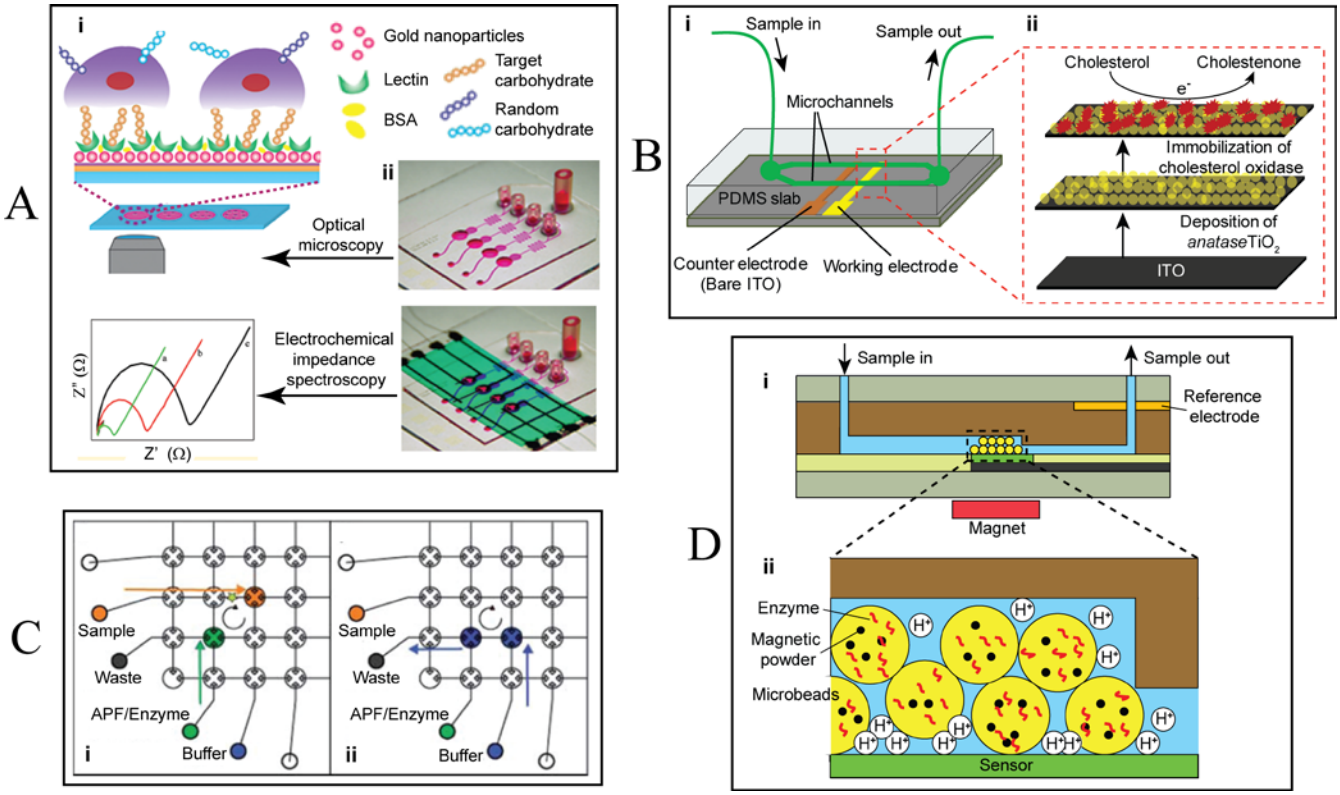

Figure 5. Microfluidic platforms for carbohydrate, lipid, & metabolite biomarker analysis. **(A)** Cao et al.'s [161] investigation into cell-surface glycan expression alterations using their microfluidic platform. i) Schematic of the lectin array in which specific lectins able to recognise target cell-surface glycans composed the surface coating of the electrode sensors. Glycan evaluation is conducted using the electrochemical impedance spectroscopy assay and optical microscopic observation, ii) Experimental setup. © American Chemical Society 2012. **(B)** Microfluidics cholesterol biosensor developed by Ali et al. [165]: i) The system comprises of two parallel microchannels connected at either end to a single inlet and outlet, which pass across the counter electrode and working electrode, ii) Close-up view of the working electrode reaction where cholesterol is oxidised by cholesterol oxidase to form cholestenone, with the electrochemical response used for cholesterol sensing. © Elsevier B.V. 2010. **(C)** Illustration of the program in the digital microfluidic automaton for performing quantitative hydrogen peroxide assays [174]. The microvalves act as reaction chambers, in addition to controlling fluid flow upon receiving signals from pneumatic channels. i) The sample is loaded into a microvalve and then mixed with aminophenyl fluorescence (APF) and horseradish peroxidase (HRP) via sequential valve actuations transferring fluid between the microvalves. Quantification is achieved by comparing the increase in fluorescence to the standard curve, ii) Washing follows in preparation for analysis of the next sample, which involves repeating the cycle again. © The Royal Society of Chemistry. **(D)** Lin et al.'s [159] microfluidic device for glucose, urea, and creatinine detection: i)

Schematic of the device, ii) Illustration of the mechanism of detection. The biomarkers react with the appropriate enzyme located within microbeads, which are themselves immobilised within the channel at the sensor surface via a magnetic field and physical obstacle. The hydrogen ion reaction product changes the sensor potential, which is used to measure analyte concentration.

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4. CONCLUSIONS & OUTLOOK

Biomarkers have immense potential to benefit healthcare and society at large, and are destined to play a major role in the future of personalised medicine [1]. While there has been significant academic and corporate investment, development of biomarkers has thus far been hindered by various challenges. Indeed, only a very small percentage of candidate biomarkers discovered have reached clinical utilisation [3, 6].

Microfluidic technology may be well suited to overcoming some of these developmental challenges [178]. As mentioned previously, inadequacies in biomarker specificity and sensitivity, as well as in characterisation and validation strategies, have been identified as major challenges [6]. The multiplexing capacity of microfluidics offers the potential for improving sensitivity and specificity through the combinatorial power generated from combining several markers for analysis [6, 12]. Microfluidics as an analytical technology has also been promoted as capable of achieving detection with high sensitivity and resolution [8]. Furthermore, the capacity for high-throughput screening, high efficiency, and low cost make it appropriate for biomarker discovery [16]. In addition, the low sample and reagent requirements, fast analytical times, portability, and the potential to achieve higher sensitivities make microfluidics very appealing for use in biomarker-based analytical devices, including for biomarker characterisation and validation purposes [86, 179]. Development of biomarker panel assays at reduced time and cost are additional benefits of microfluidics that could aid biomarkers to reach the clinic [16]. While microfluidic biomarker platforms offer great promise and are increasingly gaining research and commercial focus, they too face their own obstacles.

Indeed, challenges faced by microfluidic devices include non-specific binding (e.g. to device surfaces) and fouling due to blockage of microchannels by sample components [178]. Such problems can lead to irreproducibility of result measurements and failure of the device. In addition, issues relating to antibody array sensitivity and durability are present [178]. Although, strategies to improve microfluidic device parameters may include employing aptamer-based biomarker capture for enhanced assay sensitivity, specificity, and durability, and label-free detection, which enables direct detection without label disturbances [18, 178]. Passivation can also be used, an approach to minimise non-specific binding via surface modification [178]. While incorporation of nanosensors into microfluidic devices may be able to increase assay sensitivity, enabling early detection of sub-millimetre tumours for

example, as well as possibly making microfluidic systems independent from off-chip systems [180].

In addition to concerns surrounding device performance, there are also various other challenges faced by promising technologies such as microfluidics that hinder the transformation into a commercialised and clinically-used device. Gutman and Kessler [181] provide a comprehensive discussion regarding the challenges of bringing diagnostics to market, with a focus on the USA; while Baratchi et al. [182] review those challenges specific to microfluidic devices. Accordingly, for microfluidic devices this includes the issue that many of the reported devices demonstrate proof-of-concept capabilities only [182]. Some of these have only analysed low complexity samples, such as spiked solutions. However, to progress towards clinical use, clinical validity and utility must be demonstrated, which requires testing using clinical samples. This may be beyond the reach of some research groups due to hindrances, such as those posed by regulatory, legal, ethical, and practical concerns [99]. Further, the high costs associated with diagnostic development, including clinical validation, also poses a substantial obstacle [16]. More widely, inadequate reimbursement for investors may limit investing [99].

Interfacing current biomarker platforms with off-chip components such as pumps, processors, and detectors e.g. microscopes, is another issue [183]. The use of off-chip components, particularly large expensive instruments, is not practical for point-of-care usage [18, 184]. Miniaturisation and integration of multiple preparative and analytical components onto a single ‘lab-on-a-chip’ device will be needed [11, 178]. One development in this direction is the emergence of biochemical analytical accessories and applications for smartphones. Such advancements have many benefits, including enhanced accessibility and availability of medical testing by increasing portability, simplifying setup and operation, and reducing testing costs [177, 183].

Furthermore, there exists a reluctance to adopt new diagnostic methods by many in the medical community (including institutions), which may impede development and implementation [86]. These are consumer-posed challenges to biomarker clinical usage. To be adopted by healthcare professionals, microfluidic platforms must clearly demonstrate substantial advantages over existing devices and other emerging candidates [86]. Overcoming this hesitance is important if these platforms are to be in widespread clinical use in the future.

Further ways of reducing the barriers to implementing diagnostic devices in the future possibly include demonstrating cost-effectiveness (e.g. in terms of public health impact), which may promote investment and advocacy/support from third parties such as government, industry, and venture capital firms [95, 99]. Strategic partnerships between research groups and medical institutions may also be important. This collaboration could be mutually beneficial, such as through facilitating clinical validation of the device via access to clinical samples, and enhancing user satisfaction via gaining feedback from medical personnel (e.g. on user-friendliness) to guide device development. Proactive regulation by regulatory authorities including cooperation with other stakeholders and clear guidelines for approval, could also assist the translation of diagnostics into the clinic [99].

Thus, it can be seen that much work remains before biomarkers live up to expectations and become 'cornerstones' of medicine [1]. Microfluidics provides an opportunity to help achieve this. However, a multi-disciplinary effort will be required for the development and implementation of both biomarkers and their microfluidic analytical devices. Such an investment is warranted given that the biomarker-microfluidics partnership has enormous potential yet to be fully realised.

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