Review

Human Sputum Proteomics: Advancing Non-Invasive Diagnosis of Respiratory Diseases with Enhanced Biomarker Analysis Methods

Norberto A. Guzman [*] and Andrea Guzman

Review

Human Sputum Proteomics: Advancing Non-Invasive Diagnosis of Respiratory Diseases with Enhanced Biomarker Analysis Methods

Norberto A. Guzman [1,2,*] and Andrea Guzman [2]

[1] Princeton Biochemicals, Inc., Princeton, NJ 08543, USA

[2] AffinityCE LLC, East Brunswick, NJ 08816, USA

* Correspondence: guzman@affinityce.com; Tel.: +1-908-510-5258

Abstract: Many ailments can be diagnosed while they are asymptomatic, meaning that the patient has no signs or symptoms of a progressing disease. If caught in their initial stage of formation, these maladies can be effectively treated, leading to successful outcomes; curative therapies can halt illnesses from advancing, thus improving the quality of life, and long-term survival of the patient. Still cutting-edge upgrades in precision technologies are necessary for early, reliable, affordable, and rapid disease detection, but also are vital for the well-being of people and the future of global public health. The emerging role and utility of non-invasive and repeatable diagnostic test approaches for detection of health conditions has been liquid biopsies based on genomic biomarkers. As such, biological fluids permit any measurable molecular indicator or signature (e.g., proteins), to provide valuable information on individual's wellness and/or disease. Among the bodily secretions used for non-invasive diagnostics is sputum, a complex viscous gel-like biopolymeric network, that has gained growing recognition as a rich source of biomarkers of airway infections, pulmonary diseases, and serves as a determinant to reveal other illnesses. As per the World Health Organization, the burden of respiratory conditions is exacerbated by factors, ranging from considerable subjection to air pollution and occupational contaminants, to tobacco smoking and second-hand smoke, in addition to poor socio-economic status. Due to the likely increase of these determinants, respiratory tract ailments are on the rise, affecting the health of many individuals, in addition to putting stress on healthcare facilities and services worldwide. We therefore highlight the need to use expectorated or induced sputum specimens as a routine sample source for testing valuable protein biomarkers to diagnose these chronic maladies, to predict inflammation and disease progression, as well as monitor the effectiveness of treatments. Further, we discuss the urgency for fast and reliable point-of-care methods to detect and quantify crucial protein biomarkers in sputum specimens, and limitations faced when dealing with their complex matrices.

Keywords: COVID-19; immunoaffinity capillary electrophoresis; lateral flow immunoassays; point-of-care testing; respiratory disease biomarkers; SARS-CoV-2 virus; sputome; sputum proteomics

1. Introduction

Pivotal studies on sputum began in the 1950-1970s, and research has progressed significantly over the past decades. Initially, investigators and clinicians examined sputum cells on stained smears and mycobacterial cultures [1–4]; today, advanced imaging techniques coupled with newly found biomarkers have the potential to evaluate the efficacy of therapies in patients with pulmonary illnesses, specifically, tuberculosis (TB) [5]. As more biomarkers are identified, the probability is greater to boost research and development of enhanced pharmaceuticals, methods to measure disease activity, and the impact of various therapeutics. However, many conditions are still

diagnosed as if they are homogeneous entities, based on criteria that has not changed in a century, including complex and heterogeneous ones, such as chronic airway diseases [6,7].

Recent milestones in drug product development are now being used to manage many diseases. Still, it is essential to note that not all chronic respiratory ailments respond favorably to existing remedies, including biological therapies. Focus has therefore shifted towards personalized medicine, highlighting the importance of biomarkers to redirect costs for alternative treatment strategies so that patients can benefit the most from them. The finding of new biological indicators has not only made it possible to identify specific sick subgroups, but also offers targeted biologic therapies to individuals suffering from severe respiratory conditions [8]. As such, proteomics can reveal molecular pathways of diseases and provide translational perspectives to inform clinical decision making. In fact, studies in three types of asthma - classic, cough-variant, and chest tightness variant - found that from the analysis of more than 1000 induced-sputum proteins, 23 secreted proteins were higher in experimental groups versus control groups [9].

Experiments performed in the last decade on an array of proteins located in the sputum of patients affected with severe pulmonary disorders, have allowed for the pinpointing of important proteins and peptides whose concentration levels alter in response to the person's state of health [10]. Certain afflictions, such as nontuberculous mycobacterial (NTM) lung disease, are hard to diagnose and cure; preliminary investigations have revealed that two proteins correlated with iron chelation were significantly downregulated in critical cases of NTM lung disease, and its treatment was associated with heterogeneous changes in its sputum protein profile [11]. In another study, the identification and characterization of specific sputum proteins, such as beta- integrin, vitamin-D binding protein, uteroglobin, profilin, and cathelicidin, used as biomarker signatures, were sufficient to differentiate active tuberculosis from non-active TB patients [12].

Oligomeric mucins, the major gel-forming constituents of extracellular mucus that includes the airways' mucus gel or sputum, are complicated to handle when trying to discern its protein constituents with proteomic approaches due to their heavily glycosylated nature (up to 90%), high molecular weight (200 kDa-200 MDa) and size (Rg 10-300 nm). Their core or interior proteins are very large and highly substituted with oligosaccharides, that only permit access to a limited and restricted portion of their proteins [13]. For instance, a large-scale label-free quantitative mapping of the sputum proteomics showed that the "core" sputum proteome was composed of 284 proteins, and the "extended" proteome contained 1666 proteins [14]. Specific studies performed on the sputum proteome of cystic fibrosis patients were associated with progressive lung function impairment, some of which might have value as biomarkers of the severity of this illness [15]. Other phlegm proteins, such as eotaxin-1, interleukin-5 (IL-5), and eosinophil peroxidase (EPX), are potential predictors of remission in patients afflicted with severe eosinophilic asthma. The confirmation of sputum biomarker values for certain airway maladies in clinic settings, is now recommended by the American Thoracic Society and the European Respiratory Society, including guidelines in the management of severe asthma [16]. Still, much remains to be done, as the currently validated biomarkers only anticipate responses in a subset of cases [17].

While sputum supernatant extract is a viable alternative to liquid biopsy specimens for the diagnosis of lung cancers [18], the study of biopsy technologies for omics (genomics, transcriptomics, proteomics, metabolomics, and others), continues to evolve. Particularly in the field of sputum proteome, or the entire complement of proteins in sputum, known as sputome. Although liquid biopsies are not yet recognized as the gold standard tool to confirm different ailments, they are primarily used as a complementary test for tissue cultures [19]. Obstacles, such as the lack of sensitivity and precision to identify some particular diseases still remain, yet trends in non-invasive tests indicate that the future of screening and monitoring of chronic illnesses is promising; new sources of sputum molecular biomarkers like extracellular vesicles, have the potential to become a non-invasive liquid biopsy diagnostic tool for pulmonary illnesses [20].

Breakthrough technological advances that allow for the simultaneous confirmation and classification of hundreds of proteins, creating optimism that an abundance of novel protein biomarkers can be validated, but progress has been slower than anticipated. While proteomics has

the possibility to improve diagnostic, prognostic, and predictive tests, its applications in the medical field have yet to be fully applied [21,22]. Also, the utilization of protein biomarker panels, rather than individual protein biomarkers, offers a more comprehensive representation of human physiology, especially in situations with elaborate differential diagnoses and in patients with multiple comorbidities [22]. In infectious diseases, such as the coronavirus disease (COVID-19) caused by severe acute respiratory syndrome (SARS-CoV-2 virus), reports indicate that proteomics signatures track symptom severity and antibody responses that may identify individuals who are more likely to suffer from persisting symptoms [23].

In this manuscript, we discuss the various procedures to collect sputum, methods and systems that disrupt/dissolve the intricate, viscous, and variable sputum/phlegm meshwork structures to release its contents, techniques to identify and characterize crucial sputum protein biomarkers, as well as the advantages-disadvantages of each approach. We also explore how innovative healthcare technologies such as point-of-care testing (POCT) can improve patient outcomes when successfully implemented.

2. Methods and Systems Utilized to Study Sputum Proteomics

Approximately 95% of epithelial surfaces are mucosal and comprised of a single layer of epithelial cells mostly covered by a gel-like layer of mucus. They form semipermeable barriers enabling nutrient absorption and waste secretion and provide a major route of entry and release for pathogens, including viruses [24]. The mucus layer contains a range of mucin glycoproteins as its main components. The mucin family of glycoproteins is classified into those that are secreted by specialized epithelial cells and form the mucus gel, and those that are embedded in the epithelial cell membrane. Specifically, mucus provides protection against dehydration, abrasion, toxins, and pathogens, and is a reservoir for antimicrobial molecules [24].

Mucus hyperproduction, hypersecretion, and/or hyperconcentration are primary symptoms of obstructive lung and other airway afflictions. Inflammation of the respiratory tract mucosa due to infectious or noninfectious conditions, results in hyperreactivity of the cough receptors [25,26]; mucus regulation is therefore valuable from a therapeutics perspective as it contributes to improved airflow, reduces symptom and disease severity, and to an extent, helps with illness prognosis [26]. Due to the complexity and viscosity of sputum/phlegm, a mix of protocols have been developed for specimen collection and dissolution, followed by evaluation of sputum protein constituents. Generally, measuring certain proteins in sputum can vary widely between repeat analyses, is susceptible to sample processing effects, changes in response to organism's conditions, and can be hard to quantify accurately and precisely by mass spectrometry, despite other proteins having high inter-individual variance [10,14]. It is thus preferable to use additional testing to obtain an accurate conclusion of a respiratory illness. For example, sputum analysis of proteins coupled with microscopic examination of mycobacteria can be applied as a screening tool for diagnosing pulmonary tuberculosis [27]. Equally, samples of proteomic signatures from other biological fluids that reflect differences in the inflammatory responses and can be linked to symptoms that vary in severity and duration, may be utilized [23].

The production of phlegm is usually minimal in a healthy person, but a diseased state can increase the amount or alter the nature of a patient's sputum. Airway mucus is composed of water (98%), salt (0.9%), globular proteins (0.8%), and high molecular weight polymers (0.3%) in subjects that are well. In "muco-obstructive lung diseases" the values of the MUC5B and MUC5AC, the predominant gel-forming mucins secreted in the respiratory tract can be up to 10-times higher or more in concentration; MUC5AC in particular, increases disproportionately [26,28]. Mucins, the key component of mucus, are large glycoproteins containing regions rich in serine and threonine residues that can bind by O-glycosylation to glycan chains [24]; mucin glycoproteins polymerize covalently via disulfide bonds and can aggregate, forming plugs, and blocking airflow. In some critically ill COVID-19 patients, with low survival outcomes, formation of sticky sputum or sticky plugs (viscous secretions) builds in their air tract, suggesting that alterations in their mucus attributes are an early warning to likely advances of their pathogenesis [29].

First introduced in the 1960s by Japanese thoracic surgeon, Shigeto Ikeda, flexible bronchial fibroscopy, or bronchoscopy [30,31], is the indispensable procedure applied by respiratory physicians to investigate the throat and airway or respiratory tract. Bronchoscopies can also be used to aspirate mucus, mucus plugs, and/or to perform bronchoalveolar lavage, however, the invasive nature of fibroscopy is still a primary limitation of the exam in gravely ill patients [25,31]. Today, sputum specimens are collected by one of the two non-invasive methods – either the expectorated or the induced procedure. The expectorated or spontaneous mucous secretion is taken by coughing, following standardized protocols to collect the best sample and avoid contaminating the specimen [32,33]. The feasibility for patients with severe airflow obstruction to expel phlegm is low, so conversely, an induced sputum sample is required with the aid of an ultrasonic wave nebulizer [34]. A specimen is taken after hypertonic saline in concentrations of 0.9% - 7% is inhaled for 15-30 minutes [35,36]; this helps the production of phlegm in children who cannot and is an ideal procedure to diagnose airway illnesses [37–39]. To prevent bronchoconstriction that would be induced by hypertonic saline inhalation, it is recommended to inhale salbutamol before sampling [40,41]. Since sputum induction and its processing has a high success rate, and is considered safe for children 6 years older, it has become a popular as a non-invasive clinical tool to assess airway inflammation [41,42].

After collecting the specimen, the sputum content must be released, which is a challenging process. The extraction protocol applied to obtain proteins from sputum specimens varies significantly, especially when used with lateral flow immunochromatographic assays, as the viscosity of phlegm interferes with the migration of the antigens on the test strip [43]. Investigators have intended to extract proteins by homogenization of the heterogeneous material, primarily for its plug-containing sputum [44], while others have obtained proteins by acetone sedimentation. Acetone-precipitated samples are then dissolved in 8M urea, reduced with Tris-(2-carboxyethyl)phosphine, and alkylated with iodoacetamide [9]. A summary of protocols to extracts proteins from sputum is found in Table 1 [9,26,33,43–48]. Despite the procedure used, fluctuations may occur in all methods due to the non-standard selective approach to sample preparation.

Table 1. Overview of Types of Procedures Applied to Release Bacterial, Viral, and Diverse Specimen Constituents from the Complex Sputum Meshwork.

Procedure Performed or Substances Added to Sputum or Bronchial Aspirate Samples	Rationale	Reference
Mechanical homogenization.	To macerate or crush the sputum in the presence of a suitable buffer, to obtain viable single cell suspensions or to disperse fragments of the sputum gathered evenly throughout the mixture.	[26,44]
Acetone sedimentation.	Acetone-precipitated samples are dissolved in 8M urea, reduced with Tris-(2-carboxyethyl)phosphine, and alkylated with iodoacetamide.	[9]
Tris(2-carboxyethyl)phosphine (TCEP), N-acetyl-L-cysteine (NALC), bovine serum albumin (BSA), and protease inhibitor cocktail.	TCEP and NALC (reducing agents) are used to break the disulfide bonds of sputum. BSA is used as a blocking agent.	[43]
Hydrogen peroxide (H_2O_2).	Enzymatic liquefaction. The peroxide solution, in the presence of endogenous catalase, is used to trigger the formation of oxygen bubble.	[45]
NaOH, sodium dodecyl sulfate (SDS), glass beads, temperature ($60^{\circ}C$).	Breakdown of sample mixture.	[46]

PureLyse® bead blender, solid phase extraction which does not require chaotropic salts or organic solvents.	A miniaturized bead beating system for mechanical pathogen lysis (effectively disrupt tough-walled microorganisms). This is a disinfection and liquefaction method.	[47]
Dithiothreitol (DTT), homogenization, centrifugation, supernatant desalting.	Release of proteins from sputum.	[48]

3. Considerations for Selection of a Safe and Cost-Effective Lateral Flow Assay

Each method described in Table 1, can free the molecular constituents of sputum, and are valuable techniques for use in a laboratory designed to perform experiments under a chemical fume hood – this effective ventilation system reduces unpleasant odors and protects against exposure to some hazardous chemicals. However, choosing the procedure that is best suited for home or point-of-care testing, diagnosing COVID-19 and other infectious diseases, is not straightforward. Multiple COVID-19 rapid home lateral flow immunochromatography test kits are now available in local pharmacies; these assays contain some solutions, such as detergents, inorganic phosphate, sodium azide, and other microorganism preservatives. It is important to state that the substances in these kits are present in very low concentrations and are therefore unlikely to cause human toxicity [49]. Nonetheless, as the market for these diagnostic products expands and access to them grows, the likely outcome will be an increase in subjection to liquid agents, possibly causing allergic reactions or topical irritations. Users should be aware of hazards associated to contact with these reagent fluids [49].

Diagnostic tests for respiratory viral infections traditionally use nasopharyngeal and oropharyngeal samples. In the case of ascertaining COVID-19, the most popular methods to detect SARS-CoV-2 are via antigen-based lateral flow immunochromatography assays or lateral flow immunoassays (LFIA) and reverse transcriptase polymerase chain reaction (RT-PCR) assays with nasal and throat swab specimens. These techniques use a sampling protocol that can be stressful, especially in children, particularly when repeated testing is required. Unfortunately, these procedures have a rate of false-negative results that might enable convalescent COVID-19 patients to meet the criteria for discharge from hospital and/or release from quarantine, resulting in the spread of disease [33].

The extraction and enrichment of the target molecule in a sample is crucial to obtain a high rate of analytical sensitivity and detection limit in LFIAs. In complex matrices, it is necessary to acquire the maximum quantity of the specific analyte, especially if the substance of interest is found in low concentrations. Recently, a rapid, and affordable pretreatment protocol for releasing constituents of sputum was published. Depicted in Figure 1, this process consists of mixing Triton X-100 and/or another detergent, Alcalase (subtilisin) and/or another proteolytic or digestion enzyme to a sputum sample, that exposes hidden or masked antigenic sites of viral specimens or lingering fragments of viral proteins present in the sputum meshwork [27].
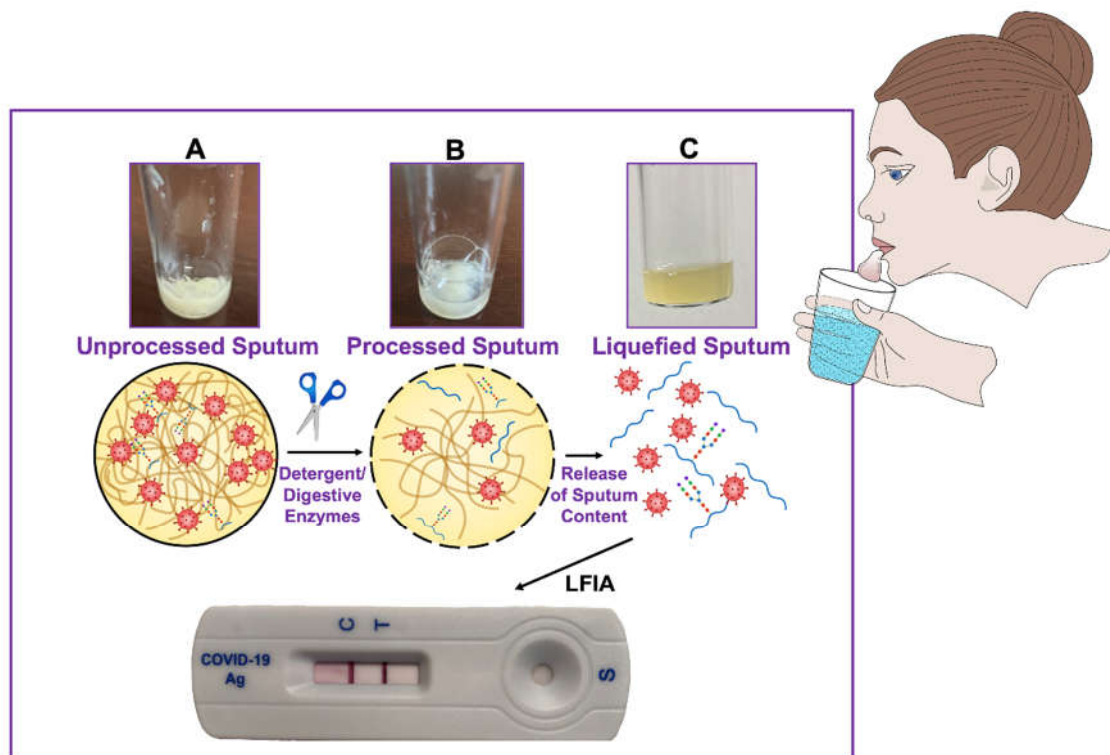
Figure 1. Representation of a sample preparation (disruption-extraction-digestion), whereby the nonionic detergent Triton X-100 and the endoprotease Alcalase are applied to the sputum specimen [33]. As depicted in panel A, the phlegm is a thick, rubbery, sticky, viscous, and gel-like biopolymeric network, which contains different types of cells, cell debris, microorganisms, and chemical-biochemical entities. After adding the detergent and the protease, some disruption occurs (panel B) as influenced by the incubation time, quantity of the proteolytic enzyme, temperature, and the pH of the solution. The incubation temperature was set at 25 degrees Celsius, and the experiment resulted in a solution containing primarily soluble material and a precipitate of insoluble components (panel C). Following decantation or centrifugation, the supernatant was tested for the presence of SARS-CoV-2 virus, or virus components, on a lateral flow immunoassay (LFIA) platform or strip.

Cumbersome and multi-step procedures can be error-prone, require skilled users, as well as additional consumables and laboratory instrumentation, hard to obtain in low-resource settings [47]. For example, the RT-PCR test, considered the gold standard test, may take 1 to 2 days to get the results, and are often expensive due to the need for trained technicians, and costly reagents. The average price of a RT-PCR test in the United States of America ranges from $50 to $200; in low- and middle-income countries, this price can make RT-PCR screening for COVID-19 and other diseases prohibitive [50,51]. Conversely, the rapid antigen test cost is about $10 to $30 in the U.S. and is less than $10 in other developed nations [52]. The antigen tests do not require trained healthcare professionals and provide results in 15 to 20 minutes. Unfortunately, many LFIA tests that use nasopharyngeal and oropharyngeal samples have poor analytical sensitivity, unless there is a high viral load; but a sputum specimen can provide a positive COVID-19 diagnostic result with a rapid antigen test, when nasopharyngeal and oropharyngeal samples yield a negative finding [33].

4. Advanced Technologies to Enhance Lateral Flow Assay Analytical Sensitivities and Specificities

It is not always possible to establish the intensity of the color-band or even detect the positive color in a LFIA platform with the naked eye. Other criteria are also necessary to confirm an affirmative outcome with an antigen-based assay. Increasing the analytical sensitivity and specificity

of LFIA tests are an inevitable requirement to broaden its application areas, and both are essential for assay optimization and signal amplification techniques [53]. Most commercially available LFIAs, offer qualitative or semi-quantitative analyses and need dedicated tools for the quantitative measure of biomarkers. To improve the analytical sensitivity and accuracy of LFIAs, efforts have been made to address these limitations. For example, a 20-minute lateral flow antigen test was designed to be read by an artificial intelligence logarithm, known as lateral flow with artificial intelligence read (LFAIR), whose results can be accessed via a smartphone application. Not only does it provide results at home, it can also digitally transmit these findings to healthcare workers and public health authorities in real-time [50].

Supplemental components have been incorporated into these devices to allow for the use of external detectors. For instance, a compact optical spectroscopy apparatus was produced from a 3D printer, carrying a micro-spectral chip, that can harness sophisticated light traces to estimate magnified detection signals and measure spectral results concurrently. This technique was created to significantly reduce misinterpretations of LFIA results [54]. Another system developed to enhance sensitivity in LFIAs, was realized by preventing the competition between specific and background antibodies that permit binding to nanosized labels. This blocking mechanism was reached via electrokinetically to drive delivery of the label once the antigen-antibody is in the test zone. The limit of detection using electrophoretically driven LFIA (eLFIA), has been proven to be 1000 times lower than that of conventionally LFIA [55]. Various post-assay upgrades based on chemical reactions to facilitate high sensitivity for LFIA have been presented in a critical review [56]. In addition, a centrifugation-assisted lateral flow immunoassay (CLFIA) has been recommended to increase sensitivity when compared to traditional LFIA based on strips [57], and magnetic enrichment applying multifunctional nanocomposite probes has also been proposed [58]. A recent publication highlights the importance of assay optimization and specificity of LFIAs that relies on the affinity, stability, and properties of the immobilized capture molecule [53].

5. Various Technologies used to Study Proteins in Sputum

Proteomics is the comprehensive study of the composition, function, interactions, and structures of all proteins contained in a cell, tissue, or individual to understand an organism's nature. This area of research encompasses a broad range of technologies aimed at determining the identity and quantity of expressed proteins in cells, their three-dimensional structure and interacting molecules [59–61]. It is estimated that there are at least one million human proteins, many of which contain alterations such as post-translational modifications (PTMs) [61–63]. In 2013, the proteomics community coined the word "proteoforms" to refer to all the unique protein molecules produced from a gene. The chemical diversity of proteins is expressed in their many proteoforms, derived from combinations of genetic polymorphisms, RNA splice variants, and posttranslational modification [60–63].

While the first analyses of proteins in biological fluids date back to the early 1970's [64], trailblazing advances to evaluate proteomic sputum have only occurred in the last decade [9–12]. The rationale for looking at phlegm as a wealth of information on lung health, is based on its' composition which encompasses mucus and microbial constituents. Researchers can therefore colonize bacteria/viruses, and particulate/inhaled matter derived from the external environment to inflammatory cell components, including cells that reside either in the airways' lumen, tissue, or cell debris in these compartments [12]. Since precision medicine extends beyond a single biomarker/treatable trait where a patient may have multiple options for treatment [6], it is ideal to use multiple techniques capable of analyzing several biomarkers, or one single platform that can identify and quantify numerous biomarkers. Table 2 summarizes the various options to study proteins in sputum, and are discussed in other publications [12,65–71]. It is apparent that the protein composition of sputum best reflects the state of lungs [48]. Additional information on sputum proteomics studies can be found in references [72–80].

Table 2. Overview of Various Techniques Used to Analyze Proteins/Peptides Extracted from Sputum.

Procedure Performed to Analyze Proteins/Peptides in Sputum	Number of Proteins/Peptides Analyzed	Reference
Two-dimensional electrophoresis gel (2-DE), and one-dimensional gel electrophoresis followed by liquid chromatography tandem Mass spectrometry (GeLC-MS/MS)	191	[65]
Mesoporous silica beads – matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)	>400	[66]
Liquid chromatography – Mass spectrometry (LC-MS)	192 - 1666	[15,16,67,68]
Capillary liquid chromatography (capLC-MS)	203	[69]
Multi-dimensional protein identification technology (MudPIT)	2210	[70]
Aptamer-based assay (SOMAscan)	1129	[71]

These references are selected illustrations of efforts to identify, quantify, and characterize proteins and peptides, including antibiotic peptides, present in sputum samples.

6. Potential Use of Point-of-Care Platforms for the Screening of Sputum Multi-Biomarker Proteins to Diagnose Infectious and Chronic Respiratory Diseases

The use of biomarkers as early warning systems in the evaluation of disease risk has increased markedly in the last decade. Making the distinction between a potential biomarker and a reliable biomarker that can be universally applied to guide critical clinical choices is a main obstacle in the field of biomarkers [81]. When referring to POCT platforms, which are designed to detect the presence of biomarkers with inexpensive and easy-to-use platforms, can they provide with certainty the detection of accurate and reliable biomarkers? Unfortunately, not always. There have been several critical roadblocks in the development of inexpensive minimal-equipment diagnostics, and even more so in the challenging faced for improving sample preparation, and biomarker analytical sensitivity and specificity. Quantitative measurement of biomarkers in complex samples is inherently difficult to do robustly, particularly when constrained to a minimal-equipment framework [82]. There are other factors that are also important to be considered as challenging in the analysis of biomarkers, such as proteins. Profiling of proteins in biological fluids has been central for decision-making in numerous diseases since the introduction of many immunoassays in the 1970s [83]. However, obstacles for comprehensive proteomic profiling include the immense size and structural heterogeneity of the proteome, and in most cases protein screens have been hampered by technical limitations, in particular limitations in sensitivity, specificity, multiplexing, and sample throughput [83]. Nonetheless, the bottleneck of sample analysis has always been sample preparation, which is often characterized by being time-consuming, labor-intensive, and error prone [84]. Particularly, when analyzing complex biological matrices such as sputum, which must be liquefied before analysis of its constituents [33,85]. Additionally, it is noteworthy to mention that many proteins are found at very low abundance in biological fluids, and the polyreactivity of affinity capture agents may have the potential to generate false results [62]. Furthermore, as numerous diseases include various pathophysiological processes, no single biomarker can be regarded as ideal in fulfilling the necessary criteria for a comprehensive diagnostic or prognostic assessment revealing optimal clinical application [86]. Therefore, a multi-component biomarker approach with the aid of crucial

biomarkers to create a detailed profile of a patient's disease, is vital to generate an accurate and reliable diagnosis and prognosis. Due to the challenges faced to collect sputum and to develop a simple method to dissolve the complex sputum matrix, emphasis should be placed if a POC testing device is intended to be manufactured for the analysis of sputum's constituents under the guidelines of ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and Robust, Equipment-free, Delivered) [87].

Currently, a gamut of technologies exists to extract and isolate proteins from intricate mixtures to further analyze them. One- and two-dimensional electrophoresis, or high-performance liquid chromatography (HPLC) are the most common uncoupling methods, but mass spectrometry (MS) is now seen as the benchmark [88,89]. Advances in quantitative MS through the application of stable isotope labelling and scanning techniques, as well as multiple reaction monitoring (MRM) have greatly enhanced the specificity and sensitivity of MS-based assays [90]. Factors including patient heterogeneity, variation in sample acquisition, storage, stability, and dynamic range of analytes, including their modification status and macromolecular interactions, all influence observation to discern biomarkers. Despite the precision and automation of these bulky instruments in the discovery and corroboration of biomarkers, they are exclusive used in research and/or central clinical laboratory settings, and do not serve the purpose of a point-of-care testing system in a doctor's office or at home for self-testing. Still, these scientific innovations have been pivotal towards the discovery of new biomarkers and corroborating the results of these biological indicators via POC tests. There is a hope that progress in the design and manufacturing of miniaturized instruments, including MS, will be a catalyst for the future of healthcare to transition from bench to bedside [91].

LFIAs, founded on an affinity-capture-migration technology occasionally yield false-negative or false-positive misdiagnoses, yet they continue to be used as a screening tool [92–95]. Conversely, immunoaffinity capillary electrophoresis (IACE), based on the principle of an affinity-capture-separation or a two-dimensional technology, are popular tests due to their ability to confirm results [62,95–100]. Even if there are doubts about the biomarker(s) separated by a capillary electrophoresis (CE) (electropherogram), the CE outlet terminal can be coupled to a mass spectrometer that also validate findings. This feature cannot be performed via lateral flow assays. Figure 2 depicts a scheme of disrupted/lysed sputum sample constituents subjected to lateral flow immunoassay and capillary zone electrophoresis.
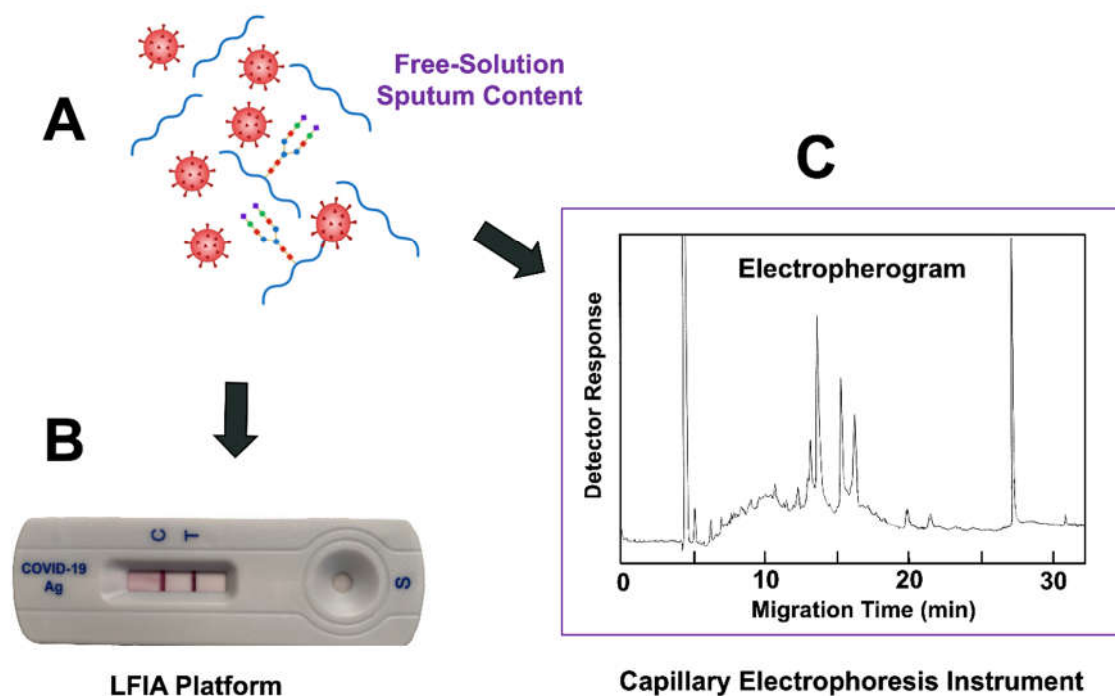
Figure 2. Diagrammatic representation of (**A**) a disrupted/lysed sputum sample treated with a detergent and a proteolytic enzyme as described in ref. [33]. This processed specimen was centrifuged to remove cellular debris. An aliquot of the supernatant constituents was tested via a LFIA platform for the detection of the SARS-CoV-2 nucleocapsid protein antigen N (**B**). Another portion underwent capillary zone electrophoresis, and the separated components of the supernatant of the entire disrupted/lysed sample were monitored at the low nm detection range (**C**) as described in ref. [96]. The electropherograms can vary significantly if distinct samples and dissimilar pretreatment procedures are applied. Supplementary experiments are under way to enrich and separate specific components via immunoaffinity capillary electrophoresis.

The implementation of a CE platform, either in microchannels or fused-silica glass as a POC device began in the 1990s [101–106]; since then, several mini handheld prototypes have been manufactured [105–109]. One palmtop bioanalyzer has a laser-induced fluorescent detector - authors affirm that this tiny device combines seamless design with low-cost construction, retailing for US$500 [105]. The palmtop bioanalyzer consists of modules, such as automated sample injection, CE separation, orthogonal LIF detection, instrument control and display, data acquisition, processing, storage, and battery power supply. Figure 3 presents the separation of standard proteins using this CE bioanalyzer, yielding results achieved in under 7 minutes.
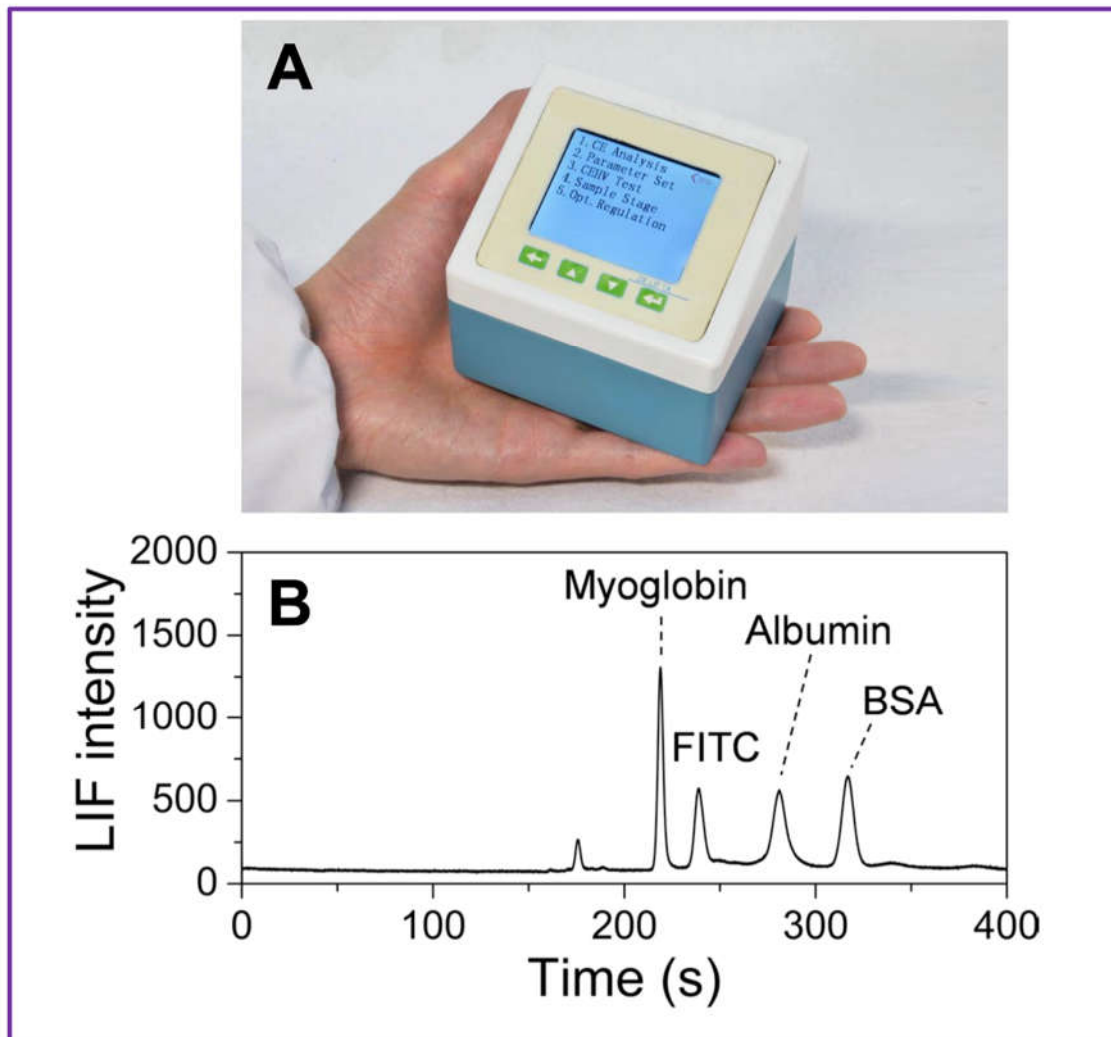

Figure 3. A handheld capillary electrophoresis instrument (**A**), shown in ref. [105]. Panel (**B**) is an electropherogram of three protein standards derivatized with fluorescein isothiocyanate (FITC), monitored by laser-induced fluorescence detection (LIF). The separation of these protein standards

was carried out in a 50 μm I.D. fused-silica capillary, 360 μm O.D., and 3.8 cm in length; the electropherogram was adapted from ref. [105].

7. Future Evolution and Benefits of a Point-of-Care Platform for the Determination of Multiple Biomarkers to Improve Early Detection and Diagnosis of Diseases

As precision medicine enters the multi-omics era, technology also needs to evolve [110]. Therefore, strengthening the healthcare system is the most ideal way to keep the population healthy and maintain a productive workforce [111]. As such, we propose a miniaturized point-of-care system based-on a two-dimensional capture-separation system capable of analyzing multiple biomarkers, that can be identified and characterized, to avoid false positive and false negative diagnostic results. This instrument is designed be portable, cost effective, generating data in a short time, having high analytical sensitivity and specificity. It can be manufactured with modular components easy to replace, and affinity-capture ligands (antibodies, antibody fragments, nanobodies, lectins, aptamers, phages, other affinity chemistry, or a combination of one or more ligands) that can be re-used multiple times. The function of this miniaturized instrument is aimed primarily as a POCT for early diagnosis and prognosis purposes of ailments, by determining protein biomarkers. Some chronic diseases have an infectious origin, such as cervical cancer (human papillomavirus – HPV) and liver cancer (hepatitis B and C viruses). Also, many patients with infectious diseases require long-term care; for example, human immunodeficiency virus (HIV) infection recognized as a chronic disease, can now be successfully managed over time [112]. Although non-communicable diseases (NCDs) are considered silent killers, they attract less attention than infectious diseases and threaten to overwhelm global health systems with their rapid rise. NCDs cause nearly 74 percent of all deaths worldwide, a majority occurring in low and middle-income countries [113–116]. The proposed portable POC model instrument with multidimensional capabilities and numerous applications is depicted in Figure 4.
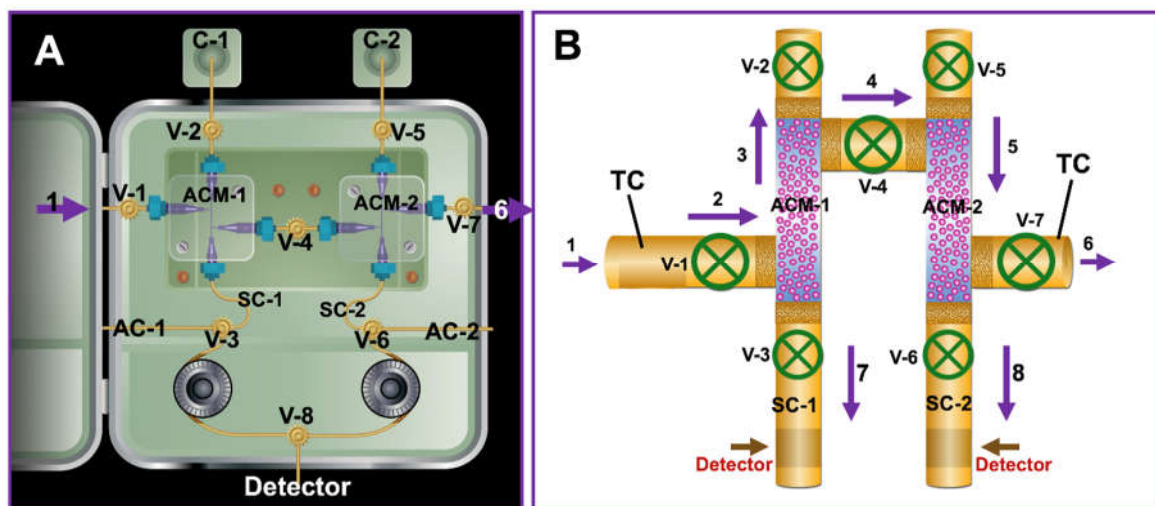

Figure 4. Diagrammatic representation of a multidimensional CE system (panel **A** and panel **B**) equipped with an analyte concentrator-microreactor (**ACM-1** and **ACM-2**) device at each transport capillary (**TC**) intersection with a separation capillary (**SC-1** and **SC-2**). Here, the ACM device has a staggered or zigzag configuration. All small arrows represent the path of the sample introduction from the inlet (**1**) to the outlet (**6**) into a waste container. Arrows **7** and **8** show the direction of separation of the analytes captured by and released from **ACM-1** and **ACM-2** in a sequential and separated mode. The green circles illustrate the various microvalves (**V-1 to V-6**) that control the passing of fluid. When the microvalves **V-2, V-3, V-5,** and **V-6** are closed and microvalves **V-1, V-4,** and **V-7** are opened, the specimen containing the target biomarkers passed through the transport capillary (**TC**) from inlet **1** to outlet **6**, and **ACM-1** and **ACM-2**. After applying a cleaning buffer, to remove unbound material, is applied, microvalves **V-1, V-5, V-6,** and **V-7** are shut, and microvalves

V-2, and **V-3** are opened. A small volume or plug of an elution buffer or solution is introduced into separation capillary **SC-1**, from container **C-1** and the targeted biomarkers captured by **ACM-1**, are released. The **ACM-1** and **ACM-2** devices are filled with a matrix containing specific affinity capturing ligands immobilized to a monolith solid support or beads, or covalently attached directly to the inner wall of the interior of each ACM. The selective affinity capturing ligands can be an antibody, antibody fragments, nanobodies, lectins, aptamers, other substances, or a combination of all these selective affinity ligands. Each ACM device captures one or more specific targeted analytes. Once the plug of an elution buffer is applied, an independent capillary electrophoresis process is initiated sequentially from the inlet side before the separation capillary 1 (**SC-1**), in the direction of the flow **7**. The process of separation of the biomarker is preferentially carried out using capillary zone electrophoresis (**CZE**), but other separation modes can be performed. The components released from **ACM-1** and separated by **CZE**, are detected/identified at the detection point where a detector (UV, fluorescence, or others) is positioned A similar but independent and sequential protocol is applied to the elution process to release biomarkers captured by **ACM-2** device, using container **C-2** (filled with an elution buffer-2, or separation buffer-2) and separation capillary **SC-2**. **Panel A** depicts an additional microvalve (**V-8**) where the two independent separation capillaries (**SC-1** and **SC-2**) are merged to share a single detector. **Panel B** illustrates these capillaries (**SC-1** and **SC-2**), each with its respective monitor. **Panel A** also shows two additional auxiliary capillaries (**AC-1** and **AC-2**) used when a separation buffer requires a different chemical composition from the one of the binding buffer, as it is required when coupled to a mass spectrometer detection system. Figure was adapted from ref. [117–120].

8. What are the Advantages of Affinity-Capture-Separation Analytical Techniques When Compared with Traditional Methods?

Traditional affinity capture immunoassays, such as enzyme-linked immunoassay (ELISA) have become the gold standard clinical diagnostic tool for the detection and quantification of protein biomarkers [121,122], and efforts have been made to use it as a platform for a point-of-care detection system for the diagnosis of infectious diseases [121,123]. ELISA continue to serve as the benchmark for diagnosis by detecting specific antigens in biological fluids for multiple applications. However, the technique has disadvantages, such as the possibility of false positive and false negative results, costly instrumentation, the requirement for trained personnel, and take longer to measure multiple analytes. In the classical double-antibody sandwich method, two different antibodies to capture and recognize the same protein antigen are needed to enhance analytical specificity, so only one target protein biomarker can be detected at a single time. Despite its limitations, ELISA remains the time-tested and best validated method for measuring individual cytokines [124]. Presently, multiplex array technologies are increasing in popularity, likely because of their ability to analyze large numbers of analytes with low sample volume compared to ELISA. When evaluating both approaches to measure cytokines, the results indicated that serum cytokine concentrations were not compatible between these two systems [125].

The limitations of traditional approaches to identify and quantify proteins have led to an evolution of new technologies for protein biomarker discovery that are highly sensitive and capable of high-throughput analyses. The advent of modernized and more sophisticated tools has granted opportunities in the exploration of biological indicators. A contemporary and prominent example of a high-throughput proteomics technology is an aptamer-based proteomics platform called SOMAscan [126]. The SOMAscan platform is a highly sensitive platform that uses slow off-rate modified DNA aptamers (SOMAmers) as high affinity proteins capture reagents to simultaneously quantify more than 1,300 human proteins in all types of protein extracts, including bodily fluids, tissue, and cells. Despite its potential, this aptamer-based proteomics technology has only been used in pilot studies and has yet to be applied to samples from population-based cohort studies [126]. As artificial single-strand oligonucleotides, aptamer possesses unique advantages compared to conventional antibodies. They can be flexible in design, have low immunogenicity, relative chemical/thermos stability, easy synthesis, as well as modifying convenience [127,128]. In contrast to

using aptamers, antibody-based methods excel in both analytical sensitivity and specificity, therefore is extensively employed to detect and quantify specific proteins [127].

An alternate multiplatform for protein research, is the highly sensitive targeted immunoassay known as the Olink proximity extension assay (Olink PEA) technology, and when combined with other platforms, offers an in-depth proteome analysis [129–131]. Also, once PEAs are coupled with next-generation sequencing (NGS) for high-throughput proteome-wide analysis, it enables parallel measurement of about 1,500 proteins with 96 samples, resulting in close to 150,000 data points per run [125]. The basic principle behind PEAs is signal generation; when two antibodies linked to corresponding DNA oligos (with the complementary sequence) find the same target, it forms a double stranded DNA (dsDNA). The presence in the reaction mix of a polymerase and other compounds allows for elongation and subsequent amplification, and each antigen will lead to a unique DNA code. The sequence then uniquely identifies a target antigen, and its abundance correlates with the protein antigen concentration [129–133].

These multi-dimensional platform technologies will continue to importantly guide in deciphering essential biomarkers and improve the substantiation of intricate diseases. For example, long-haul COVID, broadly defined as a non-monolithic, multisystemic disease that impacts multiple organ systems; it is like a disease with many subtypes that can have many different risk factors and distinct biologic mechanisms that may respond differently to treatments [134]. Because long COVID is a heterogeneous entity, it is unlikely that a single or a few biomarkers will explain its complexity. A creative rethinking of the approach for biomarker development is to harness the power of artificial intelligence to analyze large numbers of variables and identify multidimensional biomarkers to classify distinct long COVID subtypes and predict prognosis and treatment responses is needed [134].

There has been a surge in recent years in the implementation of proteomics in the study biological fluids, revealing disease mechanisms that are linked to chronic and infectious diseases. The human proteome contains thousands of protein mixtures, the consensus that most remain unexplored in their associations to multiple diseases. Even with first-rate attributes of immunoaffinity-based technologies, weakness such as insufficient specificity and antibody cross-reactivity continue [33,135]. These limitations are lessened via the Olink system, using complementary DNA oligonucleotides that attach to the antibody pairs to decrease this undetailed cross-reactivity. Still, evaluations in juxtaposing the Olink and SomaScan platforms found the matching range of coefficients varied greatly among all proteins compared. It is believed that cross-reactivity of aptamers and antibodies is a factor in the low correlation between investigations for proteomic analysis, and their cross-reactive likelihood increases as amino acid homology elevates [135,136].

Even with assays of varying benefits and drawbacks, more analysis is needed to establish the potential to integrate data across proteomics platforms fully. Investigators should be careful to treat discovery platforms appropriately, confirming key results with additional methods, using comparisons to reference standards to obtain exact protein quantification [136], specifically to acquire valid clinical biomarkers. Reliable clinical biomarkers are still limited, restricted by the suboptimal methods biomarker discovery [127,128]. When using modern technologies in the diagnosis process, careful consideration must be taken to guarantee safe, efficient, and trusted care, and avoid diagnostic errors that may lead to devastating consequences for the patient.

How can we take advantage of these emerging technologies to create an error-free, point-of-care instrument that yields accurate diagnostic tests, based on a selection of biomarkers taken from a wide range of concentrations in biological fluids, that are also affordable and accessible to everyone? Section 7 describes the added value of the two-dimensional capillary electrophoresis technique, that allows for the targeting of more than one selective biomarker in a biological fluid or tissue extract to be accurately, promptly detected and quantified at a low cost. These excellent characteristics are ideal for the future design of a portable, point-of-care instrument. Figure 5 highlights two examples validating the advantages of this two-dimensional affinity-capture separation technology. Figure 5-A shows the separation and identification of multiple biomarkers using a single microchip electrophoresis system, simultaneously in the same preconcentrator, while a dozen immobilized

antibodies target 12 distinct antigen biomarkers. During a 9-minute cycle, 12 biomarkers were analyzed, during which all analytes were reliably affinity captured, labeled with a fluorescent chromophore, eluted, separated, and detected with a laser-induced fluorescence detector [137]. The covalently attached antibodies served both as selective capture agents and as a pre-analysis clean-up system. With the aid of a semi-automated miniaturized immuno-separation system, it was possible to repeat an evaluation cycle of 6 samples/hour, to identify and quantify 12 biomarkers taken from detergent-extracted micro-dissected skin material related to mild and chronic skin lesions [137]. Figure 5-B presents a microextraction-preconcentration area containing two immobilized affinity-capture ligands that can effectively be used 45 times and exhibited an excellent repeatability and stability. In this case, the immobilized affinity-capture ligands used were aptamers, instead of antibodies, capable of trapping two distinct analytes. The process of capture, elution, separation, and detection was performed using a conventional capillary electrophoresis method coupled to a mass spectrometer [138]. The limits of detection of the analytes were 1 pg/mL.
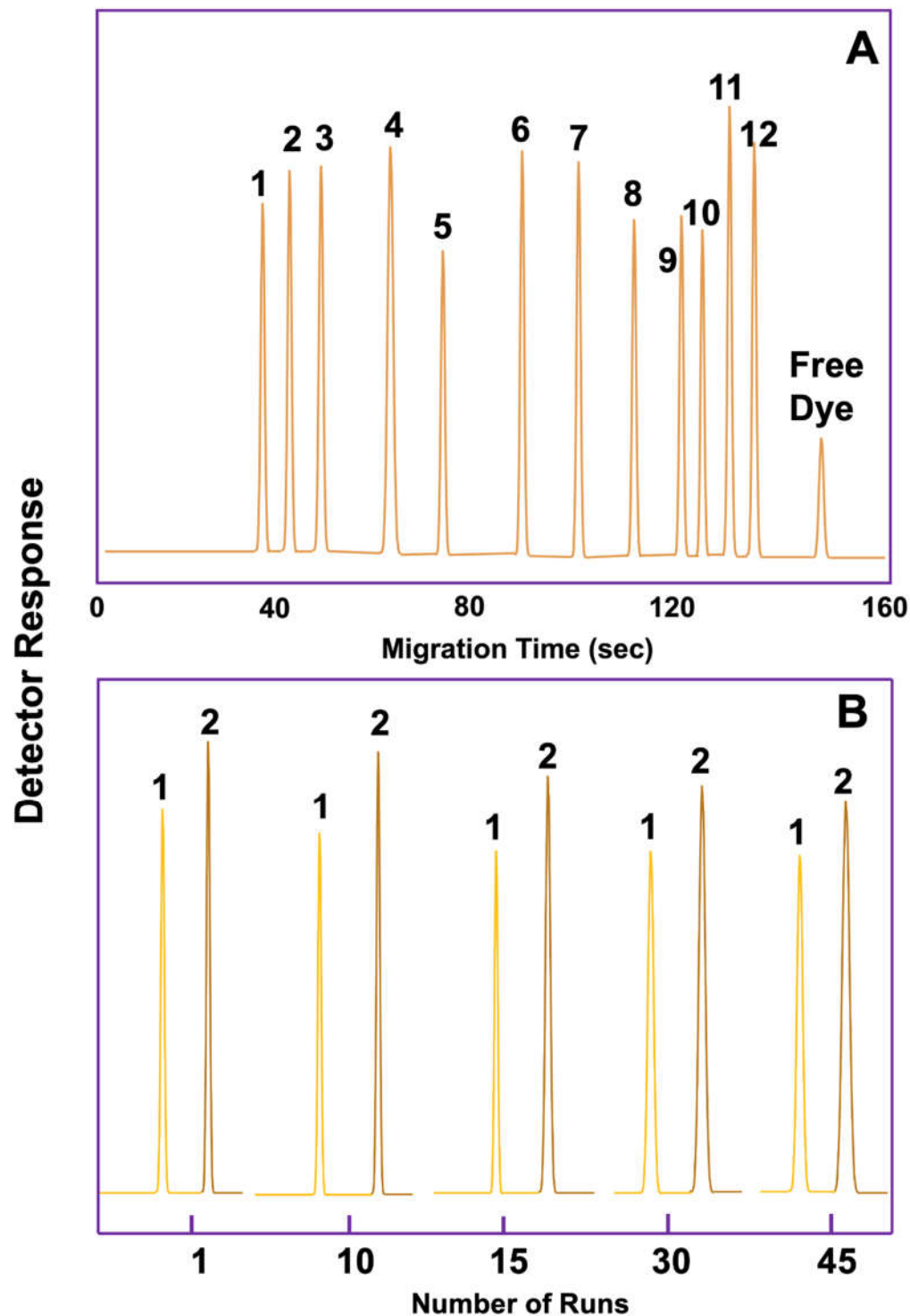
Figure 5. Diagrammatic representation of a multi-biomarker analysis based on affinity-capture-separation analytical technology performed with a microchip electrophoresis (**panel A**). The peaks in the electropherogram resolved in the following order **1**, TGF-β (transforming growth factor beta); **2**, IL-6 (interleukin-6); **3**, IL-1β (interleukin-1 beta); **4**, IFNγ (interferon gamma); **5**, MIP-1α (macrophage inflammatory protein 1 alpha); **6**, MCP-1 (macrophage chemoattractant protein 1); **7**, TNF-α (tumor necrosis factor-alpha); **8**, CGRP (calcitonin gene-related peptide); **9**, NY (neuropeptide Y); **10**, IL-8 (interleukin-8); **11**, VIP (vasoactive intestinal peptide); **12**, SP (substance P). A small amount of free dye was always present. All antibodies directed to the targeted biomarkers were co-immobilized to disposable glass fiber disks and inserted within the extraction port of the microchip. The captured analytes were labeled with a 635 nm light-emitting laser dye and electroeluted into the separation

channel as described in refer. [137]. On **panel B**, single stranded aptamers were used as affinity-capture ligands for the selective recognition of two molecular weight compounds (**1**, aflatoxin B1, and **2** ochratoxin A). These two analytes were co-immobilized via covalent bonds on the surface of the inlet end of the capillary. The separation, detection and quantification were performed by capillary coupled to a mass spectrometer. The figures were adapted and modified from ref. [137,138].

Although Figure 5 does not show an example of the separation and identification of a sputum specimen constituents, it illustrated the advantages of using at least two immobilized affinity-capture ligands in the same ACM device. This allowed the use of one of them to capture and separate a known substance as an internal standard to improve accuracy and can additionally serve to monitor the life cycle of an ACM device after multiple uses. A second immobilized affinity-capture ligand is applied as a bait to attract selectively a specific target biomarker. It is promising to think that the two-dimensional affinity-capture-separation techniques, carried out in microchip electrophoresis or capillary zone electrophoresis, can be used as a miniaturized point-of-care instrument. Such portable instrument will enable the quantification of two or more biomarkers, containing immobilized affinity-capture ligands immobilized to the pre-concentrators that can be used multiple times. Results can be obtained in short analysis times, with significant improvements in analytical sensitivity and specificity. It is foreseeable that such multi-dimensional miniaturized instrument can be cost effective and be ideal for point-of care use in countries with limited healthcare systems.

9. Discussion and Future Perspective

There is growing evidence regarding the utility and merits of two-dimensional affinity-capture separation analytical systems to address biomedical challenges [139–158]. For example, coupling microchip electrophoresis or conventional capillary instruments to specialized detectors such as radiometric, electrochemical, laser-induced fluorescence, and/or mass spectrometry offers enhanced analytical sensitivity capable of reaching limits of detection in the lower picogram or at the nanogram scale. Such features, enable biomarkers analysis found at low concentrations in biological fluids. The affinity capture agents can be a variable of substances or a combination of them; the most popular are antibodies, antibody fragments, nanobodies, lectins, aptamers, protein A/G, and multimodal peptides, including many others that have a high affinity binding to one or more moieties of another molecule. These agents can act as selective capture molecules or preanalytical concentrators and can effectively serve as a clean-up process in removing excess amounts of non-targeted analytes. Preselection of the affinity capture ligands and optimization of immobilization also guarantee that equal capture and release will occur for all analytes during a set window of time [137,138]. The immobilization of the affinity capture ligands can be performed onto glass or plastic beads, monolith structures, magnetic particles, or directly onto surface of the inlet side of the channel or capillary. The analyte concentrator-microreactor (ACM) device has this term because of its dual functions to concentrate and clean; it is also used as a microreactor that permits to perform biochemical reactions (e.g., enzymes immobilized to cleave proteins with the ACM device or area) [159,160].

Regarding applications, the analytical technology of affinity-capture-separation can be used beyond solving biomedical problems; for example, it can be extended for purposes to identify and characterize analytes in complex matrices related to food products, pharmaceutical products, environmental monitoring, space, and other research areas. These qualities of miniaturized analytical technologies offer unique benefits that cannot be matched by current traditional larger-scale equipment. Miniaturization of analytical instruments makes it possible to decrease demands in working space, reagents, and power [161,162].

In the last decade, there has been a marked improvement in the availability of laboratory and point-of-care testing for the diagnosis of microbial infections [163–166]. Unfortunately, balancing requirements for affordable, easy of use, sensitive POC testing is difficult. Raising the expectations for POC testing by requiring POCTs to match, or exceed, the accuracy and performance of centralized laboratory testing should be a contemporary goal [163]. POC testing is generally supported by two types of technologies: handheld single use devices, and small bench-top/portable analyzers. In

addition to manufacturing an ideal POC testing instrument, reagents must be considered. A key component for successful, high accuracy testing are the affinity capture ligands, and today there are many options beyond commercially available compounds such as antibodies, lectins, and aptamers. Recently, a new technique involving biopanning with phage-displayed peptides offers the ability to identify small peptides that can be used in a similar manner to antibodies for on-target capture of biomarkers [167]. When detecting the presence of microorganisms in samples, using capture ligands to selectively extract one protein of interest from a complex mixture, offers the advantage of revealing a single biomarker to indicate the presence of bacteria or virus. Biopanning for phage-display peptides allows for the development of affinity ligands from virtually any organism, permits ease in utilizing these peptides with affinity capture mass spectrometry, and holds great potential to diagnose emerging pathogens [167]. This in-vitro, antigen-driven, affinity selection, employing phage display biopanning, is now a commonly used technique to isolate monoclonal antibodies [168].

POC testing can deliver rapid diagnostic in environments where results can be used to direct clinical management during patient visits, and where centralized laboratories are limited [163]. The two-dimensional affinity-capture separation POC testing instrument that we discuss in this review can be designed not only to work in normal environments, but can also function in extreme environments, including harsh temperatures, pressure, and radiation as required in space and deep marine conditions. Evolving technology in healthcare continues to shape the way we provide care to patients. Advances in artificial intelligence, data generated in proteomics research, and the more mainstream use of telemedicine allows patients, families, and their caregivers to improve diagnostic quality, anywhere in the world [95,118,155,156,169]. Sputum tests offer hope in determining protein and nucleic acid biomarkers for the diagnosis of respiratory infectious diseases, pulmonary diseases, and lung cancer far in advance of clinical symptoms [33,170]. Sputum biomarkers have detected other diseases; for example, autoantibodies in subjects with rheumatoid arthritis [171]. Another publication reported that immunoglobulin A could be detected in sputum at an early stage of COVID-19 patients [172]. In addition, another study demonstrated the detection of antibodies against *Pseudomonas aeruginosa* in the sputum of cystic fibrosis patients, and that the sensitivity of the assay was greater in sputum than in serum [173]. In immunodeficient patients with COVID-19, regardless of treatment, persistent viral positivity was obtained in sputum samples where contemporaneous nasopharyngeal swab samples were negative [174]. Persistent viral positivity in sputum, by PCR or antigen test, present infection control challenges, especially in a group with frequent hospital attendances in clinical spaces with other vulnerable patients. Monitoring of sputum samples is therefore recommended in situations where patients can provide them [33,174].

In conclusion, we emphasize the importance of protein biomarkers as crucial molecules to discern the complexity of diseases. Proteomics continues to be at the forefront of scientific discovery, providing invaluable insights into the intricacy of living organisms at a molecular level. Proteins are not only the product of the transcription and translation of genetic information - the process of protein biosynthesis underlies a variety of processes at genome and transcriptome level, such as splicing, recombination, gene shuffling, and more. Also, a plethora of different co-and post-translational modifications can occur during the lifetime of a protein molecule. These processes lead to the formation of numerous protein species out of a single gene, for which now the term "proteoform" has been coined, encapsulating the array of molecular expressions now employed in place of previously used names such as "protein forms", "protein isoforms", "protein species", and "protein variants" [61–63,175–180]. A major advantage of a two-dimensional affinity-capture separation technique is that polyclonal antibodies are ideally suited for proteoforms retrieval. The high-affinity selection of target protein biomarkers coupled with the high-resolution characteristic of capillary electrophoresis and/or high-resolution mass spectrometry can provide information to monitor minor differences in molecular changes of structural related proteins [62,176,180]. Mass spectrometry is expected to be used for the discovery and characterization of new protein biomarkers, but should not be a routine method when biomarkers are known and validated with affinity-capture separation capillary electrophoresis. The utilization of one or two internal standards to control the performance of separation of the captured and released known targeted biomarkers, in microchip electrophoresis

or conventional capillary electrophoresis, should be sufficient to obtain an accurate diagnosis for those biomarkers known to be affected in a specific disease. We are confident that the two-dimensional, portable, miniaturized point-of-care testing instrument described in this article, for use with biological samples obtained by non-invasive methods such as sputum, saliva, urine, and sweat, will be mainstream and scaled when its advantages and definite savings are realized.

References

1. Barberis, I.; Bragazzi, N.L.; Galluzo, L.; Martini, M. The history of tuberculosis: from the first historical records to the isolation of Koch's bacillus. J. Prev. Med. Hyg. 2017, 58, E9-E12.
2. Schluger, N.W. The acid-fast bacilli smear: Hail and farewell. Am. J. Resp. Crit. Care Med. 2019, 199, 692.
3. Singhal, R.; Myneedu, V.P. Microscopy as a diagnostic tool in pulmonary tuberculosis. Int. J. Mycobacteriol. 2015, 4, 1-6.
4. Fireman, E. Induced sputum: opening a new window to the lung. Sarcoidosis Vasc. Diffuse Lung Dis. 2001, 18, 263-271.
5. Zhang, F.; Zhang, F.; Dong, Y.; Li, L.; Pang, Y. New insights into biomarkers for evaluating therapy efficacy in pulmonary tuberculosis: A narrative review. Infect. Dis. Ther. 2023, 12, 2665-2689.
6. Agustí, A.; Bafadhel, M.; Beasley, R.; Bel, E.H.; Faner, R.; Gibson. P.G.; Louis, R.; McDonald, V.M.; Sterk, P.J.; Thomas, M.; Vogelmier, C.; Pavord, I.D.; on behalf of all participants in the seminar. Precision medicine in airway diseases: moving to clinical practice. Eur. Respir. J. 2017, 50, 1701655.
7. de Souza, V.M.L.; Carvalho, L. Heterogeneity in lung cancer. Pathobiology 2018, 85, 96-107.
8. Bonnesen, B.; Jensen, J-U.S.; Mathioudakis, A.G.; Corlateanu, A.; Sivapalan, P. Promising treatment biomarkers in asthma. Front. Drug Saf. Regul. 2023, 3, 1291471.
9. Cao, C.; Li, W.; Hua, W.; Yan, F.; Zhang, F.; Huang, H; Ying, Y.; Li, Na.; Lan, F.; Wang, S.; Chen, X.; Li, J.; Liu, J.; Lai, T.; Bao, Z.; Cao, Y.; Zhao, Y.; Huang, G.; Huang, L.; Huang, Y.; Wu, P.; Peng, C.; Chen, Z.; Chung, K.F.; Zhong. N.; Ying, S.; Shen, H. Proteomic analysis of sputum reveals novel biomarkers for various presentations of asthma. J. Transl. Med. 2017, 15, 171.
10. D'Amato, M.; Iadarola, P.; Viglio, S. Proteomic analysis of human sputum for the diagnosis of lung disorders: where are we today? Int. J. Mol. Sci. 2022, 23, 5692.
11. Hull, R.C.; Huang, J.T.J.; Barton, A.K.; Keir, H.R.; Ellis, H.; Cookson, W.O.C.; Moffatt, M.F.; Loebinger, M.R.; Chalmers, J.D. Sputum proteomics in nontuberculous mycobacterial lung disease. Chest 2022, 161, 1180-1191.
12. Bishwal, S.C.; Das, M.K.; Badireddy, V.K.; Dabral, D.; Das, A.; Mahapatra, A.R.; Sahu, S.; Malakar, D.; Singh, I.I.; Mazumdar, H.; Patgiri, S.J.; Deka, T.; Kapfo, W.; Liegise, K.; Kupa, R-u.; Debnath, S.; Bjowmik, R.; Debnath, R.; Behera, R.K.; Pillai, M.G.; Deuri, P.; Nath, R.; Khalo, K.P.; Sing, W.A.; Pandit, B.; Das, A.; Bhattacharya, S.; Behera, D.; Saikia, L.; Khamo, V.; Nanda R.K. Sputum proteomics reveals a shift in Vitamin D-binding protein and antimicrobial protein axis in tuberculosis patients. Scientific Reports 2019, 9, 1036.
13. Kesimer, M.; Sheehan, J.K. Mass spectrometry analysis of mucin core proteins. Methods Mol. Biol. 2012, 842, 67-79.
14. Burg, D.; Schofield, J.P.R.; Brandsma, J.; Staykova, D.; Folisi, C.; Bansal, A.; Nicholas, B.; Xian, Y.; Rowe, A.; Corfield, J.; Wilson, S.; Ward, J.; Lutter, R.; Fleming, L.; Shaw, D.E.; Bakke, P.S.; Caruso, M.; Dahlen, S.-E.; Fowler, S.J.; Hashimoto, S.; Horváth, I.; Howarth, P.; Krug, N.; Montuschi, P.; Sanak, M.; Sandström, T.; Singer, F.; Sun, K.; Pandis, I.; Auffray, C.; Sousa, A.R.; Adcock, I.M.; Chung, K.F.; Sterk, P.J.; Djukanović, R.; Skipp, P.J.; The U-Biopred Study Group. Large-scale label-free quantitative mapping of the sputum proteome. J. Proteome Res. 2018, 17, 2072-2091.

15. Maher, R.E.; Barrett, E.; Beynon, R.J.; Harman, V.M.; Jones, A.M.; McNamara, P.S.; Smith, J.A.; Lord, R.W. The relationship between lung disease severity and the sputum proteome in cystic fibrosis. Respir. Med. 2022, 204, 107002.

16. Moermans, C.; Brion, C.; Bock, G.; Graff, S.; Gerday, S.; Nekoee, H.; Poulet, C.; Bricmont, N.; Henket, M.; Paulus, V.; Guissard, F.; Louis, R.; Schleich, F. Sputum Type 2 markers could predict remission in severe asthma treated with anti-IL-5. Chest 2023, 163, 1368-1379.

17. Nolasco, S.; Crimi, C.; Campisi, R. Personalized medicine in asthma: Current approach and future perspectives. J. Pers. Med. 2023, 13, 1459.

18. Qin, L.; Guo, T.; Yang, H.; Deng, P.; Gu, Q.; Liu, C.; Wu, M.; Lizaso, A.; Li, B.; Zhang, S.; Chen, Z.; Hu, C. The utility of sputum as an alternative liquid biopsy specimen for next-generation sequencing-based somatic variation profiling. Ann. Transl. Med. 2022, 10, 462.

19. Lone, S.N.; Nisar, S.; Masoodi, T.; Singh, M.; Rizwan, A.; Hashem, S.; El-Rifai, W.; Bedognetti, D.; Batra, S.K.; Haris, M.; Bhat, A.A.; Macha, M.A. Liquid biopsy: a step closer to transform diagnosis, prognosis and future of cancer treatments. Molecular Cancer 2022, 21, 79.

20. Wang, N.; Wang, Q.; Du, T.; Gabriel, A.N.A.; Wang, X.; Sun, L.; Li, X.; Xu, K.; Jiang, X.; Zhang, Y. The potential roles of exosomes in chronic obstructive pulmonary disease. Front. Med. (Lausanne) 2020, 7, 618506.

21. Hortin, G.L.; Carr, S.A., Anderson, N.L. Introduction: Advances in protein analysis for the clinical laboratory. Clin. Chem. 2010, 56, 149-151.

22. Harti, J.; Kurth, F.; Kappert, K.; Mülleder, M.; Hartman, G.; Raiser, M. Quantitative protein biomarker panel: a path to improved clinical practice through proteomics. EMBO Molecular Med. 2023, 15, 4.

23. Captur, G.; Moon, J.C.; Topriceanu, C.-C.; Joy, G.; Swading, L.; Hallqvist, J.; Doykov, I.; Patel, N.; Spiewak, J.; Balswin, T.; Hamblin, M.; Menacho, K.; Fontana, M.; Treibel, T.A.; Manisty, C.; O'Brien, B.; Gibbons, J.M.; Pade, C.; Brooks, T.; Altmann, D.M.; Boyton, R.J.; McKnight, A; Maini, M.K.; Noursadeghi, M.; Mills, K.; Heywood, W.E.; UK COVIDsortium Investigators. Plasma proteomics signature predicts who will get persistent symptoms following SARS-CoV-2 infection. eBiomedicine 2022, 85, 104293.

24. Wallace, L.E.; Liu, M.; van Kuppeveld, F.J.M.; de Vries, E.; de Haan, C.A.M. Respiratory mucus as a virus-host range determinant. Trends microbiol. 2021, 29, 983-992.

25. Farzan, S. Cough and sputum production. Clinical Methods: The History, Physical, and Laboratory Examinations, 1990, 3rd Edition, Chapter 38, Walker, H.K.; Hall, W.D.; Hurst, J.W. (Editors), Butterworths, Boston.

26. Charriots, J.; Volpato, M.; Petit, A.; Vachier, I.; Bourdin, A. Methods of sputum and mucus assessment for muco-obstructive lung diseases in 2022: Time to "unplug" from our daily routine! Cells 2022, 11, 812.

27. Dao, T.L.; Hoang, V.T.; Ly, T.D.A.; Lagier, J.C.; Baron, S.A.; Raoult, D.; Parola, C.; Courjon, J.; Marty, P.; Chaudet, H.; Gautret, P. Sputum proteomic analysis for distinguishing between pulmonary tuberculosis and non-tuberculosis using matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS): preliminary results. Clin. Microbiol. Infect. 2021, 27, 1694.e1-1694.e6.

28. Radicioni, G.; Ceppe, A.; Ford, A.A.; Alexis, N.E.; Barr, R.G.; Bleecker, E.R.; Christenson, S.A.; Cooper, C.B.; Han, M.K.; Hansel, N.N.; Hastie, A.T.; Hoffman, E.A.; Kanner, R.E.; Martinez, F.J.; Ozkan, E.; Paine III, R.; Woodruff, P.G.; O'Neal, W.K.; Boucher, R.C.; Kesimer, M. Airway mucin MUC5AC and MUC5B concentrations and the initiation and progression of chronic obstructive pulmonary disease: an analysis of the SPIROMICS cohort. Lancet Respir. Med 2021, 9, 1241-1254.

29. Wang, Y.; Zhang, M.; Yu, Y.; Han, T.; Zhou, J.; Bi, L. Sputum characteristics and airway clearance methods in patients with severe COVID-19. Medicine (Baltimore) 2020, 99, 46, e23257.

30. Panchabhai, T.S.; Methta, A.C. Historical perspectives of bronchoscopy. Connecting the dots. Ann. Am.Thorac. Soc. 2015, 12, 631-641.

31. Aravena, C.; Metha, A.C.; Almeida, F.A.; Lamb, C.; Maldonado, F.; Gildea, T.R. Innovation in rigid broncoscopy – past, present, and future. J. Thorac. Dis. 2023, 15, 2836-2847.

32. Elsevier health. Specimen collection: Sputum (pediatrics), January 2024; https://elsevier.health/en-US/preview/specimen-sputum-peds.

33. Guzman, N.A.; Guzman, D.E. A COVID-19 infection with positive sputum and negative nasopharyngeal/oropharyngeal rapid antigen-based testing: A case report and pilot study. Medi. Clin. Case Rep. J. 2023, 1, 77-84.

34. Dragonieri, S.; Bilov, A.; Capuano, A.; Scarlata, S.; Carpagnano, G.E. Methodological aspects of induced sputum. Adv. Respir. Med. 2023, 91, 397-406.

35. Paggiaro, P.L.; Chanez, P.; Holz, O.; Ind, P.W.; Djukanović, T.; Maestrelli, P.; Sterk, P.J. (Working group). Sputum induction. Eur. Respir. J. 2020, 20, 3s-8s.

36. Taube, C.; Holz, O.; Mücke, M.; Jörres, R.A.; Magnussen, H. Airway response to inhaled saline in patients with moderate to severe chronic obstructive pulmonary disease. Am. J. Respir. Crit. Care Med. 2001, 164, 1810-1815.

37. Bart, I.Y.; Mourits, M.; van Gent, R.; van Leuken, M.H.; Hilbink, M.; Warris, A.; Wever, P.C.; de Vries E. Sputum induction in children is feasible and useful in a bustling general hospital practice. Glob. Pediatr. Health 2016, 3, 2333794X16636504.

38. Rueda, Z.V.; Bermúdez, M.; Restrepo, A.; et al. Induced sputum as an adequate clinical specimen for the etiological diagnosis of community-acquired pneumonia (CAP) in children and adolescents. Inter. J. Infect. Dis. 2022, 116, 348-354.

39. Gibson, P.G.; Grootendorst, D.C.; Henry, R.; Pin, I.; Rytila, P.L.; Wark, P.; Wilson, N.; Gjukanović, R. Sputum induction in children. Eur. Respir. J. 2002, 20, 44s-46s.

40. Weiszhar, Z.; Horvath, I. Induced sputum ana lysis: step by step. Breathe 2013, 9, 301-306.

41. Goncalves, B.; Eze, U.A. Sputum induction and its diagnostic applications in inflammatory airway disorders: a review. Front. Allergy 2023, 4, 1282782.

42. Suri, R.; Marshall, L.J.; Wallis, C.; Metcalfe, C.; Shute, J.K.; Bush, A. Safety and use of sputum induction in children with cystic fibrosis. Pediatr. Pulmonol. 2003, 35, 309-313.

43. Kang, A.; Yeom, M.; Kim, H.; Yoon, S-W.; Jeong, D-G.; Moon, H-J.; Lyoo, K-S.; Na, W.; Song, D. Sputum processing method for lateral flow immunochromatography assays to detect coronaviruses. Immune Netw. 2021, 21, 1, e11.

44. Barber, C.; Lau, K.; J.A. Ward.; Daniels, T.; Watson, A.; Staples, K.J.; Wilkinson, T.M.A.; Howarth, P.H. Sputum processing by mechanical dissociation: A rapid alternative to traditional sputum assessment approaches. Clin. Respir. J. 2021, 15, 800-807.

45. Clemente, A.; Alba-Patiño, A.; Santopolo, G.; Rojo-Molinero, E.; Oliver, A.; Borges, M.; Aranda, M.; del Castillo, A.; de la Rica, R. Immunodetection of lung IgG and IgM antibodies against SARS-CoV-2 via enzymatic liquefaction of respiratory samples from COVID-19 patients. Anal. Chem. 2021, 93, 5259-5266.

46. Hiraiwa, M.; Kim, J-H.; Lee, H-B.; Inoue, S.; Becker, A.L.; Weigel, K.W.; Cangelosi, G.A.; Lee, K-H.; Chung, J-H. Amperometric immunosensor for rapid detection of Mycobacterium tuberculosis. J. Micromech. Microeng. 2015, 25, 055013.

47. Ferguson, T.M.; Weigel, K.M.; Becker, A.L.; Ontengco, D.; Narita, M.; Tolstorukov, I.; Doebler, R.; Cangelosi, G.A.; Niemz, A. Pilot study of a rapid and minimally instrumented sputum sample preparation for molecular diagnosis of tuberculosis. Sci. Rep. 2016, 6, 19541.

48. Fu, Y.R.; Guan, S.Z.; Zhang, S.Y.; Li, M. Proteomic analysis of sputum in patients with active pulmonary fibrosis. Clin. Microbiol. Infect. 2012, 18, 12, 1241-1247.

49. Johnson-Arbor, K.; Reid, N.; Smolinske, S. Human toxicity from COVID-19 rapid home test kits. Am. J. Emerg. Med. 2022, 57, 215-216.

50. Richardson, S.; Kohn, M.A.; Bollyky, J.; Parsonnet, J. Validity of at-home rapid antigen lateral flow assay and artificial intelligence read to detect SARS-CoV-2. Diagn. Microbiol. Infect. Dis. 2022, 104, 115763.

51. Filchakova, O.; Dossym, D.; Ilyas, A.; Kuanysheva, T.; Abdizhamil, A.; Bukasov, R. Review of COVID-19 testing and diagnostic methods. Talanta 2022, 244,123409.

52. Lee, H. At home COVID-19 antigen test kits: Where to buy and what you should know. 2023; https://www.nytimes.com/wirecutter/reviews/at-home-covid-test-kits/.

53. Omidfar, K.; Riahi, F.; Kashanian, S. Lateral flow assay: A summary of recent progress for improving assay performance. Biosensors (Basel) 2023, 13, 837.

54. Chiu, W-H.; Kong, W-Y.; Chueh, Y-H.; Wen, J-W.; Tsai, C-M.; Hong C.; Chen, P-Y.; Ko, C-H. Using an ultra-compact optical system to improve lateral flow immunoassay results quantitatively. Heliyon 2022, 8, e12116.

55. Panferov, V.G.; Ivanov, N.A.; Brinc, D.; Fabros, A.; Krylov, S.N. Electrophoretic assembly of antibody-antigen complexes facilitates 1000 times improvement in the limit of detection of serological paper-based assay. ACS Sensors 2023, 8, 1792-1798.

56. Panferov, V.G.; Zherdev, A.V.; Dzantiev, B.B. Post-assay chemical enhancement for highly sensitive lateral flow immunoassay: A critical review. Biosensors 2023, 13, 866.

57. Shen, M.; Chen, Y.; Zhu, Y.; Zhao, M.; Xu, Y. Enhancing the sensitivity of lateral flow immunoassay by centrifugation-assisted flow control. Anal. Chem. 2019, 91, 4814-4820.

58. Le, T.S.; He, S.; Takahashi, M.; Enomoto, Y.; Matsumura, Y.; Maenosono, S. Enhancing the sensitivity of lateral flow immunoassay by magnetic enrichment using multifunctional nanocomposite probes. Langmuir 2021, 37, 6566-6577.

59. Cho, W.C.S. Proteomics technologies and challenges. Geno. Prot. Bioinfo. 2007, 5, 77-85.

60. Al-Amrani, S.; Al-Jabri, Z.; Al-Zaabi, A.; Alshekaili, J.; Al-Khabori, M. Proteomics: Concepts and applications in human medicine. World J. Biol. Chem. 2021, 12, 57-69.

61. Smith, L.M.; Agar, J.N.; Chamot-Rooke, J.; Danis, P.O.; Ge, Y.; Loo, J.A.; Paša-Tolić, L.; Tsybin, Y.O.; Kelleher, N.L., The Consortium for Top-Down Proteomics. Sci. Adv. 2021, 7, eabk0734.

62. Guzman, N.A.; Guzman, D.E. Immunoaffinity capillary electrophoresis in the era of proteoforms, liquid biopsy and preventive medicine. A potential impact in the diagnosis and monitoring of disease progression. Biomolecules 2021, 11, 1443.

63. Seydel, C. Diving deeper into the proteome. Nat. Methods 2022, 19, 1036-1040.

64. Huang, L.; Shao, D.; Wang, Y.; Cui, X.; Li, Y.; Chen, Q.; Cui, J. Human body-fluid proteome: quantitative profiling and computational prediction. Brief. Bioinformat. 22, 315-333.

65. Nicholas, B.; Skipp, P.; Mould, R.; Rennard, S.; Davies, D.E.; O'Connor, C.D.; Djukanović, R. Shotgun proteomic analysis of human-induced sputum. Proteomics 2006, 6, 4390-4401.

66. Terracciano, R.; Preianò, M.; Palladino, G.P.; Carpagnano, G.E.; Barbaro, M.P.F.; Pelaia, G.; Savino, R.; Maselli, R. Peptidome profiling of induced sputum by mesoporous silica beads and MALDI-TOF MS for non-invasive biomarker discovery of chronic inflammatory lung diseases. Proteomics 2011, 11, 3402-3414.

67. Gallagher, T.; Riedel, S.; Kapcia III, J.; Caverly, L.J.; et al. Liquid chromatography mass spectrometry detection of antibiotic agents in sputum from persons with cystic fibrosis. Antimicrob. Agents Chemother. 2021, 65, e00927.

68. Mateos, J.; Estévez, O.; González-Fernández, A.; Anibarro., L.; Pallerés, A.; Reljic, R.; Gallardo, J.M.; Medina, I.; Carrera, M. High-resolution quantitative proteomics applied to the study of the specific protein signature in the sputum and saliva of active tuberculosis patients and their infected and uninfected contacts. J. Proteom. 2019, 195, 41-52.

69. Casado, B.; Iadarola, P.; Pannell, L.K.; Luisetti, M.; Corsico, A.; Ansaldo, E.; Ferrarotti, I.; Boschetto, P.; Baraniuk, J.N. Protein expression in sputum of smokers and chronic obstructive pulmonary disease patients: a pilot study by CapLC-ESI-Q-TOF. J. Proteome Res. 2007, 6, 46.

70. Pattison, S.H.; Gibson, D.S.; Johnston, E.; Peacock, S.; Rivera, K.; Tunney, M.M.; Pappin, D.J.; Elborn, J.S. Proteomic profile of cystic fibrosis sputum cells in adults chronically infected with Pseudomonas aeruginosa. Eur. Respir. J. 2017, 50, 1601569.

71. Asamoah, K.; Chung, K.F.; Kermani, N.Z.; Bodinier, B.; Dahlen, S.-E.; Djukanovic, R.; et al. Proteomic signatures of eosinophilic and neutrophilic asthma from serum and sputum. eBioMedicine 2024, 19, 1-12, 104936.

72. HaileMariam, M.; Yu, Y.; Singh, H.; Teklu, T.; Wondale, B.; Worku, A.; Zewude, A.; Mounaud, S.; Tsitrin, T.; Legesse, M.; Gobena, A.; Pieper, R. Protein and microbial biomarkers in sputum discern acute and latent tuberculosis in investigation of pastoral Ethiopian cohort. Front. Cell. Infect. Microbiol. 2021, 11, 595554.

73. Dong, T.; Santos, D.; Yang, Z.; Yang, S.; Kirkhus, N.E. Sputum and salivary protein biomarkers and point-of-care biosensors for the management of COPD. Analyst 2020, 145, 1583-1604.

74. Dennis, E.G.; Hornbrook, M.M.; Ishizaka, K. Serum proteins in sputum of patients with asthma. J. Allergy Clin. Immunol. 1964, 35, 464-471.

75. Houston, C.J.; Alkhatib, A.; Einarsson, G.G.; Tunney, M.M.; Taggart, C.C.; Downey, D.G. Diminished airway host innate response in people with cystic fibrosis who experience frequent pulmonary exacerbations. Eur. Respir. J. 2013, in press, https://doi.org/10.1183/13993003.01228-2023.

76. Ali, M.M.; Mukherjee, M.; Radford, K.; Patel, Z.; Capretta, A.; Nair, P.; Brennan, J.D. A rapid sputum-based lateral flow assay for airway eosinophilia using RNA-cleaving DNAzyme selected for eosinophil peroxidase. Angew. Chem. Int. Ed. 2023, 62, e202307451.

77. Gharib, S.A.; Nguyen, E.V.; Lai, Y.; Plampin, J.D.; Goodlett, D.R.; Hallstrand, T.S. Induced sputum proteome in healthy subjects and asthmatic patients. J. Allergy Clin. Immunol. 2011, 128, 1176-1184, e6.

78. Rezaeeyan, H.; Arabfard, M.; Rasouli, H.R.; Shahriary, A.; Gh, B.F.N.M. Evaluation of common protein biomarkers involved in the pathogenesis of respiratory diseases with proteomic methods: a systematic approach. Immun. Inflamm. Dis. 2023, 11, e1090.

79. Polachek, A.; Vree Egberts, W.; Fireman, E.; Druckman, I.; Stark, M.; Paran, D.; Kaufman, I.; Wigler, I.; Levartovsky, D.; Caspi, D.; Pruijn, G.; Elkayam, O. Sputum anticitrullinated protein antibodies in patients with long-standing rheumatoid arthritis. J. Clin. Rheumatol. 2018, 24, 122-126.

80. Devlin, R.B.; Alexis, N.E.; Carter, J.; Ghio, A.J.; Richards, J.H. Iron-binding and storage proteins in sputum. Inhal. Toxicol. 2004, 14, 387-400.

81. Bodaghi, A.; Fattahi, N.; Ramazani, A. Biomarkers: Promising and valuable tools towards diagnosis, prognosis and treatment of Covid-19 and other diseases. Heliyon 2023, 9, e13323.

82. McNerney, M.P.; Zhang, Y.; Silverman, A.D.; Jewett, M.C.; Styczynski, M.P. Point-of-care biomarker quantification enabled by sample-specific calibration. Sci. Adv. 2019, 5, eaax4473.

83. Smith, J.G.; Gerszten, R.E. Emerging affinity-based proteomic technologies for large-scale plasma profiling in cardiovascular disease. Circulation 2017, 135, 1651-1664.

84. Ling, X.; Li, G. Accelerating sample preparation for the analysis of complex samples. LC-GC 2022, 40, 374-376.

85. Ferguson, T.M.; Weigel, K.M.; Becker, A.L.; Ontengco, D.; Narita, M.; Tolstorukov, I.; Doebler, R.; Cangelosi, G.A.; Niemz, A. Pilot study of a rapid and minimally instrumented sputum sample preparation method for diagnosis of tuberculosis. Sci. Rep. 2016, 6, 19541.

86. Kim, S.-H.; Weiß, C.; Hoffman, U.; Borggrefe, M.; Akin, I.; Behnes, M. Advantages and limitations of current biomarker research: From experimental research to clinical application. Curr. Pharm. Biotechnol., 2017, 18, 445-455.

87. St. John, A.; Price, C.P. Existing technologies for point-of-care testing. Clin. Biochem. Rev. 2014, 35, 155-167.

88. Verrills, N.M. Clinical proteomics: Present and future prospects. Clin. Biochem. Rev. 2006, 27, 99-116.

89. Papatheocharidou, C.; Samanidou, V. Two-dimensional high-performance liquid chromatography as a powerful tool for bioanalysis: The paradigm of antibiotics. Molecules 2003, 28, 5056.

90. Kitteringham, N.R.; Jenkins, R.E.; Lane, C.S.; Elliott, V.L.; Park, B.K. Multiple reaction for quantitative biomarker analysis in proteomics and metabolomics. J. Chromatogr. B 2009, 877, 1229-1239.

91. Banerjee, S. Empowering clinical diagnostics with mass spectrometry. ACS Omega 2020, 5, 2041- 2048.

92. Di Nardo, F.; Chiarello, M.; Cavalera, S.; Baggiani, C.; Anfossi, L. Ten years of lateral flow immunoassay technique applications: Trends, challenges and future perspectives. Sensors 2021, 21, 5185.

93. 93. Sena-Torralba, A.; Álvarez-Diduk, R.; Parolo, C.; Mercoçi, A. Toward next generation lateral flow assays: Integration of nanomaterials. Chem. Rev. 2022, 122, 14881-14910

94. Deng, Y.; Jiang, H.; Li, X.; Lv, X. Recent advances in sensitivity enhancement for lateral flow assay. Microchim. Acta 2021, 188, 379.

95. Guzman, N.A.; Guzman, D.E.; Blanc, T. Advancements in portable instruments based on affinity- capture-migration and affinity-capture-separation for use in clinical testing and life applications. J. Chromatogr. A 2023, 1704, 464109.

96. Guzman, N.A. Determination of sputum constituents by capillary electrophoresis. In preparation.

97. Guzman, N.A.; Phillips, T.M. Immunoaffinity capillary electrophoresis: A new versatile tool for determining protein biomarkers in inflammatory processes. Electrophoresis 2011, 32, 1565-1578.

98. Esene, J.E.; Burningham, A.J.; Tahir, A.; Nordin, G.P.; Wooley, A.T. 3D printed microfluidic devices for integrated solid-phase extraction microchip electrophoresis of preterm birth biomarkers. Anal. Chim. Acta 2024, 1296, 342338.

99. Pont, L.; Benavente, F.; Barbosa, J.; Sanz-Nebot, V. On-line immunoaffinity solid-phase extraction capillary electrophoresis mass spectrometry using Fab' antibody fragments for the analysis of serum transthyretin. Talanta 2017, 170, 224-232.

100. Vergara-Barberán, M.; Pont, L.; Salim, H.; Giménez, E.; Benavente, F. On-line aptamer affinity solid-phase extraction capillary electrophoresis-mass spectrometry for the analysis of protein biomarkers in biological fluids and food: A tutorial. Adv. Sample Prep. 2023, 7, 100082.

101. Effenhauser, C.S.; Manz, A.; Widmer, H.M. Glass chips for high-speed capillary electrophoresis separations with submicrometer plate heights. Anal. Chem. 1993, 65, 2637-2642.

102. Garcia, C.D.; Henry, C.S. Direct detection of renal function markers using microchip CE with pulsed electrochemical detection. Analyst 2004, 129, 579-584.

103. Ávila, M.; Floris, A.; Staal, S.; Ríos, Á.; Eijkel, J.; van den Berg, A. Point of care creatinine measurement for diagnosis of renal disease using disposable microchip. Electrophoresis 2013, 34, 2956- 2961.

104. Eijkel, J. Chip-based capillary electrophoresis platforms: toward point-of-care applications. Bioanalysis 2015, 7, 1385-1387.

105. Pan, J.-Z.; Fang, P.; Fang, X.-X.; Hu, T.-T.; Fang, J.; Fang, Q. A low-cost palmtop high-speed capillary electrophoresis bioanalyzer with laser induced fluorescence detection. Sci. Rep. 2018, 8, 1791.

106. Nguyen, V.D; Nguyen, H.Q.; Bui, K.H.; Ko, Y.S.; Park, B.J.; Seo, T.S. A handheld-type total integrated capillary electrophoresis system for SARS-CoV-2 diagnostics: Power, fluorescence detection, and data analysis by smartphone. Biosen. Bioelectron. 2022, 195, 113632.

107. Harrison, D.J.; Fluri, K.; Seiler, K.; Fan, Z.; Effenhauser, C.S.; Manz, A. Micromachining a miniaturized capillary electrophoresis-based chemical analysis system on a chip. Science 1993, 261, 895- 897.

108. 108. Kubáň, P.; Kubáň, P. Novel developments in capillary electrophoresis miniaturization, sampling, detection and portability: An overview of the last decade. Trends Anal. Chem. 2023, 151, 116941.

109. 109. He, M.; Xue, Z.; Zhang, Y.; Huang, Z.; Fang, X.; Qu, F.; Ouyang, Z.; Xu, W. Development and characterizations of a miniature capillary electrophoresis mass spectrometry system. Anal. Chem. 2015, 87, 2236-2241.

110. 110. Annese, V.F.; Hu, C. Integrating microfluidics and electronics in point-of-care diagnostics: Current and future challenges. Micromachines 2022, 13, 1923.

111. 111. Behera, P.P.; Kumar, N.; Kumari, M.; Kumar, S.; Mondal, P.K.; Arun, R.K. Integrated microfluidic devices for point-of-care detection of bio-analytes and disease. Sens. Diagn. 2023, 2, 1437-1459.

112. 112. Choi, B.C.K.; Morrison, H.; Wong, T.; Wu, J.; Yan, Y-P. Bringing chronic disease and infectious disease epidemiology back together. J. Epidemiol. Community Health 2007, 61, 832.

113. 113. Peeling, R.W.; Mabey, D. Point-of-care tests for diagnosing infections in the developing world. Clin. Microbiol. Infect. 2010, 16, 1062-1069.

114. 114. Goldstein, S. Chronic, noncommunicable diseases (NCDs): A silent scourge threatening to overwhelm global health. Fogarty International Center at the NIH 2011, 10, 5; https://www.fic.nih.gov/News/GlobalHealthMatters/Sept-Oct-2011/Pages/chronic-disease.aspx

115.   115. Priyadarshani, W.V.D.; de Namor, A.F.D.; Silva, S.R.P. Rising of a global silent killer: critical analysis of chronic kidney disease if uncertain aetiology (CKDu) worldwide and mitigation steps. Environ. Geochem. Health 2023, 45, 2647-2662.

116.   116. Marrif, H.I.; Al-Sunousi, S.I. Pancreatic ß cell mass death. Front. Pharmacol. 2016.7, 83.

117.   117. Guzman, N.A.; Guzman, D.E. An emerging microscale immuno-analytical diagnostic tool to see the unseen. Holding promise for precision medicine and P4 medicine. J. Chromatogr. B 2016, 1021, 14-29.

118.   118. Guzman, N.A.; Guzman, D.E. From a central laboratory to the bedside: a point-of-care instrument for monitoring wellness and disease using two-dimensional immunoaffinity capillary electrophoresis technology. Arch. Biomed. Res. 2018, 1, 1.

119.   119. Guzman, N.A. Disease detection system and method. U.S. Patent Number 10,408,789. Granted on September 10, 2019.

120.   120. Guzman, N.A. Method and system for simultaneous determination of multiple measurable biomarkers during the development of a communicable disease. U.S. Patent Number 11,740,204. Granted on August 29, 2023.

121.   121. Thiha, A.; Ibrahim, F. A colorimetric enzyme-linked immunosorbent assay (ELISA) detection platform for a point-of-care dengue detection system on a lab-on-compact-disc. Sensors (Basel) 2015, 15, 11431-11441.

122.   122. Alhajj, M.; Zubair, M.; Farhana, A. Enzyme linked immunosorbent assay. StatPearls [Internet] 2023, https://www.ncbi.nlm.nih.gov/books/NBK555922.

123.   123. Bar-Haim, E.; Rotem. S.; Elia, U.; Bercovich-Kinori, A.; Israeli, M.; Chen-Gihon, I.; Israeli, O.; Erez, N.; Achdout, H.; Zauberman, A.; Aftalion, M.; Mamroud, E.; Chitlaru, T.; Cohen, O. Early diagnosis of pathogen infection by cell-based activation immunoassay. Cells 2019, 8, 952.

124.   124. Leng, S.X.; McElhaney, J.E.; Walston, J.D.; Xie, D.; Fedarko, N.S.; Kuchel, G.A. ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. J. Gerontol. 2008, 63A, 879-884.

125.   125. Ungaro, C.T.; Wolfe, A.S.; Brown, S.D. Comparison of serum cytokine measurement techniques between ELISA vs Multiplex. FASEB J. 2020, 34; doi:10.1096/fasebj.2020.34.s1.00254.

126.   126. Kim, C.H.; Tworoger, S.S.; Stampfer, M.; Dillon, S.T.; Gu, X.; Sawyer, S.J; Chan, A.T.; Libermann, T.A.; Eliassen, A.H. Stability and reproducibility of proteomic profiles measured with an aptamer-based platform. Sci. Rep. 2018, 8, 8382.

127.   127. Li, Y.; TAM, W.W.; Yu, Y.; Zhuo, Z.; Xue, Z.; Tsang, C.; Qiao, X.; Wang, X.; Li, Y.; Tu, Y., Gao, Y. The application of aptamer in biomarker discovery. Biomark. Res. 2023, 11, 70.

128.   128. Huang, J.; Chen, X.; Fu, X.; Li, Z.; Huang, Y.; Liang, C. Advances in aptamer-based biomarker discovery. Front. Cell. Dev. Biol. 2021, 9, 659760.

129.   129. Petrera, A.; von Toerne, C.; Behler, J.; Huth, C.; Thorand, B.; Hilgendorff, A.; Hauck, S.M. Multiplatform approach for plasma proteomics: Complementary of Olink proximity extension assay technology to mass spectrometry-based protein profiling. J. Proteome Res. 2021, 20, 751-762.

130.   130. Lind, L.; Arnlöv, J.; Lindahl, B.; Siegbahn, A.; Sundström, J.; Ingelsson, R. Use of a proximity extension assay proteomic chip to discover new biomarkers for human atherosclerosis. Atherosclerosis 2015, 242, 205-210.

131.   131. Carlyle, B.; Kitchen, R.R.; Mattingly, Z.; Celia, A.M.; Trombetta, B.A.; Das, S.; Hyman, B.T.; Kivisäkk, P.; Arnold, S.A. Technical performance evaluation of Olink proximity extension assay for blood- based biomarker discovery in longitudinal studies of Alzheimer's disease. Front. Neurol. 2022, 13, 889647.

132.   132. Wik, L.; Nordberg, N.; Broberg, J.; Björkesten, J.; Assarsson, E.; Henriksson, S.; Grundberg, I.; Petterson, E.; Westerberg, C.; Liljeroth, E.; Falck, A.; Lundberg, M. Proximity extension assay in combination with next-generation sequencing for high-throughput proteome-wide analysis. Mol. Cell. Proteomics 2021, 20, 100168.

133.   133. htttps://www.bioxpedia.com/olink-proteomics/.

134.   134. Al-Aly, Z.; Topol, E. Solving the puzzle of Long COVID. Science 2024, 383, 830-832.

135.   135. Palstrom, N.B.; Matthlesen, R.; Rasmussen, L.M.; Beck, H.C. Recent developments in clinical plasma proteomics – Applied to cardiovascular research. Biomedicines 2022, 10, 162.

136.   136. Raffield, L.M.; Pratte, K.A.; Jacobson, S.; Gillenwater, L.A.; Ampleford, E.; Barjaktaveric, I.; Basta, P.; Clish, C.B.; Comellas, A.P.; Cornell, E.; Curtis, J.L.; Doerschuk, C.; Durda, P.; Emson, C.; Freeman, C.M.; Guo, X.; Hastie, A.T.; Hawkins, G.A.; Herrera, J.; Johnson, W.C.; Labaki, W.W.; Liu, Y.; Master, B.; Miller, M.; Ortega, V.E.; Papanicolaou, G.; Peters, S.; Taylor, K.D.; Rich, S.S.; Rotter, J.I.; Auer, P.; Reiner, A.P.; Tracy, R.P.; Ngo, D.; Gerszten, R.E.; O'Neal, W.K.; Bowler, R.P; NHLBI Trans-Omics for Precision Medicine (TOPMed) Consortium. Comparison of proteomic assessment methods in multiple cohort studies. Proteomics 2020, 20, e1900278.

137.   137. Phillips, T.M. Analysis of inflammatory biomarkers from tissue biopsies by chip-based immunoaffinity CE. Electrophoresis 2007, 28, 3041-3048.

138. 138. Wang, A.; Liu, J.; Yang, J.; Yang, L. Aptamer affinity-based microextraction in-line coupled to capillary electrophoresis mass spectrometry using porous layer/nanoparticle-modified open tubular column. Anal. Chim. Acta 2023, 1239, 340750.

139. 139. Nge, P.N.; Pagaduan, J.V.; Yu, M.; Woolley, A.T. Microfluidic chips with reversed-phase monoliths for solid phase extraction and on-chip labeling. J. Chromatogr. A 2012, 1261, 129-135.

140. 140. Almughamsi, H.M.; Howell, M.K.; Parry, S.R.; Esene, J.E.; Nielsen, J.B.; Nordin, G.P.; Woolley, A.T. Immunoaffinity monoliths for multiplexed extraction of preterm birth biomarkers from human blood serum in 3D printed microfluidic devices. Analyst 2022, 147, 734-734.

141. 141. Yang, W.; Yu, X.; Sun, X.; Woolley, A.T. Microdevices integrating affinity columns and capillary electrophoresis for multi-biomarker analysis in human serum. Lab. Chip. 2010, 10, 2527-2533.

142. 142. Pero-Gascon, R.; Pont, L.; Sanz-Nebot, V.; Benavente, F. On-line immunoaffinity solid-phase extraction capillary electrophoresis-mass spectrometry for the analysis of serum transthyretin. Methods Mol. Biol. 2019, 1972, 57-76.

143. 143. Tu̇ma, P. Progress in on-line, at-line, and in-line coupling of sample pretreatment with capillary and microchip electrophoresis over the past 10 years: A review. Anal. Chim. Acta 2023, 1261, 341249.

144. 144. Kašička, V. Recent developments in capillary and microchip electroseparations of peptides (2021- mid-2023). Electrophoresis 2024, 45, 165-198.

145. 145. Guzman, N.A. Determination of immunoreactive gonadotropin-releasing hormone in serum and in urine by on-line immunoaffinity capillary coupled to mass spectrometry. J. Chromatogr. B Biomed. Sc. Appl. 2000, 749, 197-213.

146. 146. Kammeijer, G.S.M.; Nouta, J.; de la Rosette, J.J.M.C.H.; de Reijke, T.M.; Wuhrer, M. An in-depth glycosylation assay for urinary prostate-specific antigen. Anal. Chem. 2018, 90, 4414-4421.

147. 147. Yu, F.; Zhao, Q.; Zhang, D.; Yuan, Z.; Wang, H. Affinity interactions by capillary electrophoresis: Binding, separation, and detection. Anal. Chem. 2019, 91, 372-387.

148. 148. Puerta, A.; Martin-Alvarez, P.J.; Ongay, S.; Diez-Masa, J.C.; de Frutos, M. Immunoaffinity, capillary electrophoresis, and statistics for studying intact alpha 1-acid glycoprotein isoforms as atherothrombosis biomarker. Methods Mol. Biol. 2013, 919, 215-230.

149. 149. Okanda, F.M.; El Rassi, Z. Biospecific interaction (affinity) CEC and affinity nano-LC. Electrophoresis 2007, 28, 89-98.

150. 150. Guzman, N.A.; Trebilcock, M.A.; Advis, J.P. The use of a concentration step to collect urinary components separated by capillary electrophoresis and further characterization of collected analytes by mass spectrometry. J. Liq. Chromatogr. 1991, 14, 997-1015.

151. 151. Guzman, N.A. Biomedical applications of on-line preconcentration-capillary electrophoresis using an analyte concentrator: Investigation of design options. J. Liq. Chromatogr. 1995, 18, 3751-3768.

152. 152. Nevídalová, H.; Michalcová, L.; Glatz, Z. Capillary electrophoresis-based immunoassay and aptamer assay: A review. Electrophoresis 2020, 41, 414-433.

153. 153. Guzman, N.A. Method and system for simultaneous determination of multiple measurable biomarkers during the development of a communicable disease. U.S. Patent Number 11,740,204. Granted on August 29, 2023.

154. 154. Guzman, N.A.; Stubbs, R.J.; Phillips, T.M. Determination of inflammatory biomarkers by immunoaffinity capillary electrophoresis. Drug Discov. Today Technol. 2006, 3, 29-37.

155. 155. Guzman, N.A.; Guzman, D.E. A two-dimensional affinity capture and separation mini-platform for the isolation, enrichment, and quantification of biomarkers and its potential use for liquid biopsy. Biomedicines 2020, 8, 255.

156. 156. Guzman, N.A.; Guzman, D.E. Improving diagnostic testing and disease analysis with IACE. Res. Features 2022; doi:10.26904/RF-141-2652756006.

157. 157. Shimura, K.; Nagai, T. Capillary isoelectric focusing after enrichment with immunochromatography in a single capillary. Sci. Rep. 2016, 6, 39221.

158. 158. Li, Y.; Miao, S.; Tan, J.; Zhang, Q.; Chen, D.D.Y. Capillary Electrophoresis: A three-year literature review. Anal. Chem. 2024, in press; https://doi.org/10.1021/acs.analchem.4c00857.

159. 159. Guzman, N.A. Consecutive protein digestion and peptide derivatization employing an on-line analyte concentrator to map proteins using capillary electrophoresis. Chapter 4, pages 101-121. In: Capillary Electrophoresis in Analytical Biotechnology (Ed. P.G. Righetti), 1996, CRC Press, Boca Raton, New York.

160. 160. Nashabeth, W.; El Rassi, Z. Enzymophoresis of nucleic acids by tandem capillary enzyme reactor-capillary zone electrophoresis. J. Chromatogr. 1992, 596, 251-264.

161. 161. Balaram, V. Current advances in the miniaturization of analytical instruments – Applications in cosmochemistry, geochemistry, exploration, and environmental sciences. Spectroscopy 2016, 31, 40-44.

162. 162. Thomas, S.; Ahmadi, M.; Nguyen, T.A; Afkhami, A.; Madrakian, T (Editors). Micro- and Nano Technology Enabled Applications for Portable Miniaturized Analytical Systems - 2022, Chapters 1-17, pages 1-424, Elsevier.

163. 163. Hansen, G.T. Point-of-care testing in microbiology: A mechanism for improving patient outcomes. Clin. Chem. 2020, 66, 124-137.
164. 164. Drain, P.K.; Heichman, K.A.; Wilson, D. A new point-of-care test to diagnose tuberculosis. Lancet Infect. Dis. 2019, 19, 794-796.
165. 165. Drain, P.K. Rapid diagnostic testing for SARS-CoV-2. N. Engl. J. Med. 2022, 386, 264-272.
166. 166. Schito, M.; Peter, T.F.; Cavanaugh, S.; Piatek, A.S.; Young, G.J.; Alexander, H.; et al. Opportunities and challenges for cost-efficient implementation of new point-of-care diagnostics for HIV and tuberculosis. J. Infect. Dis. 2022, 205 (Suppl. 2), S169-S180.
167. 167. Johnson, E.M.; Ellis Jr., W.R.; Powers, L.S., Wysocki, V.H. Affinity capture mass spectrometry of biomarker proteins using peptide ligands from biopanning. Anal. Chem. 2009, 81, 5999-6005.
168. 168. Panagides, N.; Zacchi, L.F.; De Souza, M.J.; Morales, R.A.V.; Karnowsi, A.; Liddament, M.T.; Owczarek, C.M.; Mahler, S.M.; Panoulis, C.; Jones, M.L.; Fercher, C. Evaluation of phage display biopanning for the selection of anti-cell surface receptor antibodies. Int. J. Mol. Sci. 2022, 23, 8470.
169. 169. Castro, G.; Schrandt, S. Improving diagnosis through technology. HealthManagent.org-The Journal 2020, 20, 472-473.
170. 170. Tockman, M.S. Advances in sputum analysis for screening and early detection of lung cancer. Cancer Control 2000, 7, 19-24.
171. 171. Willis, V.C.; Demourelle, M.K.; Derber, L.A.; Chartier-Logan, C.J.; Parish, M.C.; Pedraza, I.F.; Weisman, M.H.; Norris, J.M.; Holers, V.M.; Deane, K.D. Sputum autoantibodies in patients with established rheumatoid arthritis and subjects at risk of future clinically apparent disease. Arthritis Rheum. 2013, 65, 2545-2554.
172. 172. Ren, C.; Gao, Y.; Zhang, C.; Zhou, C.; Hong, Y.; Qu, M.; Zhao, Z.; Du, Y.; Yang, L.; Liu, B.; Wang, S.; Han, M.; Shen, Y.; Liu, Y. Respiratory mucosal immunity: Kinetics of secretory immunoglobulin A in sputum and throat swabs from COVID-19 patients and vaccine recipients. Front. Microbiol. 2022, 13, 782421.
173. 173. Kelemen, K.; Cslszér, E.; Barta, I.; Antus, B. Detection of antibodies against Pseudomonas in the sputum of cystic fibrosis patients: A pilot study. Eur. Respirat. J. 2012, 40, P1449.
174. 174. Chan, M.; Linn, M.M.N.; O'Hagan, T.; Guerra-Assunção, J.A.; Lackenby, A.; Workman, S.; Dacre, A.; Burns, S.O.; Breuer, J.; Hart, J.; Tadros, S.; Lowe, D.M. Persistent SARS-CoV-2 PCR positivity despite anti-viral treatment in immunodeficient patients. J. Clin. Immunol. 2023, 43, 1083-1092.
175. 175. Tholey, A.; Schlüter, H. Top-down proteomics and proteoforms – special issue (Editorial). Proteomics 2024, 24, 2200375.
176. 176. Nedelkov, D. Human proteoforms as new targets for clinical mass spectrometry protein tests. Exp. Rev. Proteom. 2017, 14, 691-699.
177. 177. Forgrave, L.M.; Wang, M.; Yang, D.; DeMarco, M.L. Proteoforms and their expanding role in laboratory medicine. Pract. Lab. Med. 2022, 28, e00260.
178. 178. Su, J.; Yang, L.; Sun, Z.; Zhan, X. Personalized drug therapy: innovative concept guided with proteoformics. Mol. Cell. Proteomics. 2024, 12, 100737.
179. 179. Cramer, D.A.T.; Yin, V.; Caval, T.; Franc, V.; Yu, D.; Wu, G.; Lloyd, G.; Langendorf, C.; Chisstock, J.C.; Law, R.H.P.; Heck, A.J.R. Proteoform-resolved profiling of plasminogen activation reveals novel abundant phosphorylation site and primary N-terminal cleavage site. Mol. Cell. Proteomics 2024, 23, 100696.
180. 180. Tamara, S.; den Boer, M.A.; Heck, A.J.R. High-resolution mass spectrometry. Chem. Rev. 2022, 122, 7269-7326.