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Circulating biomarkers from tumour bulk to tumour machinery: promises and pitfalls

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Abstract

In this paper, we provide a working classification for circulating biomarkers according to their potential clinical application. We broadly divided biomarkers into four groups: (i) biomarkers of cancer risk, (ii) biomarkers of tumour–host interactions, (iii) biomarker of tumour burden, and (iv) function-related biomarkers. We hope this classification will provide a framework to which the results of future studies can be added. We also discuss the promises and pitfalls in the optional use of biomarkers in oncology.

Keywords: Biomarkers; Classification; Cancer; Pitfalls

1. Biomarkers: where are we?

Molecules associated with the presence of cancer have been extensively investigated and used in clinical practice for more than 30 years and have been defined as 'tumour markers'. This definition is inaccurate since almost all of the so-called tumour markers are also expressed in normal tissues, although produced and/or released in smaller amounts than from tumours. Therefore, from a semantic point of view, the more general term 'marker' or 'biomarker' should be used, avoiding the customary association with the word 'tumour'. In general, the amount of biomarkers released into the circulation is roughly proportional to the tumour burden. Therefore, biomarkers have been used mainly as indicators of tumour extension, finding their most prominent application in the monitoring of cancer patients [1].

In the last decade, basic research has highlighted numerous pathways of cancer cell regulation, identifying many molecules potentially implicated in well-known biological mechanisms. This remarkable progress is leading to the discovery of an increasing number of biomarkers measurable in cancer tissue and biological fluids [2].

These new biomarkers present a striking dissimilarity from the classical 'tumour markers'. The latter were discovered through their prevalent expression in cancer and are therefore mere indicators of tumour extension. The former are being discovered via their relation to the known biological mechanisms of cancer cells, which are potentially related to the clinical behaviour of the malignancy (i.e., aggressiveness, expression of metabolic pathways); interestingly, these pathways may be targets for diverse anticancer agents. These new biomarkers provide the ground for an array of different applications [3] and highlight the need for a new working classification that will take into account their potential clinical use.

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2. Working classification of circulating biomarkers according to their potential clinical application

Every classification is in some way arbitrary, reflecting the judgement of its designer, and has only provisional value, since it is expected to be modified and even outstripped by the progress of knowledge. However, the classification proposed here provides a framework upon which the results of basic research on biomarkers may be matched with their potential clinical applications. Biomarkers may be broadly subdivided into four groups: (i) biomarkers of cancer risk, (ii) biomarkers of tumour–host interactions, (iii) biomarkers of tumour burden, and (iv) function-related biomarkers (see Table 1).

2.1. Biomarkers of cancer risk

An increased risk of developing a malignancy may be related to both genetic and environmental factors. Genetic factors have been extensively evaluated, leading to the identification of several genetic markers of familial cancer [4]. Genetic testing for inherited germline mutations, also called cancer susceptibility tests, include, for example, the detection of mutations in the *BRCA1* and *BRCA2* genes associated with familial breast and ovarian cancer [5], or in *MLH1* and *MSH* genes associated with hereditary non-polyposis colon cancer [6]. These markers have a very high positive predictive value, but they concern only a small percentage of all malignancies. However, improvements in the available tests [7] and recent evidence of risk reduction obtained by follow-up interventions [8,9] are expected to accelerate the dissemination of these tests in clinical practice.

Environmental factors related to the development of sporadic cancers have been studied using classical epidemiological approaches. In addition, the biological background of the action of recognised (i.e., smoking habits) or suspected (i.e., diet) factors has been studied, leading to the identification of biomarkers potentially related to increased risk of developing a malignancy. Several molecules have been looked at from this point of view, including steroid hormones [10] and their metabolic products, insulin-like growth factors and their binding proteins [11,12], as well as polymorphisms of diverse genes [13,14]. Research on these biomarkers offers an important perspective for the identification of groups of people at higher risk for developing a cancer who might eventually be submitted to more stringent screening interventions and/or chemoprevention programmes, thus greatly broadening the possible clinical applications of biomarkers.

2.2. Biomarkers of tumour–host interactions

These include those biomarkers related to either damage in diverse organs or the immune response of the host to molecules overexpressed or overexposed by the tumour. The former group includes several common items in clinical chemistry or haematology, such as tissue-specific enzymes, acute-phase proteins, markers of inflammation, haemoglobin. Alterations in these items may indicate extensive invasion of the patient's organs and can be helpful in monitoring those with advanced disease. These biomarkers, which are measurable with reproducible and cheap methods, have been successfully used in association with more specific markers for monitoring the response to chemotherapy of advanced breast [15] and prostate cancers [16]; these investigations have shown that integrated biomarker information on host–tumour status is more accurate in predicting the response to therapy than the information obtained from markers of tumour bulk only [17].

Biomarkers related to the immune response of the host are interesting in that they could theoretically represent a naturally occurring system of boosting molecules expressed by the malignancy [18]. Antibodies against *MUC1* gene products [19], p53 mutated protein [20] and overexpressed erbB2/neu protein [21] have been investigated, but the results so far are mixed.

2.3. Biomarkers of tumour burden

These include the classical 'tumour markers', which have been extensively investigated, leading to a sound consensus on their possible clinical impact and to reasonable agreement about the cost-effectiveness of their routine use [22]. Most of them were discovered through their distinct association with malignant tissue. However, progress in basic research has provided information on the putative functions of some of them. Prostate-specific antigen (PSA) is a clear example. In seminal plasma, PSA has been identified as the enzyme responsible for the proteolysis of semenogelin, resulting in the liquefaction of the seminal gel and promoting the release of progressively motile spermatozoa [23]. In addition, PSA seems to elicit anti-angiogeneic properties. In a mouse model of metastatic cancer, daily PSA treatment (i) inhibited endothelial cell proliferation, migration and invasion, (ii) inhibited endothelial cell response to epidermal growth factor 2 (EGF-2) and vascular endothelial growth factor (VEGF), and (iii) resulted in a 40% reduction in the mean number of lung tumour nodules compared with phosphate-buffered saline [24]. Likewise, carcinoembryonic antigen (CEA) and mucin-like biomarkers seem to modulate the immune response against the tumour [25] or to regulate the adhesive properties of tumour cells [26]. These findings, if confirmed, may throw new light on areas of application for some classical, tumour burden-related biomarkers. However, the clinical usefulness of these markers is at present restricted to their coarse association with tumour spread. They are indeed effective in distinguishing advanced

Table 1
Working classification of circulating biomarkers according to their potential clinical application

Biomarker groups	Subgroup	Some examples
Biomarkers of cancer risk	Biomarkers of genetic risk	*BRCA1* and *BRCA2*, *MSH*, *MLH1*
	Biomarkers of environmental risk	Steroid hormones, insulin-like growth factors and their binding proteins, gene polymorphisms (i.e. lactate dehydrogenase gene)
Biomarkers of tumour–host interactions	Biomarkers of organ damage	Tissue-specific enzymes, acute-phase proteins, inflammation markers, haemoglobin
	Biomarkers of immune response	Auto-antibodies against: MUC1 protein, p53 mutated protein, overexpressed erbB2/neu protein
Biomarkers of tumour burden		Carcinoembryonic antigen, prostate-specific antigen, mucin markers (CA15.3, CA19.9, CA125), alpha-fetoprotein, human chorionic gonadotropin, cell-free DNA
Function-related biomarkers	Biomarkers of oncogene or oncosuppressor gene deregulation	p53, soluble HER2neu, soluble epidermal growth factor receptor, *APC*, *RAR*, p73, FHIT, RASSF1A, LKB1, VHL, *BRCA1* promoter hypermethylation
	Biomarkers of cell-cycle regulation	Cyclin D1 mRNA, p14, p15, p16 promoter hypermethylation
	Biomarkers related to DNA repair	MGMT and hMLH1 promoter hypermethylation
	Biomarkers related to the apoptotic (programmed cell death) pathway	Survivin, M30 antigen
	Biomarkers of extracellular matrix modification	TIMP2, MMP, uPA, DAPK1, E-cadherin, TIMP3 promoter hypermethylation
	Biomarkers of neoangiogenesis	Vascular endothelial growth factor, fibroblast growth factor
	Biomarkers related to detoxification	GSTP1 promoter hypermethylation

from localised disease, but they are not helpful in distinguishing different locoregional stages. Their circulating concentration is broadly associated with the quantity of tumour cells, but it is also affected by diverse tumour-unrelated variables, including body mass, metabolism and secretion rates, the possible occurrence of benign diseases, and the random presence in the bloodstream of molecules interfering with the assay. Recently, it has been shown that cell-free DNA can be shed into the bloodstream not only as a consequence of tumour cell death, but also as a result of active release [27,28]. Accordingly, non-apoptotic or 'long' DNA is also detectable in stools from individuals with colorectal diseases [29], and it can be found in larger amounts in patients with cancers of different origin than in healthy persons. These findings have prompted the development of assays to quantitate circulating DNA as a putative biomarker both for an early cancer diagnosis in high-risk individuals and for prognostic assessment [30].

2.4. Function-related biomarkers

Understanding of the many biochemical pathways of tumour cell regulation has led to the discovery of many biomarkers related to specific functions. Unlike the biomarkers in the aforementioned categories, the discovery of these substances provides insight into specific cellular mechanisms, including oncogene or oncosuppressor gene deregulation, neoangiogenesis, cell-cycle regulation, adhesion, invasion or extracellular matrix modification [31]. The interest in this category of biomarkers has shifted rapidly from tumour tissue to other biological fluids, because with these it is possible to obtain information on the genotype or phenotype of the tumour simply with a blood test. Measurements of chromosomal aberrations, point mutations and loss of heterozygosity can be obtained from saliva, sputum and urine with novel assays [32]. *p53* and *ras* mutations have been identified in the urine and stool of cancer patients [33,34], and the detection of the anti-apoptotic protein, survivin, in urine – if interesting preliminary results [35] are validated – appears to offer a simple, non-invasive diagnostic test for identifying patients with new or recurrent bladder cancers. Moreover, epigenetic changes, such as DNA methylation, which is predominant in the promoter region, play an important part in many processes, including DNA repair, genome stability and the regulation of chromatin structure. Changes in methylation have been shown for several genes involved in cell-cycle control, apoptosis, angiogenesis and detoxification processes [36–42]: *APC*, *RAR*, *p73*, *FHIT*, *RASSF1A*, *LKB1*, *VHL*, *BRCA1*, *p14*, *p15*, *p16*, *MGMT*, *hMLH1*, *DAPK1*, *E-cadherin*, *TIMP3* and *GSTP1* are detectable in serum/plasma specimens by methylation-specific polymerase chain reaction (MSP). Some of these might represent potential tools for early

detection and disease monitoring [43], in association with markers of tumour burden or other functional alterations. The clinical applications of these biomarkers have been rapidly expanding over a widening range of possibilities, including prognosis and the individualisation of therapies, thanks to their prediction of responsiveness to molecular-targeted anticancer agents. These markers may also express a 'functional' interplay between the tumour and the patient's internal environment. Recently, Tagliabue and colleagues [44] have shown that wound fluid and post-surgical serum contain growth factors that induce proliferation in Her2/neu-overexpressing breast carcinomas, thus suggesting a biological basis for the known correlation between surgical removal of the tumour and the growth of metastases.

3. The role of biomarkers in the assessment of clinical outcome

Biomarkers from all the above-mentioned categories may provide valuable information on clinical outcomes of interest, such as (i) prognosis, (ii) prediction of the response to certain therapies and (iii) therapy monitoring.

Tumour-bulk biomarkers and biomarkers related to tumour–host interactions have been traditionally used in therapy monitoring, while biomarkers related to definite cell functions have been used as prognostic or predictive factors; oestrogen receptors and cell kinetic factors have been used for over 30 years and are classical examples. New, function-related biomarkers are expected to play a key role in this scenario. These biomarkers have mainly been determined in tumour tissue. However, they have recently been shown to have a predictive role when measured in the blood; for example, an increased serum concentration of shed Her2/neu is predictive of reduced responsiveness to endocrine treatments in patients with advanced breast cancer [45]. In addition, given that serum concentrations of shed Her2/neu appear to be correlated with Her2/neu tissue expression, its circulating concentration is now under investigation as a putative serological surrogate biomarker of tumour Her2/neu status for assigning patients to treatment with the monoclonal antibody, trastuzumab [46,47].

Standard criteria for response evaluation in solid tumours are based on the measurable variation of tumour volume [48]. This well-established principle has limitations in three particular circumstances: (i) when disease is non-measurable or non-evaluable, (ii) when using molecular-targeted anticancer agents, which tend to stabilise the malignancy rather than eradicating it, and (iii) in chemoprevention trials, when the assessment of the true endpoint requires long-term studies with a large number of patients. In these conditions, biomarkers could be extremely helpful in clinical decision-making. Traditional tumour bulk-related biomarkers correlate well with the failure of treatment, but are less strictly associated with its success [49], thus impairing their possible role as surrogate markers. Function-related biomarkers are a promising tool, particularly in monitoring the effectiveness of molecular-targeted agents, for both cancer treatment and chemoprevention [50]. Knowledge of the biological mechanisms related to the molecular target of a given drug may also confirm the research on biomarkers showing the development of alternative pathways that may circumvent the effectiveness of the agent. These biomarkers, which may be defined '*escape biomarkers*', may be helpful in designing a therapeutic strategy tailored to the individual modulation of the target biochemical machinery. For example, drug resistance observed with anticancer agents that inhibit enzymes involved in critical biosynthetic pathways is frequently related to gene-amplification mechanisms. Several examples have been already reported. Thymidylate synthase (TS) concentrations are predictive of the response to TS inhibitors [51]; amplification of the M2 subunit of dihydrofolate reductase is associated with resistance to hydroxyurea [52]; methotrexate resistance is correlated with dihydrofolate reductase gene amplification [53]; resistance to ABL tyrosine kinase inhibitor, STI571, has been shown to be related to increased expression of the target protein due to oncogene amplification in haematopoietic cell lines [54]. In addition, cells may develop resistance by accumulating compensating mutations of other genes, inducing either gain or loss of cell functions. From a biochemical perspective, this complex and multifactorial gene deregulation has a common end-result of increasing the expression of proteins that may be identified as biomarkers of developed resistance (escape biomarkers) and eventually used as new molecular targets for different anticancer agents. In fact, diverse biological mechanisms of cell regulation are strictly interconnected. For example, transfection of human osteosarcoma cells with the Ras oncogene that were then implanted in mice induced the growth of large neovascularised tumours, due to both an increased expression of VEGF and a decrease of thrombospondin [55]. Therefore, molecular-targeted anticancer agents directed against oncogene products are also expected to inhibit angiogenesis. The administration of the monoclonal antibody trastuzumab, which targets HER2/neu tyrosine kinase signalling, also inhibits the production of certain angiogenic factors (transforming growth factor-β (TGFβ), angiopoietin 1, plasminogen-activator inhibitor I and VEGF) by cancer cells [56]. It may be that the malignancy escapes the suppression of angiogenesis by switching on a different angiogenic loop and increasing the expression of diverse angiogenic factors such as β-fibroblast growth factor (β-FGF) or interleukin 8 (IL-8) [57]. The clinical assessment of these patients might lead to the conclusion that

they have become resistant to trastuzumab and would suggest the discontinuation of their treatment. In contrast, if such patients were to be monitored for appropriate escape biomarkers, it would be possible to highlight the specific cause of the apparent clinical failure and to design a rescue therapeutic strategy by adding a second molecular-targeted agent specifically directed towards the new angiogenic target. In conclusion, awareness of the complex biochemical background to the mechanisms of action of molecularly targeted drugs may allow the design of a comprehensive strategy of biochemical monitoring.

4. Pitfalls in the optimal use of biomarkers in oncology

The growing availability of biomarkers that are effective in providing information on an expanding array of biochemical pathways may be of value in clinical oncology. For the proper implementation of a biomarker in clinical practice, evidence-based consensus on its effective clinical usefulness is needed. However, clinical studies on biomarkers frequently report conflicting results. Several factors may be responsible for the discrepancies observed among different clinical studies and must be considered in order to design appropriate clinical trials using new biomarkers [58,59].

(1) Several methods are often available for the determination of the same marker, such as gene expression, mRNA transcription, protein concentration or biological activity. Studies on HER2/neu determination, using either immunohistochemistry (IHC) or fluorescent *in situ* hybridisation (FISH), provide a good example of this problem. Discrepancies between p53 determination by immunohistochemistry and molecular biological techniques is another classical example [60].
(2) For each type of assay, the analytical procedure may differ in several respects, such as the reagents used, assay architecture and signal detection. Press and colleagues, reviewing the results obtained using 28 different antibodies for HER2/neu IHC, showed that 18 of them detected HER2/neu expression when the oncogene was amplified 5-fold or more, while only 12 of these 18 still provided a positive staining when the antigen was amplified only 2- to 5-fold [61].
(3) Biomarkers frequently present a complex molecular structure that may change its arrangement according to its functional status. Oestrogen receptor (ER) concentrations obtained with a commercially available enzyme immunoassay were shown to differ in the same tissue sample when cytosol was prepared using buffers of different ionic strengths; this was due to the different reactivity of one of the monoclonal antibodies in the kit for the different functional states that ER can form in the various buffers [62].
(4) Any given biological function should be assessed using a panel of biomarkers. Rarely, if ever, does one biomarker provide exhaustive information on a given biochemical mechanism. However, for practical and cost reasons, we tend to oversimplify our assays and use the smallest possible number of biomarkers. The tissue ER concentration has been used for more than 30 years to select patients with breast cancer for hormone therapy. However, we now know that oestrogen regulation of the cancer cell is a complex process involving more than 400 different genes [63]. Nonetheless, we still use ER as the only criterion for decisions on endocrine therapy. The key point is therefore to establish the optimal number of biomarkers capable of maintaining a satisfactory level of information about the target biological mechanism. Needless to say, the complexity of the statistical evaluation involved in translating the markers into clinical practice sharply increases with the number of parameters considered.
(5) For practical reasons, biomarker values are usually classified with reference to a positive/negative cut-off point. This conventional oversimplification probably does not reflect the complexity of biomarker biology, since many biological phenomena are expected to occur in a continuous rather than in a discrete fashion. Several studies show continuous (either monotonous or non-monotonous) correlations between diverse biomarkers and clinical outcome [64–68]. In addition, biomarkers may interact with each other, thus modifying the information that they provide when evaluated individually [69]. The use of dichotomous decision criteria may reduce the value of the biomarker when continuous monotonous correlations occur and cause even more meaningful biases in the case of non-monotonous correlations, since cases with different associations with prognosis are arbitrarily combined. The use of positive/negative cut-off points presents several pitfalls [70]. Dichotomous criteria may eventually be used only after the biomarker has been analysed on its original measurement scale, in order to explore its correlation with clinical outcome and maximise knowledge of the underlying biological complexities [71]. Surprisingly, in spite of published evidence and recommendations, most published studies have categorised biomarkers with reference to cut-off points [72].
(6) The standardisation of the pre-analytical phase is rarely considered, although it may be a major source of variability when comparing the results

of different studies. Sample collection, handling, storage and preparation must be standardised in order to preserve the integrity of antigens. Immunohistochemical results may be affected by several pre-analytical variables, including the type of fixative, time of fixation, inclusion method, time of paraffin-embedding, thickness of the sections, storage of unstained slides and the methods for antigen retrieval [73]. Standardisation of the pre-analytical phase is not restricted to tissue biomarkers. Several investigators have shown very different VEGF concentrations in different blood derivatives, including serum, plasma collected with different procedures and whole blood haemolysate [74,75]. Moreover, the concentrations of VEGF changed significantly according to the procedure used to prepare the serum or plasma [75]. Clotting time increased the release of VEGF, which reached a plateau between 2 and 4 h. The percentage increase in VEGF at 2 h ranged from 118% to 4515% (median 327%) with reference to that found in samples centrifuged within 10 min of withdrawal. However, in spite of these findings, several investigators have published promising results on serum VEGF as a cancer biomarker, providing scarce, if any, information on the standardisation of the pre-analytical phase. Surprisingly, the standardisation of sample preparation has not been considered in a recent Review article in which the potential clinical role of VEGF is extensively discussed [76].

Investigators are continually striving to improve the design and implementation of clinical trials on new therapeutic cancer agents. Not surprisingly, several initiatives aimed at standardising the methodology for planning, performing and reporting of clinical trials and the criteria for peer-reviewing these clinical studies have been endorsed by the Editors of different journals [77,78]. In contrast, efforts to standardise the use of biomarkers in clinical studies have only been considered anecdotally. When scrutinising the published studies on the clinical evaluation of biomarkers, international criteria of analytical validation (i.e., NCCLS National Committee for Clinical Laboratory Standard) are only applied or even quoted on an infrequent basis [79]. The uncritical use of laboratory data obtained with non-standardised approaches bears the risk of increasing confusion about the potential clinical usefulness of new biomarkers. In the new circumstances set by the development of molecularly targeted therapies, unrecognised analytical biases may ultimately impair the identification of the real effectiveness of anticancer agents. Tentative criteria for the choice of biomarkers and for their standardisation and use in clinical trials have recently been proposed in [80,81], with the aim of reaching a consensus on a specific and comprehensive methodological approach that takes into account the clinical needs related to the new agent, the biological background, the development and standardisation of laboratory methods and decision criteria that consider biological complexities. The issue of low methodological quality in diagnostic and screening tests was discussed at the 1999 Cochrane Colloquium in Rome. On the basis of the success of the CONSORT experience, a working group has developed a checklist and a flow diagram aimed at improving the quality and completeness of reporting studies on diagnostic accuracy [82]. The validation of the Standard for Reporting of Diagnostic Accuracy (STARD) initiative is expected to contribute to a continuous improvement in the quality of the design of studies on diagnostic tests, including those on cancer biomarkers.

5. Biomarkers: where are we going?

In a recent overview, Ranshoff [83] proposes a schematic separation into two categories of the approaches presently available for the discovery of new cancer biomarkers: (i) hypothesis-driven research and (ii) discovery-based research. The former concerns molecules believed to be involved in cancer biology. In this approach, putative candidate genes or proteins are considered one by one as possible biomarkers. The use of multiple biomarkers requires the standardisation of separate assay methods for every one of them, leading to a high level of complexity when several biomarkers are considered in association. As well, the statistical approaches needed to evaluate interactions are increasingly complicated when the number of biomarkers increases.

In contrast, the 'product' of discovery-based research consists of gene-expression patterns or mass-spectroscopic peaks, which may be used to select and identify new candidate genes or proteins by conventional methods. However, the patterns themselves may be used as 'pattern biomarkers'. In other words, expression 'signatures' or algorithm-interpreted proteomic patterns may became tools for clinical decision-making without our necessarily understanding which proteins or genes account for the cancer-specific patterns. Among the circulating biomarkers, serum proteomic patterns appear of special interest. Preliminary data on diverse malignancies show that these techniques appear to detect early as well as advanced disease with similar efficiency [84], suggesting a possible role for these pattern biomarkers as candidate tools for cancer screening.

The analysis of proteomic patterns in serum for prostate cancer diagnosis showed a sensitivity ranging from 83% to 100%, with a remarkable specificity of 78–100%. However, on examining carefully the papers reporting these data, Diamandis [85] identified some

inconsistencies that highlight the poor level of standardisation that still affects these methodological approaches. In fact, two authors [86,87] had even identified very different distinguishing peaks when extracting serum samples with the same chip and using the same instrument for peak identification. The same peak (*m*/*z* ratio of 7820) was identified as distinguishing cancer from non-cancer patients by Adam and colleagues [86] and as distinguishing healthy men from those with benign prostate hyperplasia, but not cancer and non-cancer patients, by Qu and colleagues [87]. Concerning prostate cancer diagnosis, Diamandis [85] points out that matrices used for sample treatment in the papers examined are not specific for any type of protein. Considering that the serum PSA concentration is approximately 1 mg/L, while the total protein is roughly 80 g/L (80 000 000 mg/L), Diamandis underlines that each PSA molecule will compete with 80 000 000 possibly non-informative molecules for binding to the same matrix. He concludes that, given this interaction, it seems unlikely that PSA, like any other low-abundance molecule in serum, might be detected by this methodology. By contrast, Petricoin [88] argues that these complex proteomic signatures, even if viewed as 'epiphenomena' produced by the host in response to the presence of cancer, may themselves represent valuable diagnostic information, providing amplification-cascade biomarkers of the tumour–host microenvironment. Irrespective of any interpretation, which is complicated due to the lack of precise information on the exact biochemical identity of the distinguishing peaks, we must emphasise the enormous diagnostic potential of these pattern biomarkers but take into account the following issues of standardisation (modified from [87]:

- sample handling and preparation may be a critical issue since the method for extracting these potential molecules from serum is very sensitive to differing experimental protocols and to varying serum storage conditions;
- distinguishing peaks identified by different investigators may be different for the same disease, even if the same method is used;
- results are not easily reproducible between laboratories, making validation difficult;
- mass spectrometry is a largely qualitative technique (the relationship between peak height and molecule abundance is not linear);
- identities and serum concentrations of distinguishing molecules are not known;
- absorption matrices are non-specific, thus favouring the extraction of high-abundance proteins/peptides rather than low-abundance;
- validated reference serum markers (i.e., PSA for prostate cancer, CA125 for ovarian cancer) are not identified by this technology.

Many researchers are seeking to overcome some of the aforementioned pitfalls [88], including (i) the development of serum/plasma reference standards (by the Human Proteome Organisation in partnership with the World Health Organisation, the American Red Cross and the Food and Drug Administration), (ii) the continuous improvement of analytical quality assurance through monitoring in-process controls (internal reference standards, release specifications, stability measures), (iii) the development of standard operating procedures for sample collection, handling and shipping, (iv) the validation of spectral data by rigorous quality testing before diagnostic pattern analysis, and (v) the improvement of mass-spectrometry platforms.

The recent, substantial increase in our knowledge regarding cancer cell biology and the rapid development of technology are broadening the appropriate settings for biomarker applications. However, this astonishing development dramatically emphasises the need for rigorous standardisation and quality control. We conclude by quoting a sentence from Ranshoff [83] that seems to summarise superbly the current perspectives on biomarkers: 'The journey to discover useful biomarkers will require imaginative exploration, fastidious validation and some good luck'.

Conflict of interest statement

None declared.

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