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REVIEW ARTICLE

Rapid emergence of multimarker strategies in laboratory medicine

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Abstract

A single biomarker has an inherent specificity and sensitivity that cannot be improved, but multiple biomarkers can be combined to achieve improved clinical performances. This is the basis of multimarker strategies that integrate different biomarkers into a single score to support medical decisions. The simplest strategy determines ratios of different biomarkers or the number of different markers above their respective thresholds. A more advanced strategy employs similar biomarkers, but uses more sophisticated algorithms. The most advanced strategy employs large numbers of biomarkers that may or may not have been previously characterized and uses sophisticated algorithms.

Keywords: *Multivariate; multiplexed diagnostics; biomarkers; combination; information technologies; algorithm*

Introduction

Multimarker strategies (MMS) are based on the observation that a single biomarker has an inherent specificity and sensitivity that cannot be improved, but that multiple biomarkers can be combined to improve their impact on medical decisions. MMS have the potential to improve early diagnosis and prognosis for several disorders, support medical decisions and assist in treatment response evaluation. MMS are made possible by the increasing numbers of available biomarkers that can be related to a pathological condition. Moreover, MMS are made practical in laboratory medicine by the already established use of multimarker technologies such as reverse transcriptase polymerase chain reaction (RT-PCR), microarrays and mass spectrometry. The rapid emergence of multimarker tests is already evident by the increasing number of citations in PubMed (Figure 1) that cover a broad spectrum of diseases (Figure 2). New biomarkers and new technologies are expected to extend this growth further. Nevertheless, while the promises and expectations are enormous, significant challenges remain.

Multimarker strategies: definitions

Biomarkers may be defined as objectively measurable indicators of normal or pathological processes or pharmacological responses to a therapeutic intervention. Therefore, the identification of reliable biomarkers is a major step forward in the management of a disease because they can elucidate the aetiology, define the diagnosis, and predict the prognosis of a disease. A reliable biomarker should be characterized by high sensitivity and specificity to be able to support a medical decision.

We define MMS as the integration of quantitative results of laboratory tests, alone or in combination with patient characteristics and medical/family history, to support a medical decision and to facilitate the

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physicians' work of interpretation of multiple sources of information from different laboratories. Within this framework, multiple approaches have been used for the selection of the most reliable model of multiple markers, and we have arbitrarily defined three major categories of MMS. The simplest MMS approach is related to a combination of assays for different markers in the same pathophysiological context with minimal, if any, mathematical manipulations. This approach is labelled minimal MMS. A second MS approach is based on more sophisticated mathematics and employs bioinformatic or dynamic statistical treatment of the results from the studied/associated markers. This approach is labelled sophisticated MMS. The third approach is based on sophisticated mathematics and complex sets of biomarkers, some or all of which may not have been previously characterized. This approach is labelled complex MMS.

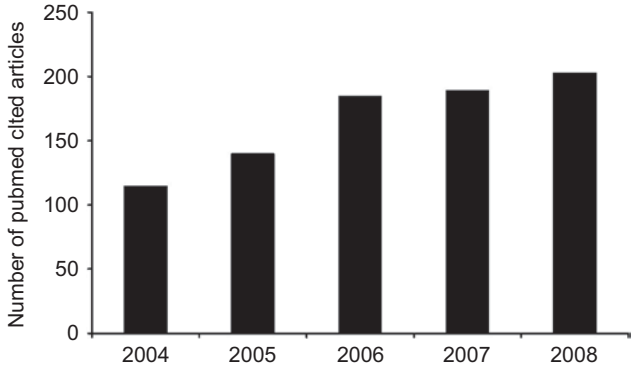

Figure 1. Evolution of the number articles related to multimarker approaches and cited in PubMed over the past 5 years (the criteria of search were multimarker strategies and multiple biomarker approach).

Multimarker strategies: minimal

Minimal MMS is based on a simple ratio between biomarkers or an analysis of the number of markers above their respective thresholds. This is an extension of common medical practice, where physicians integrate biomarkers to establish a diagnosis, such as recording body temperature with clinical signs, serum markers and urinary biochemistry for assessing liver function. Minimal MMS merely formalizes this type of integration. Because of the simplicity of this strategy, the biomarkers are usually already known to be closely related to the pathophysiology of the disease in question. The range of published studies and clinical applications using this strategy is quite diverse.

Because of the large number of documented biomarkers and because of its multifactorial origin, cardiovascular disease is especially well suited to the minimal strategy. An excellent example is the screening and diagnosis of primary aldosteronism (PA). PA is a form of endocrine hypertension in which aldosterone production is inappropriate and at least partially autonomous of the renin–angiotensin system (Mulatero et al. 2004). PA was previously believed to account for less than 1% of hypertensive patients, but recent studies using a simple ratio between plasma aldosterone/plasma renin activity or plasma aldosterone/plasma direct renin ratios as a screening test demonstrated that PA accounts for up to 12% of hypertensive patients (Campbell et al. 2009). Thus a simple MMS could identify a treatable and potentially curable form of hypertension. This result has been corroborated in additional studies. In a retrospective study from Mulatero et al., the wide use of the aldosterone-to-renin ratio (ARR) as a screening test in hypertensive patients from five continents led to a marked increase in the

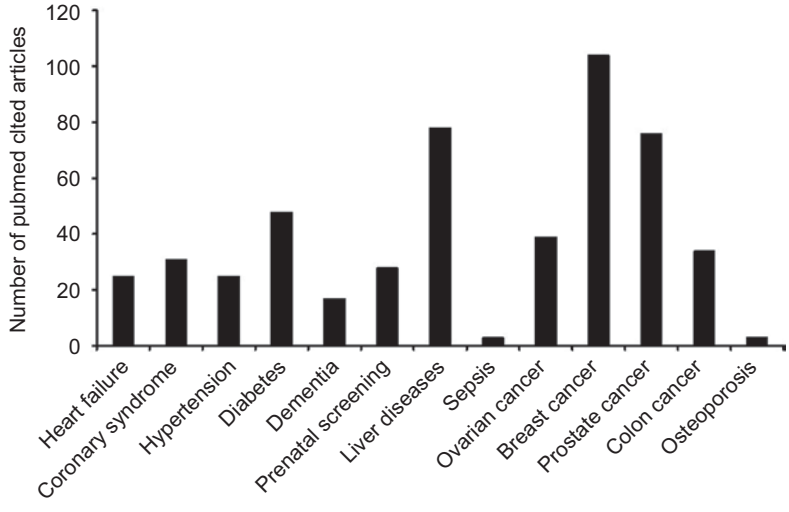

Figure 2. Number of articles related to multimarker approaches and cited in PubMed for some frequent disorders (the criteria of search were multimarker strategies and multiple biomarker approach).

detection rate of PA. In another study, the ARR was also positively associated with different measures of vascular stiffness (Lieb et al. 2009), indicating even greater benefits from this test.

In addition to the simple ratio, another minimal MMS is to categorize cardiovascular patients based on multiple elevated biomarkers. Sabatine et al. (2002) selected one marker of damage to cardiac tissue, cardiac troponin I (cTnI), one marker of excessive stretching of cardiac tissue, B-type natriuretic peptide (BNP), and one marker of inflammation, C-reactive protein (CRP) and found in a study of 1635 patients with non-ST acute coronary syndrome that those with one, two and three elevated biomarkers had a 2.1-, 3.1- and 3.7-fold increase, respectively, in the risk of death, myocardial infarction (MI) or congestive heart failure (CHF) by 6 months (Sabatine et al. 2002) (Figure 3).

In a cohort of 216 patients with acute coronary syndrome (ACS), Kavsak et al. evaluated the clinical value of a combination of N-terminal pro-BNP (NT-proBNP), interleukin (IL)-6, IL-8 and monocyte chemotactic protein (MCP)-1 determined early after symptom onset for prediction of death or CHF (Kavsak et al. 2007). The MMS including IL-6, MCP-1 and NT-proBNP was an independent predictor of long-term risk of death or HF, highlighting the importance of identifying leukocyte activation and recruitment in ACS patients.

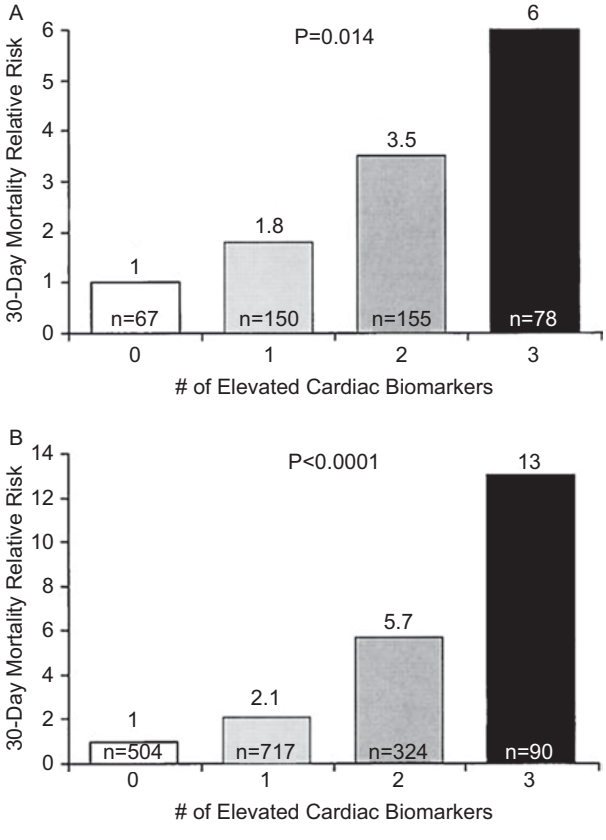

Figure 3. Relative 30-day mortality risks in OPUS-TIMI 16 (A) and TACTICS-TIMI 18 (B) in patients stratified by the number of elevated cardiac biomarkers (adapted from Sabatine et al. 2002).

In addition, McCann et al. reported that for risk stratification of patients admitted with ischaemic-type chest pain, the measurement of heart fatty acid-binding protein and NT-proBNP at the time of admission adds useful prognostic information to that provided by the measurement of baseline and 12-h cardiac troponin T (cTnT) (McCann et al. 2009). Furthermore Arant et al. used a multiple biomarker approach to investigate CRP, IL-6, serum amyloid A and haemoglobin levels in 595 women referred for coronary angiography and found that women with three or four abnormal biomarkers were approximately 10–20 times more likely to die (Arant et al. 2009).

Furthermore, non-invasive markers of subclinical atherosclerosis, namely carotid intimal media thickness (IMT), flow-mediated dilatation of the brachial artery, augmentation index or pulse wave velocity, may be useful in the prediction of cardiovascular risk particularly in primary prevention settings. The combination of these non-invasive tests has been shown to improve their prognostic accuracy compared with each other alone (Ikonomidis et al. 2008). Therefore, a combination of an established inflammatory marker such as CRP or a vascular marker such as IMT with novel biochemical and vascular markers of cardiovascular disease may offer additive prognostic information for adverse outcome.

The benefits of minimal MMS are not limited to cardiovascular diseases. Dementia, which afflicts approximately 40% of people older than 90 years, is another candidate. Hansson et al., for example, studied the association between cerebrospinal fluid biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment and found that a combination of total tau and Aβ42 at baseline yielded a sensitivity of 95% and a specificity of 83% (Hansson et al. 2006). The combination of total tau and Aβ42/phosporylated tau 181 ratio yielded similar results. Moreover, the results obtained by Fagan et al. (2007) also showed the association of the CSF tau/Aβ42 ratio or the phosphorylated tau 181/Aβ42 ratio to cognitive decline in non-demented older adults.

Successes with minimal MMS have also been reported in oncology. The prostate-specific antigen (PSA) assay is single-analyte measurement that is limited by frequent false-positive and -negative results and may lead to an excessive number of biopsies. A study from Sunami et al. reported that combining a DNA assay for methylation of microsatellites with the PSA assay gave a sensitivity of 89%, which represents a significant improvement (Sunami et al. 2009). Moreover, in non-small cell lung cancer, a combination of circulating mRNA has been studied; Sheu et al. combined detection of carcinoembryonic antigen (CEA), cytokeratin (CK)-19 and c-met

mRNAs in blood and reported promising results (Sheu et al. 2006).

Multimarker strategies: sophisticated

The premise behind this strategy is that the analytes in question vary in importance and/or independence. Simple ratios or the number of analytes above their thresholds do not adequately model the disease state and/or response to treatment. Adequate modelling requires the more sophisticated approach of using an algorithm-based index or score. One example of sophisticated MMS is the homeostasis model assessment (HOMA) from the University of Oxford (Matthews et al. 1985). This model is based on the fact that the steady-state basal plasma glucose and insulin concentrations are determined by their interaction in a feedback loop. This computer model is used to predict the homeostatic concentrations that arise from varying degrees in β-cell deficiency and insulin resistance and is available for community use.

In another example, Zairis et al. (2009) investigated the combined prognostic value of admission serum levels of BNP, cTnI and high-sensitivity (hs)-CRP in 577 patients hospitalized because of CHF and, using a multivariate Cox regression analysis, concluded that increasing numbers of elevated biomarkers gradually increased the risk of 31-day cardiac death (Zairis et al. 2009). A third example, also related to cardiovascular disease, was published by Mockel et al. (2008). In this study, 432 unselected patients admitted to the emergency department (ED) with ACS were enrolled and monitored for major adverse cardiovascular events (MACE) and cTnI, NT-proBNP, hsCRP, placental growth factor, lipoprotein-associated phospholipase A(2) and D-dimers were measured. This study demonstrated that a MMS defined synergistically by logistic regression and by classification and regression tree (CART) analysis can stratify patients into risk groups ranging from very low risk (0% MACE) to very high risk (39.5% MACE) based on admission values.

A fourth example is a predictive model to assess the risk of epithelial ovarian cancer (EOC) in women with a pelvic mass (Moore et al. 2009). Preoperative serum levels of HE-4 and CA-125 were measured in 531 patients with benign tumours, EOC and non-ovarian cancers. Indexes based on algorithms using quantitative values of HE-4 and CA-125 were generated and utilized to categorize patients into low- and high-risk groups. In a postmenopausal group, the sensitivity was 92.3% and in a premenopausal group the sensitivity was close to 77% (Table 1). A fifth example, also related to ovarian cancer, was published by Amonkar et al. (2009) and presents a multivariate index to distinguish women with ovarian cancer from those with benign conditions. The sera representing 176 cases and 187 controls from women presenting for surgery were examined using high-throughput, multiplexed immunoassays. The design of an 11-analyte profile, composed of CA-125, CA 19-9, epidermal growth factor receptor (EGFR), CRP, myoglobin, apolipoprotein A1, apolipoprotein CIII, macrophage inflammatory protein (MIP)-1α, IL-6, IL-18 and tenascin C was identified and appears informative for all stages and common subtypes of ovarian cancer and offered approximately 90% sensitivity and 90% specificity.

A fifth example is the FibroTest (BioPredictive, Paris, France), which combines six markers, α2-macroglobulin, haptoglobin, apolipoprotein A1, γ-glutamyl transpeptidase, total bilirubin and alanine transaminase (ALT), with the age and sex of the patient to provide a non-invasive alternative to biopsies for diagnosing liver fibrosis (Le Calvez et al. 2004). In studies of more than 7985 subjects who had undergone both FibroTest and biopsy, the mean standardized area under the receiver operating characteristic (ROC) curve was 0.84 (95% confidence interval (CI) 0.83–0.86) (Halfon et al. 2008). Moreover, when biopsy and marker results are discordant, a reason can be identified in more than two-thirds of cases and, in those cases, biopsy failure is greater than sevenfold more common than the diagnostic failure of the markers (Poynard et al. 2004).

Table 1. Distribution of patients into low-risk and high-risk groups: benign vs. EOC and LMP Tumors (adapted from Moore et al. 2009).

		Low risk		High risk						
Menopausal status	Disease	*n*	%[a]	*n*	%[a]	Total (*n*)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
Combined	Benign	263	93.9	89	39.9	352	88.7	74.7	60.1	93.9
	Cancer	17	6.1	134	60.1	151				
	Total	280	100	223	100	503				
Premenopausal	Benign	151	95.0	51	66.2	202	76.5	74.8	33.8	95.0
	Cancer	8	5.0	26	33.8	34				
	Total	159	100	77	100	236				
Postmenopausal	Benign	112	92.6	38	26.0	150	92.3	74.7	74.0	92.6
	Cancer	9	7.4	108	74.0	117				
	Total	121	100	146	100	267				

[a]Percentage of cases within low-risk group and within high-risk group.
PPV, positive predictive value; NPV, negative predictive value.

Multimarker strategies: complex

Computational, 'omics and multiplexing technologies have come together to enable the study and analysis of large numbers of analytes. It is possible, for example, to analyse all of the gene transcripts in a tissue and cluster the responses according to the endpoint(s) in question. As the numbers of analytes get larger, the studies are driven less by hypothesis and more by empirical analysis, and the complexity grows. However, if the core principle of MMS is that combining biomarkers can improve sensitivity and specificity, complex MMS has the potential to make the largest impact on diagnostics by combining the most biomarkers.

In the study from Ridker et al., for example, 35 factors were assessed in 24 558 initially healthy US women aged 45 years or older who were followed up for a median of 10.2 years for incidence of cardiovascular events (Ridker et al. 2007). The minimization of the Bayes Information Criterion was used in the derivation cohort to develop the best-fitting parsimonious prediction models. The model presented in this study, named the Reynolds risk score, demonstrated highly improved accuracy for global cardiovascular risk prediction that reclassified 40–50% of women at intermediate risk into higher- or lower-risk categories. More recently, a Reynolds risk score for men has been proposed (Ridker et al. 2008).

Another example of complex MMS is the simultaneous testing using a multiplexed panel of serum biomarkers that may present a promising new approach for the early detection of squamous cell carcinoma of the head and neck (SCCHN) (Linkov et al. 2007). Concentrations of 60 cytokines, growth factors and tumour antigens were measured in the sera of 116 SCCHN patients before treatment (active disease group), 103 patients who were successfully treated (no evidence of disease group) and 117 smoker controls without evidence of cancer. A multimarker panel of 25 biomarkers was found to offer the highest diagnostic performances (sensitivity of 84.5%, specificity of 98% and 92% of patients in the active disease group correctly classified from a cross-validation serum set).

Twenty-five biomarkers is a large number for a single diagnostic, but such complexity is becoming the standard in gene expression profiling tests. The MammaPrint (Agendia Inc., Huntington Beach, CA, USA) 70-gene signature and Oncotype DX (Genomic Health Inc., Redwood City, CA, USA) 21-gene signature, for example, are beginning to have a significant impact on the clinical practice of breast cancer (Slodkowska & Ross 2009). MammaPrint was developed by screening 25 000 gene expression events across 98 primary breast cancers and using an unsupervised, hierarchical clustering algorithm (Glas et al. 2006, Slodkowska & Ross 2009) (Figure 4). The test was subsequently validated in multiple studies. It was shown, for example, to distinguish patients correctly who needed adjuvant chemotherapy from those who did not (Slodkowska & Ross 2009). It was also compared with clinicopathological scoring by Adjuvant! software in ER-positive and -negative patients and shown to have significantly better hazard ratios (Glas et al. 2006). Oncotype DX was developed through a similar approach, and the final signature contains 21 genes that predict breast cancer recurrence and whether an early-stage, node-negative, estrogen-receptor-positive tumour will respond to chemotherapy (Cronin et al. 2007). The test has also been validated in numerous studies. In a study published in 2004, researchers analysed 668 of 675 tumour blocks from patients with node-negative, tamoxifen-treated breast cancer and were able to categorize the patients as having low, intermediate or high risk of recurrence (Paik et al. 2004). In another study published in 2006, researchers calculated recurrence scores for 651 patients and found that those with a high recurrence score had a large benefit from chemotherapy whereas those with a low recurrence score derived minimal, if any, benefit (Paik et al. 2006).

Challenges

These are early days for MMS and challenges remain. On the side of difficulty, the combination of biomarkers is only useful if the correlations between the biomarkers are weak. A simulation done by Pepe and Thompson, for example, shows that two weakly correlated biomarkers generate a sensitivity of 80%, but if the correlation increased to a moderate level, the sensitivity drops to 70% (Pepe et al 2008). Wang et al. write that most current biomarkers for cardiovascular disease participate in pathways that are known to be associated with atherosclerotic cardiovascular disease, such as those involved in inflammation and cholesterol biosynthesis, and the available biomarkers provide information that is often correlated with what is already known or being measured (Wang et al. 2006). The same conclusions were reached recently by Melander et al. (2009). Moreover, Apple et al. recently showed that among seven biomarkers (myeloperoxidase, soluble CD40 ligand, placental growth factor, matrix metalloproteinase 9, hsCRP, cTnI and NT-proBNP) the most clinically accurate biomarker for the early diagnosis of MI is the use of cTnI alone, rather than a multiple-biomarker approach, when an analytically robust cardiac troponin assay based on the 99th percentile is used (Apple et al. 2009). On the other hand, these pessimistic conclusions are countered with results from Zethelius et al. who demonstrated that the use of multiple biomarkers may improve the prediction of death from cardiovascular causes (Zethelius et al. 2008), and the previously mentioned results from Kavsak et al. (2007).

These studies demonstrate that an important challenge for MMS is the selection of the biomarkers (Lindahl

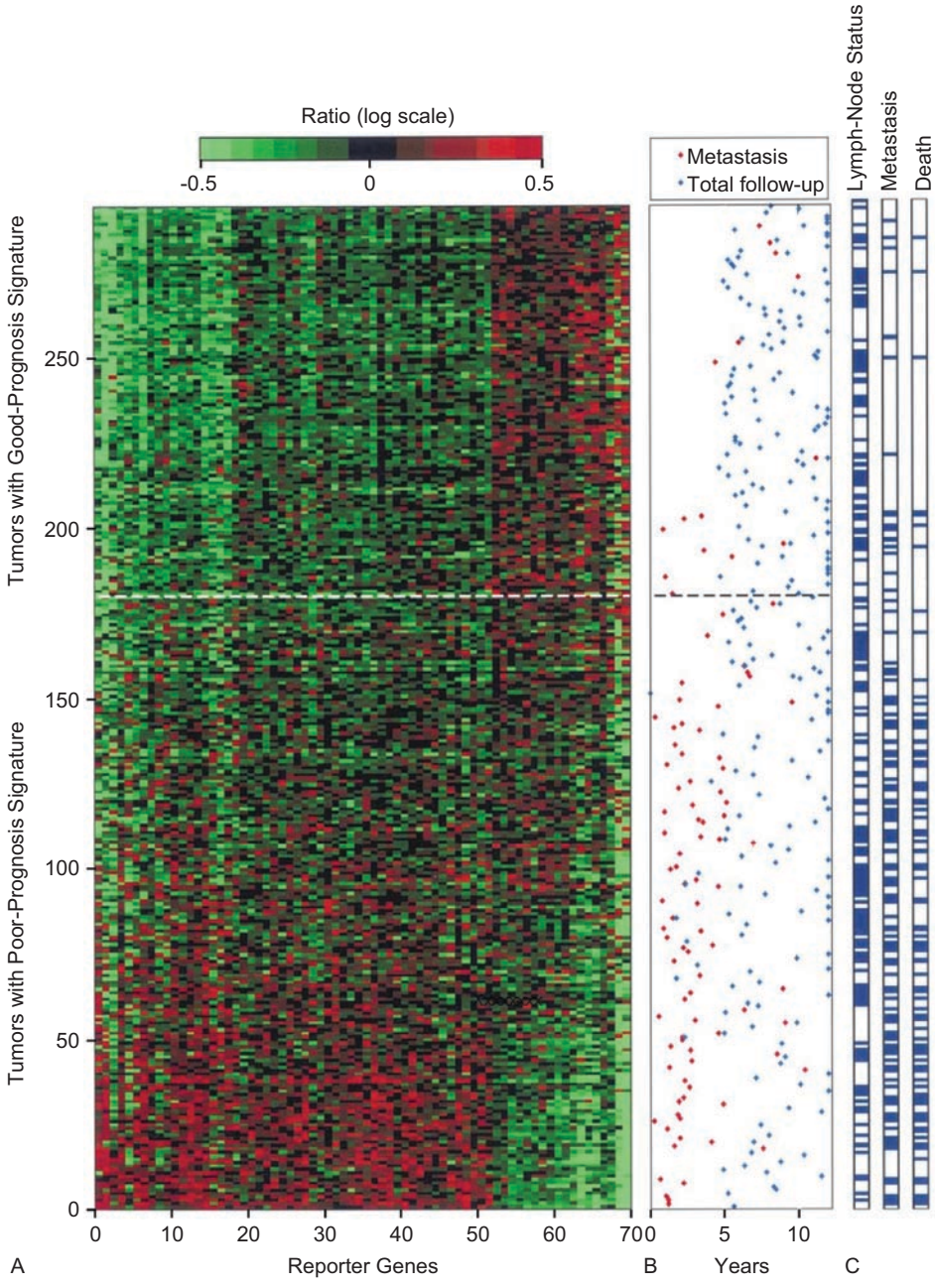

Figure 4. The MammaPrint test from Agendia.

2009). On the other hand, in a recent study of prognostic gene signatures for non-small cell lung cancer, researchers found more than 500 000 possible six-gene panels that could accurately stratify individuals with stage I cancer into two groups with different survival outcomes (Boutros et al. 2009). These apparently opposite findings indicate that the process for developing multimarker panels is far from mature and the variation presents a challenge to the industry.

Therefore, the process of model selection for MMS and cross-validation of a set of biomarkers remains extremely important. The weighting criteria applied to every observation and the relative importance assigned to one marker against another in the final diagnosis will influence the reliability of MMS for diagnosis or prognosis especially in a complex system where the use of too large a number of biomarkers may affect the prediction power. Different tools can be used to evaluate the reliability of biomarkers sets, to cross-validate the choice of the selected biomarkers and to estimate the generalization error of a given model. Leave-one-out cross-validation, Jack-knife system and area under the receiver operating curve may be used for such processes. Indeed, these tools may contribute to the evaluation of the model efficiency

(Ziol et al. 2005), to improvement of the selection of markers (Sepehri et al. 2008), to assessment of the reliability (Roberts & McNamee 2005) and to building predictive scores (Harima et al. 2009).

The reliance on statistics raises a philosophical question about the underlying science. Large panels of biomarkers may contain elements that have not been characterized in any meaningful way. Some elements may have no known function or no known relevance to the disease in question. This is unsatisfying from the point of view of biology, because without knowing the relevance of individual biomarkers, we cannot use theory to guide the development of improved tests. We are relegated to continuing a high level of empiricism, which is expensive and time-consuming. Nonetheless, despite the encumbrances, this level of empiricism appears to be highly functional, as demonstrated by Oncotype DX and MammaPrint. These tests were developed by empirical and statistical methods to such an extent that none of the 91 genes on the two tests are the same. Yet, in a comparison of 295 samples of breast cancer, the two tests were in agreement in 239 samples (81%); in particular, 81 of the 103 samples with an Oncotype DX recurrence score of low or intermediate were classified as having a good MammaPrint profile (Fan et al. 2006). This indicates that both tests are tracking the same underlying biology, even though the specific relationship of all of the biomarkers to breast cancer is unknown.

In addition, the challenges facing MMS are not solely technical or scientific. MMS, for example, require new regulation. To meet this challenge in the USA, the Food and Drug Administration (FDA) started the process of creating a new diagnostic category entitled '*in vitro* diagnostic multivariate index assay (IVDMIA)'. In contrast to conventional biomarkers, an IVDMIA requires an algorithm to calculate the diagnostic score. The linear classifier is the preferred algorithm. When the number of diagnostic genes is n, each tumour is represented by a point in an n-dimensional space made from gene expression values. Diagnostic algorithms (linear classifier) make an (n-1)-dimensional plane in the n-dimensional space to separate two patient groups. Calculation of the diagnostic score is achieved by dimension reduction. Currently, the final IVDMIA guidelines are still in process and are more than 1 year overdue. The complexity of the tests and perhaps, as shown in this review, the breadth of strategies, is proving daunting. Meanwhile, in Europe, no new regulatory guidelines are in progress; only the CE-mark is required, and this is not a measure of effectiveness or utility. In the absence of clear regulatory guidelines, developers face additional risks. The pathologists, clinical scientists and the commercial developers welcome the implementation of regulatory principles for MMS devices or algorithms.

Conclusions

MMS overcome the inherent limitations of single biomarkers and enable specificity and sensitivity that would otherwise be unobtainable. Multiple studies have demonstrated this principle across a wide range of disease states, and these successes are beginning to transform laboratory medicine. The pessimists would point out that MMS have not be standardized, that multimarker panels have not been proven in prospective studies and that regulation is yet to be determined. The optimists, however, would point out the potential to usher in an entirely new era of diagnostics.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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