EXPERT REVIEWS

Found in transcription: gene expression and other novel blood biomarkers for the early detection of breast cancer

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Early detection of a growing breast tumor is of key importance for patient survival. Despite limitations, mammography screening has improved the detection of breast tumors, however many tumors are not detected. This is especially true for younger women and women with high breast density. Novel diagnostic blood biomarkers either generated by the tumor and released into the blood, or generated by nontumor cells as a response to the tumor presence, can now potentially help improve the accuracy of early-stage breast cancer detection. They include multicomponent biomarkers, circulating tumor cells and RNA expression of peripheral blood. These novel biomarkers and their potential use will be presented and discussed in this review, with special emphasis on gene expression-based markers.

Keywords: circulating tumor cells • diagnostic biomarker • miRNA • peripheral response • RNA

Breast cancer has the highest incidence and mortality among women afflicted with cancer in the world. Over 1.1 million women were diagnosed in 2002 with breast cancer and more than 400,000 women died from the disease in the same year [1]. Breast cancer is also the most prevalent cancer in the world with 4.4 million survivors up to 5 years following diagnosis [1]. However, there has been a gradual reduction in mortality beginning in 1990 when the rate in the USA began to decline by 2.3% annually [201]. In addition to the increased utilization of adjuvant systemic therapies, this improvement in survival has been attributed to the early detection through mammography screening programs [2]. While the introduction of mammography screening has contributed to reduced mortality, there is potential for further gains when considering the limited sensitivity of mammography. In a review of historical mammography screening trials, it was concluded that the overall sensitivity with mammography alone was only in the range of 60–66% [3]. Using the more advanced digital technology in the Digital Mammography Imaging Screening Trial (DMIST) the overall sensitivity, as defined by a 12-month follow-up period, revealed only a slight improvement to 70% sensitivity compared with the 66% sensitivity with film screen technology [4]. The sensitivity of mammography is further reduced in younger women below the age of 45–50 years, which is the age when screening is usually initiated, and in women with high breast density. In a comparative study incorporating data from five prospective studies [5–9] and including 3571 screened high-risk women with a mean age of 41 years, the sensitivity of mammography was only 40% [10]. Despite these limitations, mammography screening has been shown to help reduce mortality due to breast cancer. However, there is clearly potential for improvement.

There are several emerging tools that can potentially help improve the accuracy of early-stage breast cancer detection. Diagnostic biomarkers are one option that has to be considered. A diagnostic biomarker is a substance most often found in a body fluid such as peripheral blood, which is either produced by the tumor or by nontumor cells as a response to the presence of a tumor. Diagnostic biomarkers include not only the traditional protein and glycoprotein markers but also novel types of markers such as autoantibodies, circulating tumor cells (CTCs), RNA and miRNA. The American Society of Clinical Oncology (ASCO) evaluated in their latest recommendations, 17 different markers

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for breast cancer, but only three of these where evaluated for screening or diagnosis of the disease and none of the three were recommended for clinical use [11]. This review will discuss the potential use of novel blood-based diagnostic biomarkers with a special focus on the use of gene-expression patterns as early diagnostic markers for breast cancer.

Distant responses

A biomarker for diagnostic purposes is most conveniently collected if it is present in a bodily fluid, such as peripheral blood. The marker is then either generated by the tumor and released into the blood, or generated by nontumor cells in close proximity or further away from the tumor as a response to the specific type of cancer. Tumor specific interactions with its environment have been shown as part of the natural history of a malignant tumor, including angiogenic and antiangiogenic factors, metalloproteases, growth factors and inflammatory factors. There are molecules released from tumors [12,13] and there are different responses to the presence of a tumor away from the tumor itself, all with potential as diagnostic biomarkers.

The blood–tumor dialogue

Breast cancer is generally considered to be a genetic disease of somatic cells. Carcinogenesis can be looked upon as microevolution, where some cells over-rule the signals from the surrounding tissue owing to alterations in the genome. The cells start growing uncontrollably and might, with time, acquire metastatic potential and spread to remote sites, often with life-threatening consequences. To be able to grow and thrive, the tumor is dependent on sufficient oxygen and nutritional supply. To achieve this, the tumor has to communicate with its surrounding non-neoplastic cells [14]. Tumors have been described as wounds that do not heal, because there are striking similarities between the molecular processes taking place in a healing wound and in growing tumors [15]. Both wounds and growing tumors need help from the immune system to remodel the surrounding tissue and to recruit new blood vessels to the site. It is believed that many tumor cells in this way exploit an already existing biological mechanism to its own benefit [16]. It becomes more and more evident that tumor growth leads to a defense response in the host, activating its immune system [17]. The importance of this response is reflected in the elevated number of spontaneous tumors occurring in immunocompromised animals [18] and humans [19]. The enormous communication between cancer cells and their environment includes a host of factors released into the intracellular compartment, including cytokines, lipids, prostaglandins, interleukins, integrins and growth factors. These factors lend themselves to investigation as surrogate markers of breast cancer. However, for the markers to be of clinical use they need to be specific to the tumor and show consistent results within a population. The amount of signal molecules from a tumor of limited size might be imperceptible, but technologies for detecting minute amounts of biomarkers are steadily improving. Another possible signal one can seek to detect is the tumor immune response. This signal might be stronger and hence easier to detect.

Responses in stroma

Stroma refers to the connective supportive framework or microenvironment of the tumor. Under normal physiological conditions, stroma serves as an important barrier to epithelial cell transformation, this is the interplay between epithelial cells and the microenvironment which maintains epithelial polarity and modulates growth inhibition [20]. However, the stromal compartment undergoes changes in response to developing malignant lesions and can have a key role in cancer initiation and progression [20,21]. These changes may include the recruitment of immune and endothelial cells, providing growth and matrix remodeling factors, as well as a new blood supply promoting tumor growth and metastasis [20–22]. The communication between tumor and stroma provides the environment for tumor development and includes factors such as hypoxia-inducing factor [12] and growth factors such as VEGF [23]. Although VEGF is produced by the tumor cells, the circulating levels are augmented by hypoxia-inducing factor stimulating production of VEGF by the stromal cells [23]. The paracrine-acting factors released as part of the stromal–lesion communication may not exert biological effects on more distant targets and the use of these factors as markers for diagnostic purpose may not be relevant.

Immune responses

The immune system responds to a growing breast tumor in many ways. The response in the tumor-draining lymph nodes is well known. In 1953, Black *et al.* implied a general knowledge that locoregional lymph nodes from breast tumors are often enlarged, suggesting a native immune response against the tumor [24]. Later, it was shown that the tumor-draining lymph nodes of breast cancer patients contain high numbers of IgG-positive B cells [25]. Higher total number of B cells has also been observed in the lymph nodes of stage II breast cancer patients compared with stage I patients [26]. It has been shown that the majority of tumor-associated antibodies (TAAs) from nodes are of the IgM isotype and are reactive with antigens whose expression is restricted to normal secretory epithelia, including normal breast epithelium [27]. It is suspected that the immunogenicity of these antigens may be the result of tumor overexpression.

Most often there are also tumor-infiltrating B cells (TIL-B) present in the breast tumor lesions. TIL-B lymphocytes were the more abundant in ductal carcinoma *in situ* (DCIS) lesions and were perivascular, clustered in aggregates and surrounded by T cells [28]. Many breast adenocarcinomas contain lymphocytic infiltrates to a varying extent. In one study including all histological subtypes of breast cancer, approximately 20% were heavily infiltrated and approximately 50% had moderate infiltrates [29]. Since all TIL-B aggregates contained $CD21^+$ follicular dendritic cells, it suggests that an anti-tumor B-cell response might develop *in situ* in tumors rather than in lymph nodes alone. Also, high levels of T cells are observed in early stages of breast cancer. Hussein *et al.* reported an increase in the density of infiltrating T cells in benign proliferative breast disease compared with normal breast tissue. This indicates that the immune system responds at a very early stage of carcinogenesis, probably due to increased load of associated antigens on the damaged ductal cells [30].

As a response to a growing tumor, TAA can often be detected in sera. This is also true for breast cancer [31,32]. It has even been shown that TAAs can be detected in patients with breast cancer months to years prior to clinical diagnosis [33–35]. Since TAAs can be detected in sera of breast cancer patients it has been speculated whether they can be used as diagnostic biomarkers. TAAs have been detected in the sera that respond to several different antigens, including HER2, p53, MUC1, endostatin, lipophilin B, HSP90, cyclin B1 and D1, fibulin, cathepsin D, and TOPO2α. However, there are several challenges with TAAs as diagnostic biomarkers that have to be addressed. One challenge is that none of the TAAs used in these studies are able to detect the same antigen in the sera of all breast cancer patients. The TAAs are detected in the range of 5–75% of the sera samples from breast cancer patients [36]. They are also often detected in women with benign changes in the breasts [37,38]. Another challenge with many of the TAAs is their lack of specificity for breast cancer. Antigens such as p53 and HER2 are not unique for breast cancer and TAAs specific for these antigens are also elevated in the sera of patients with many other types of cancer [39–41]. Combining several TAAs appears to improve the accuracy. Receiver operating curves for a combination of TAAs against p53, HER2, IGFBP-2 and TOPO2α were constructed and gave an AUC of 0.63. Although still rather low, it is an improvement from the AUC of 0.48 achieved with anti-p53 alone [36]. Nevertheless, tumor marker determination may complement patient staging – high levels of TAA in patients thought to have localized disease suggest the presence of unsuspected metastatic disease. The sensitivity of tumor markers is significantly higher in patients with advanced disease and is related to the site of recurrence [42].

Changes in blood cell populations

Several research groups report that the neutrophil–lymphocyte ratio can be used as a diagnostic or prognostic marker for various disease states, such as epithelial ovarian cancer, colorectal cancer, acute coronary syndromes and systemic inflammations [43–46]. The ratio is calculated based on blood cell count with disease states having higher counts of neurophil granulocytes (neutrophilia) and reduced counts of lymphocytes (lymphocytopenia), compared with controls. The method appears to have limited sensitivity and specificity as a standalone tool, but might serve as a complementing early diagnostic tool.

Circulating tumor cells

The presence of CTCs was first described more than a century ago [47] but has only recently become of greater interest [48–51]. CTCs are frequently associated with the presence of axillary lymph node metastasis, and markers of CTCs have been used for the prognosis for short disease-free interval [52] or progression-free survival [53] and as predictors of poor clinical outcome [54–57]. Recently, CTCs were detected in lymph node-negative breast cancer patients [58]; however, the sensitivity of the markers used varied between 29 and 77%, suggesting that CTC markers still lack the necessary sensitivity for use as diagnostic markers. None of the CTC markers were detected in healthy controls, suggesting high specificity. As yet, it is unclear whether CTC markers are positive for patients with DCIS and whether they are able to differentiate between different forms of cancer. A few studies have explored the potential use of CTCs as an aid to assist breast cancer diagnosis [59,60]. Combining a reverse transcription (RT)-PCR-based marker for the mammaglobin and B305D-C genes, Reinholz *et al.* achieved a sensitivity and specificity of 70.5 and 81%, respectively [59]. The ten samples collected from DCIS patients did not have significantly different levels of these markers, indicating that these tumors do not shed malignant cells into the circulation. Using a combination of RT-PCR-based CTC-enriched markers for the cytokeratin-19, carcinoembryogenic antigen, c-Met, Her2/neu and mammaglobin genes, a sensitivity of 80.6% and a specificity of 83.8% was achieved [60]. Sensitivity and specificity increased both with tumor–node–metastasis staging and tumor size. At stage I the sensitivity was 68% and increased to 96 and 100% in stage IIb and III, respectively. No DCIS samples were included in this study. These results suggest that CTC markers can be useful in the more advanced stages of breast cancer, while their value is more limited in the earlier stages of the disease. Since cell shedding increase with disease development these results are also what can be expected.

Inflammation

Epidemiologic studies have shown that chronic inflammation predisposes individuals to various types of cancer. It is estimated that underlying infections or inflammatory responses are linked to 15–20% of all deaths from cancer worldwide [61]. The hallmarks of cancer-related inflammation include the presence of inflammatory cells and inflammatory mediators such as chemokines, cytokines and prostaglandins, in tumor tissues. These signs of inflammation are also present in tumors such as breast cancer, for which a firm causal relationship to inflammation has not been established. Indeed, inflammatory cells, such as macrophages, and mediators are present in the microenvironment of most, if not all, tumors irrespective of the trigger for development [62]. Key mediators of the inflammatory response include transcription factors, such as nuclear factor (NF)-κB and signal transducer and activator of transcription (STAT)3, and cytokines such as IL-1β, -6 and -23, and TNF-α [63–68]. The potential use of NF-κB as a diagnostic biomarker is discussed in the next section. It is not clear whether the amount of any of the other indicators for cancer-related inflammation is altered in peripheral blood and have potential for use as diagnostic biomarkers.

Cachexia

Cachexia, the massive loss of both adipose tissue and skeletal muscle mass, is a significant factor contributing to the poor performance status and high mortality rate of cancer patients [69]. The dramatic metabolic changes that occur during tumor growth are triggered by the proteolysis-inducing factor [70], and by proinflammatory cytokines such as TNF-α [71] and IL-6 [72]. Whereas proteolysis-inducing factor is produced by the tumor [13], cytokines are released as a consequence of interactions between host cells and tumor cells. As is the case for inflammation, initiation

of transcription by NF-κB is also a key factor in all mechanisms involved in cachexia [72,73]. Wasting does not result from a general downregulation of muscle proteins, but rather a controlled process with degradation of selected proteins dependent upon the particular mechanism facilitating the wasting state [74]. Since cachexia is a systemic response to cancer it is expected that systemic signals are released from the tumor.

Interestingly, it was shown that the level of the key factor NF-κB is significantly increased in whole blood lysate from women with breast cancer [75] and the level was not affected by chemotherapy treatment [75]. Whether this increase in NF-κB levels is related to cachexia, cancer-related inflammation or both is not clear. Since the women in this study had advanced stage cancer (stage III and IV), it remains to be determined if the elevated level can also be found in breast cancer patients at an earlier stage of the disease. Additional performance characteristics of NF-κB as a diagnostic biomarker have not been evaluated, however, an increased level of NF-κB in blood is not unique for breast cancer and it is to be expected that NF-κB will have low specificity for the detection of breast cancer.

Biomarkers

The majority of studies to identify useful breast cancer biomarkers have focused on only one or very few potential candidate markers. Given the multiplicity of pathophysiological processes implicated in breast cancer, the diagnostic accuracy may be further improved by combining several markers. Such an approach has the potential to create a more robust marker profile characteristic for the disease.

Protein-related markers

In their latest update, ASCO recommended the use in practice of eight different protein-related tumor markers [11]. These markers include CA 15-13, CA 27-29, carcinoembryonic antigen, estrogen receptor (ER), progesterone receptor, HER2, urokinase plasminogen activator (uPA) and plasminogen activator inhibitor (PAI)-1. They are recommended for monitoring during therapy (CA 15-13, CA 27-29 and carcinoembryonic antigen), for treatment planning (ER, progesterone receptor and HER2) and recurrence risk prediction (uPA and PAI-1) [11], and this is very much in line with the evaluation of existing breast tumor markers carried out by Duffy [76]. However, none of them are recommended for diagnostic use [11].

Several other protein-related markers have been suggested for clinical use, including kallikrein 14 [77], p53, cathepsin D and cyclin E [11], but they all show limited specificity and/or sensitivity for breast cancer, even proteins that seem to be the ultimate regulators of cell function and the largest target for therapeutic interventions.

Attempts have been made to identify novel serum-based diagnostic protein biomarkers using proteomic approaches. Using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry and Ciphergen Protein-Chip® arrays on serum samples collected from 103 breast cancer patients and 25 women with benign breast diseases, three biomarkers were identified that could discriminate the two classes with high sensitivity (93%) and specificity (91%) [78]. There was no significant correlation between concentration of the three biomarkers and tumor size. The study included four DCIS samples but it is not clear how well they were predicted. In another study, also using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry, four models were built using seven markers to detect breast cancer using serum samples. In this study, samples from 49 breast cancer patients (stages I–IV), 59 patients with benign breast diseases and 33 healthy women were included. The best model was able to predict breast cancer from benign disease and healthy controls with a sensitivity of 79.6% and a specificity of 77.4% [79].

mRNA

The uniform chemical nature of mRNA makes transcriptome studies less of a challenge than both proteome and metabolome studies. However, the use of gene expression has been debated and a technology measuring small differences in selected transcripts from a large pool of mRNA species can generate a vast amount of data that is very sensitive to minimal differences in sample and data processing. Technical problems, such as lack of reproducibility between experiments and between laboratories, use of different methods for analysis of data, lack of standardized preprocessing protocols methods for data analysis and study design limitations, such as lack of test set validation, and limited sample sizes have all hampered earlier acceptance of gene-expression technology. However, extensive studies have now shown that with careful control in experimental design, microarray technology and RT-PCR are reproducible techniques both between experiments within a laboratory and between laboratories [80,81]. A technology that was once the domain of research has matured such that reproducibility, stability, precision and repeatability are now acceptable for commercial use for clinical purposes.

Use of mRNA from tumor tissue as a useful prognostic or predictive breast cancer marker has been presented in several papers [82–90]. The 70-gene expression profile of Mammaprint™, cleared by the US FDA, is under evaluation by ASCO, while the Clinical Laboratory Improvements Amendments (CLIA)-approved RT-PCR-based 21-gene assay profile of Oncotype DX™ has been recommended by ASCO to be used for prediction of recurrence in patients treated with tamoxifen [11]. A third commercial test, the H/I assay, measures the ratio of the expression of *HOXB6* and *IL17BR* genes using RT-PCR. It is used as a marker for risk of recurrence in node-negative, ER-positive patients [89,90]. MapQuant Dx™ Genomic Grade is a test to accurately measure tumor grade based on the expression of 97 genes [91,92]. However, these are all markers based on gene expression in tissue and are not useful for diagnostic purposes.

Peripheral blood is an ideal surrogate tissue as it is readily obtainable and provides a large biosensor pool in the form of gene transcripts. Peripheral blood has the potential to reflect responses to changes in the immediate and distant environments, in the form of detectable alterations in the levels of selected RNA transcripts. Several studies have examined how gene-expression profiles of blood samples are affected by technical variables such as collection, transportation, storage of blood samples, RNA extraction and choice of microarray platform, and have reported that all these factors besides biological effects can affect the gene-expression profile [93–96]. Again, these challenges can be met by close attention

to study design to remove all bias in study populations, and by implementation of quality control procedures to ensure consistency in technical variables.

It has been shown that approximately 80% of the genes encoded in the human genome are expressed in peripheral blood cells. When compared with other tissues, the expression of over 80% of the genes expressed in blood were also expressed in these tissues [97]. Tissues included in the comparison were brain, colon, heart, kidney, liver, lung, prostate, spleen and stomach, but unfortunately not breast tissue [97]. In the study, it was shown that transcripts considered specific for heart and pancreatic islet β cells were also expressed in blood. The similar response of blood-derived RNA compared with tissue-derived RNA led to the hypothesis that blood cells could act as sentinels of disease [97] and could therefore be useful in a diagnostic setting. This further supports the idea that expression of a selected set of genes in blood has the potential to act as a multicomponent biomarker for diseases such as breast cancer.

Whether a potential breast cancer signature reflects changes in gene expression directly related to a certain immune response or if the changes reflect, for instance, changes in blood cell populations are still to be investigated.

Blood constitutes multiple cell types and the relative proportions of the different types of cells often vary significantly from time to time and from subject to subject. This relative ratio may contribute to a significant proportion of the observed variation in the blood transcriptome. A few studies have used microarrays to analyze blood from healthy volunteers. They found that inter-individual sample variation was associated with donor sex and age, the time of day the sample was taken, the proportion of blood cell subsets [95,98–100] and defined exposure [101–106].

Substantial differences in gene-expression profiles are identified between individuals. This provides a challenge for the identification of disease-related changes among the background of inter-subject variation. Despite this, the potential use of blood-based gene-expression profiling in the diagnosis of cancer, including breast cancer, has been described by several independent groups [107–113]. Sharma *et al.* identified 37 genes that predicted breast cancer patients from healthy females with an accuracy of 82% [114]. The genes were identified by comparing gene-expression patterns in blood samples from 24 women with breast cancer to the patterns of 32 healthy women. Interestingly, three of the normal subjects were pregnant and were predicted as having cancer. This may not be surprising since the mammary gland epithelium of pregnant women undergoes extensive proliferation and neovascularization, processes that are similar to those in growing breast tumors.

Last year, the first commercial diagnostic tests based on gene expression in blood were launched. The ColonSentry™ is a RT-PCR-based assay of seven genes for colorectal cancer screening and the BCtect™ is also a RT-PCR-based assay using 96 genes to detect breast cancer at an early stage of the disease.

miRNA

miRNAs are small (~22 nt) regulatory RNA molecules that function to modulate the activity of specific mRNA targets and play important roles in a wide range of physiologic and pathologic processes [115,116]. In a study the prognostic performance of miRNA profiles has been found to be at least as good as the mRNA profiles in the corresponding cancer tissue [117]. Several miRNAs show aberrant expression profiles in breast cancer, among them mir-125b, mir-145, mir-21 and mir-155 [118]. miRNAs identified whose expression correlated with estrogen and progesterone receptor expression, tumor stage, vascular invasion or proliferation index [118].

It was recently shown that miRNAs are present in plasma in a stable form protected from endogenous RNase activity [119]. Serum levels of a miRNA typically expressed in prostate cancer, miR-141, could distinguish patients with prostate cancer from healthy controls. With a serum level set for 100% specificity a sensitivity of 60% was achieved [119]. Although further studies are required to establish in more detail how specific miR-141 is for prostate cancer and if the results can be reproduced, the results still indicate that diagnostic information can be found within the population of serum miRNAs. Whether miRNAs characteristic for breast tumors can be detected in serum of breast cancer patients will hopefully be explored in the near future.

Expert commentary

Existing tumors and other biomarkers have been based on the quantification of single molecules. Tumor markers, such as the prostate-specific antigens CA 15-13 and CA 27-29, are typical examples of single molecule markers. With the realization that cancers are complex diseases, and that breast cancer may comprise a set of several molecular-defined diseases with some common clinical features but differences in outcome [88], it is becoming more acceptable to consider the use of approaches that simultaneously assay multiple biological markers and their interactions. Gene expression technology is the most amenable for technology, exploratory and clinical use today. In addition, technologies for measuring miRNAs and technologies within proteomics and metabolomics are rapidly developing and may soon be amenable for discovery searches to find novel, clinically useful, diagnostic biomarkers.

Five-year view

It is expected that mammography will continue to be the main screening tool for breast cancer, with diagnostic biomarkers playing a role as adjunctive tests to improve the overall sensitivity and specificity of screening/diagnostics tools available to a patient. Advances in proteomics and perhaps also in metabolomics are likely to help identify proteins and small molecules as diagnostic markers. The assembly of several TAAs may have improved sensitivity compared with single TAA analysis, but the specificity of these markers has been low, suggesting that the clinical utility of these markers is, at present, limited. Since TAA sensitivity for breast cancer detection improves with stage progression, with low sensitivity associated with early stage breast cancer the likely arena is development of prognostic markers.

The discovery of small-regulatory miRNA has generated a lot of interest in many areas of research. The discovery of high stability in sera makes miRNA an interesting molecule for further exploration as a potential diagnostic marker and it is likely that we will know more about its clinical potential within the 5 years.

We expect the greatest developments within diagnostic markers to be with the use of RNA and gene expression. The understanding of the technology, the challenges of handling a labile molecule such as RNA, and the handling of large amounts of data have improved significantly within the last 5 years. The information stored in this biological material in peripheral blood can now be better utilized and the first commercial cancer diagnostic products are now available on the market. It is now time for scientists and clinicians within the field to evaluate these tests.

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Key issues

- Biomarkers to aid mammography with improved sensitivity and specificity in the early detection of breast cancer are needed. This is especially true for younger women and women with high breast density.
- Although great attempts have been made to identify single diagnostic biomarkers, protein related biomarkers and circulating tumor cell-based biomarkers, none have been recommended by American Society of Clinical Oncology for clinical use or received US FDA approval.
- Since breast cancer is a complex disease with many features in common with other cancer types and other diseases, it is likely that multiple markers will be required to adequately differentiate the disease and improve sensitivity.
- The special features with the whole-blood transcriptome make it a potentially very useful source to find diagnostic breast cancer markers.
- The first commercial products based on gene expression in blood for cancer detection give a clear indication of the usefulness of the blood transcriptome.
- To achieve reproducible and reliable results from gene-expression studies, great care has to be used in all steps in the development of diagnostic biomarker based on gene expression.

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•• of considerable interest

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