Clinical Relevance of Circulating Nucleosomes in Cancer

Stefan Holdenrieder,[a] Dorothea Nagel,[a] Andreas Schalhorn,[b] Volker Heinemann,[b] Ralf Wilkowski,[c] Joachim von Pawel,[d] Hannelore Raith,[e] Knut Feldmann,[e] Andreas E. Kremer,[a] Susanne Müller,[a] Sandra Geiger,[a] Gerhard F. Hamann,[f] Dietrich Seidel,[a] and Petra Stieber[a]

[a]Institute of Clinical Chemistry, [b]Internal Medicine III, and

[c]Institute of Radiotherapy and Radiooncology, University Hospital Munich, Munich, Germany

[d]Department of Oncology and

[e]Department of Laboratory Medicine, Asklepios Hospital Gauting, Munich, Germany

[f]Department of Neurology, Dr. Horst-Schmidt-Klinik, Wiesbaden, Germany

Nucleosomes, complexes of DNA and histone proteins, are released during cell death into the blood circulation. Elevated serum and plasma levels have been found in various forms of cancer, but also in autoimmune diseases and acute situations such as stroke, trauma, and during sepsis. Here, the clinical relevance of circulating nucleosomes for diagnosis, staging, prognosis, and therapeutic monitoring of cancer is reviewed. Several studies have shown that levels of nucleosomes are significantly higher in serum and plasma of cancer patients in comparison to healthy controls. However, because of elevations of nucleosome levels in patients with benign diseases relevant for differential diagnosis, they are not suitable for cancer diagnosis. Concerning tumor staging, nucleosome levels correlate with tumor stage and presence of metastases in gastrointestinal cancer, but not in other tumor types. Prognostic value of circulating nucleosomes is found in lung cancer in univariate analyses, but not in multivariate analyses. Circulating nucleosomes are most informative for the monitoring of cytotoxic therapy. Strongly decreasing levels are mainly found in patients with remission of disease, whereas constantly high or increasing values are associated with progressive disease during chemo- and radiotherapy. In addition, therapy outcome is already indicated by the nucleosomal course during the first week of chemo- and radiotherapy in patients with lung, pancreatic, and colorectal cancer as well as in hematologic malignancies. Despite their non-tumor-specificity, kinetics of nucleosomes are valuable markers for the early estimation of therapeutic efficacy and may be helpful to adapting early cancer therapy in the future.

Key words: **nucleosomes; DNA; cancer; apoptosis; serum; plasma; diagnosis; prognosis; therapeutic monitoring**

Address for correspondence: Stefan Holdenrieder, M.D., Institute of Clinical Chemistry, University Hospital of Munich, Ludwig Maximilians University, Marchioninistrasse 15, 81366 Munich, Germany. Voice: +49-89-7095-3231; fax: +49-89-7095-3240. Stefan.Holdenrieder@med.uni-muenchen.de

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Structural and Functional Characteristics of Nucleosomes

Human nuclear chromatin makes up about 99% of DNA and is organized in a multistep manner. In its secondary structure, it is arranged as a chain of nucleosomes consisting of a central core protein formed by an octamer of the double-represented histones H2A-H2B and H3-H4 and 147 base pairs (bp) of double-stranded DNA twisted around this complex.[1–3] The 206-kDa disk-like nucleosomes are connected by so-called linker DNA, which varies in length between 10 and 100 bp. A further histone H1 is located at these linking sites outside of the nucleosomes and stabilizes the chain in its tertiary structure as chromatin fibers.[2,3] The arrangement in multinucleosomal order plays an essential role not only for organization and stablization of DNA, but also for regulation of transcription of genetic information, DNA replication, and repair processes. The access of transcription factors to relevant DNA sequences is mainly coordinated by nucleosomal histones, which can be modified at their tails by adding or cleaving acetyl-, methyl-, phosphor-, ubiquitin-, and ADP-ribose groups.[1,2,4] Histone acetylation facilitates the decondensation of the chromatin, the unfixing of nucleosomal connections between DNA and histones, and promotes the transcription process, whereas deacetylation and condensation suppress it.[1,2,4,5] The interaction between transcription factors and specific promotor regions is further regulated by genetic and epigenetic modifications of the DNA.[6] ATP-dependent chromatin-remodeling factors enable a flexible and dynamic structure of the nucleosomal organization, being necessary for the active involvement of DNA in transcription processes. These factors disrupt the close connection of DNA to histones, transfer a histone octamer to another DNA molecule, or slide the core particle along the DNA.[1,2,5]

Origin of Circulating Nucleosomes

Apoptotic cell death during physiological cell regeneration is supposed to be a major source of nucleosome release.[7,8] Although most of liberated nucleosomes are engulfed and digested by macrophages and neighboring cells, parts of them enter the blood circulation.[8,9] In cases of enhanced cell death—such as degenerative, autoimmune, inflammatory, ischemic, traumatic and toxin-mediated diseases or malignant tumors—those elimination systems can be overloaded or impaired, leading to higher levels of circulating nucleosomes.[7–10] Besides apoptosis, oncosis, or mixed forms between these extreme forms of cell death may account for the demise of damaged cells, depending on the type and intensity of the stimulus as well as on the energy level and type of the affected cells.[10–13] Most of the circulating DNA in plasma and serum is sized as small mono- and oligonucleosomal fragments of about 180 bp and multiples thereof.[14] This supports the theory that apoptosis, which is associated with internucleosomal cleavage of chromatin by activated endonucleases, presents a major mechanism of nucleosome liberation. In contrast, high-molecular-weight DNA fragments are observed after oncotic cell death (e.g., after acute damaging events).[8,12] In addition, the active secretion of nucleosomes by mono- and polymorphonuclear cells in blood is still debated.[7,15]

Metabolism of Circulating Nucleosomes

Whether cell-free DNA circulates in blood as naked DNA, associated with histones as nucleosomes, bound to other plasma proteins, packed in apoptotic bodies or in diverse forms may vary inter- and intraindividually. The typical apoptotic ladder pattern found frequently in gel electrophoresis of plasma and serum samples suggests that the main part of circulating DNA is organized in multimeric complexes as

mono- and oligonucleosomes.[8,14] It could be shown that nucleosomes are removed *in vivo* from circulation in a biphasic, saturable, and concentration-dependent manner with an initial half-life of 4 minutes.[16,17] Various systems are thought to be involved in these degradation and elimination processes such as (1) degradation by circulating endonucleases,[18] (2) immunologic complexing by anti-nucleosome antibodies,[19] (3) phagocytosis and lysosomal degradation by cells of the reticuloendothelial system,[20] (4) metabolization of nucleosomes in the liver,[17] and (5) direct renal elimination in the form of liposomes.[7] The elimination of nucleosomes can furthermore be delayed during acute inflammations as they bind to acute phase proteins.[21]

Pathophysiological Relevance of Circulating Nucleosomes

Up to now, little has been known about the role of nucleosomes in the pathogenesis of diverse diseases. In systemic lupus erythematosus, the antigenic potential of circulating nucleosomes in blood or on the surface of antigen-presenting cells stimulates the early production of anti-nucleosome antibodies. These antibodies form complexes with circulating nucleosomes and aggregate at the glomerular basal membrane in the kidneys, where they promote disease progression.[19] In cancer, circulating nucleosomes are suspected to carry metastatic information, as the injection of DNA to mice led to the generation of new tumor manifestations.[22] Further, nucleosomes liberated from tumor cells stimulate the expression of interleukin-8 in endothelial cells, which, in turn, promote local neoangiogenesis in tumor tissue and allow progression of disease.[23] Finally, nucleosomes are supposed to play an important role in tumor cells' ability to evade immunosurveillance by inhibition of natural killer cell–mediated tumor cell lysis.[24] Most theories about the pathophysiological contribution of circulating nucleosomes to the development and progression of cancer sound promising and may open new diagnostic and therapeutic ways.

Clinical Aspects of Circulating Nucleosomes

Several enzyme-linked immunosorbent assays (ELISAs) have been developed to quantify circulating nucleosomes in serum, plasma, and other body fluids using mainly monoclonal mouse antibodies directed specifically against the DNA and histone component, respectively. Starting 10 years ago, our group modified an existing cellular-based nucleosome assay for its application in plasma and serum.[25] Besides evaluating the assay thoroughly and methodically, an extensive work-up of preanalytics and potentially influencing factors was performed. Careful handling and rapid stabilization of the samples were found to be prerequisites to obtaining reliable results.[25] After following a strict preanalytical protocol, it was shown that shaking, rolling, and storing of serum did not affect levels of circulating nucleosomes, which enabled postal shipment of stabilized samples.[26] In a comparative study with the current "gold standard" for DNA quantification by real-time PCR, a good correlation between both methods was found, particularly in the longitudinal courses of single patients, suggesting that both methods are valuable for the follow-up of patients after cell death–inducing events.[27]

Although most clinical studies focus on the quantification of plasma and serum DNA, some studies have analyzed the relevance of circulating nucleosomes for diagnostic, staging and prognostic purposes in cancer as well as in noncancer diseases. Because of their nonspecific pathophysiological background, nucleosomes were supposed to appear in circulation in various acute and chronic diseases.

Relevance of Circulating Nucleosomes in Noncancerous Diseases

Several acute diseases were reported to produce high levels of circulating nucleosomes in plasma and serum: In patients with acute bacterial infections, nucleosomes were correlated with disease stage and the course of acute-phase protein values.[28,29] In addition, nucleosome levels were elevated in sera of septic patients and demonstrated a strong correlation to the severity of the disease.[30]

Further, high levels of nucleosomes were observed in sera of patients after cerebral stroke, particularly in those with large volumes of stroke lesions. Nucleosome concentrations rose quickly during the first days after ischemia, reaching a maximum after 3–5 days, followed by a slow decrease. This increase was shown to be faster and stronger in patients with severe functional deficits than in those with only slight deficits.[31] Moreover, when nucleosomes, S100 protein, neuron-specific enolase, C-reactive protein, and leukocytes in combination with radiological infarction volume and clinical scores were tested on their prognostic relevance for the one-year recovery period after acute stroke, nucleosomes on day 3 after the event were found to be the only independent prognostic biomarker in addition to the clinical Barthels score.[32] Other studies reported on the prognostic significance of the initial level of circulating DNA for survival and multiorgan failure after stroke.[33] In addition, elevated DNA levels caused by trauma, burns, or graft rejection after transplantation were associated with poor prognosis.[34–36] Severe chest pain, myocardial infarction, exhaustive exercise, and preeclampsia could also provoke elevated DNA and nucleosome levels.[37–40]

Finally, nucleosome levels rose significantly during autoimmune diseases as compared with findings in healthy controls. Because antinucleosome antibodies are produced only in patients with systemic lupus erythematosus, but high nucleosome values were observed in various autoimmune diseases, it was assumed that nucleosomes may undergo specific qualitative processing (e.g., in antigen-presenting cells) to achieve their high antigenicity in this disease.[41]

Relevance of Circulating Nucleosomes for Diagnosis and Staging of Cancer

Several studies detected higher nucleosome levels in individuals with diverse forms of cancer, particularly in those with advanced stages.[28,29,42,43] Similar results were obtained for circulating DNA in cancer patients.[6,44–48] However, most studies used healthy donors as controls, but not patients with organ-specific benign diseases, who are more relevant for differential diagnosis. In a comprehensive study, we investigated nucleosome levels in sera of 418 patients with cancer, including colorectal and various other gastrointestinal kinds of cancer, lung cancer, breast cancer, ovarian and other gynecologic cancers, renal and prostate cancer, and lymphoma, as well as sera of 63 healthy individuals and 109 patients with relevant organ-specific benign diseases.[29] Nucleosome concentrations in sera of healthy donors were found to be generally fairly low. In contrast, pretherapeutic serum levels in various malignancies were significantly higher. However, various benign diseases, particularly infectious diseases, often were also associated with elevated serum levels of nucleosomes, limiting the diagnostic capacity for cancer. While nucleosome levels were able to distinguish significantly between healthy donors and patients with malignant diseases, the difference between benign and malignant diseases did not reach the level of significance (Fig. 1A). Sensitivity for cancer detection was calculated to be 64% at 95%, specificity for healthy donors, whereas it was only 11% at 95% specificity for benign diseases. Only in the subgroup of lung cancer patients was a significant difference calculated between benign and malignant groups, and sensitivities were higher (90% at 95% specificity for healthy donors and

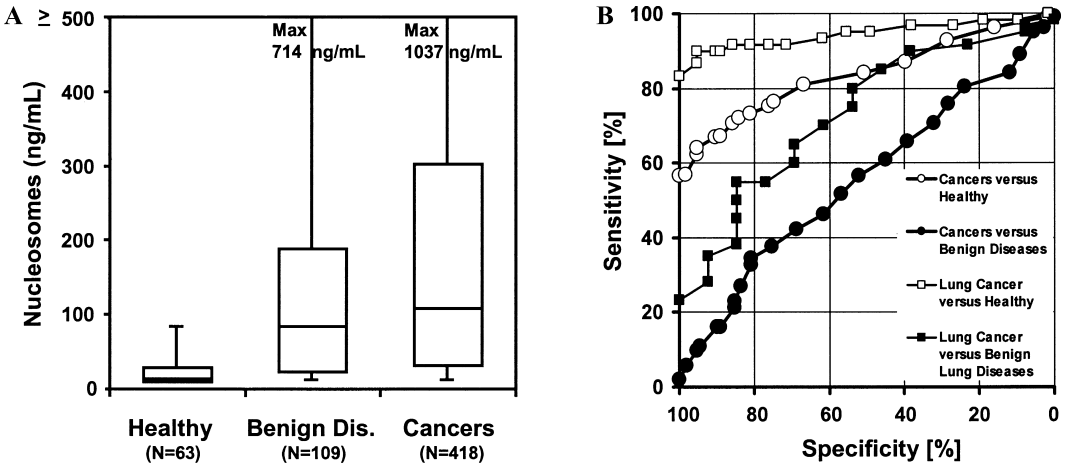

Figure 1. Diagnostic sensitivity of nucleosomes for cancer detection. **(A)** Boxplots for nucleosome values in healthy controls, patients with benign diseases, and patients with various forms of cancer. **(B)** Profiles of sensitivity and specificity for the detection of various kinds of cancer and of lung cancer when compared with healthy or respective benign diseases as control groups.

35% at 95% specificity for those with benign lung diseases; Fig. 1B). Among the various types of cancer, medians, percentiles, and ranges of nucleosome values were comparable, but lung cancer was associated with significantly higher levels and prostate cancer with lower ones.[29,49]

Regarding tumor staging, nucleosome values correlated with tumor stage and the presence of distant metastases in patients with gastrointestinal cancer. In other subtypes only a tendency or no correlation was found at all. In some tumor entities, such as lung and breast cancer, high nucleosome values were already observed in early stages. No association was found between nucleosome levels and lymph node involvement, cell differentiation, age, or gender.[29,49]

Relevance of Circulating Nucleosomes for Prognosis of Cancer

Only few data are currently available about the prognostic relevance of pre-therapeutic values of circulating nucleosomes in cancer patients. Concerning cell-free DNA, some studies reported a prognostic value in patients with lung and breast cancer,[50,51] whereas others could not confirm these findings in the same tumor types.[52,53] These discrepant results may be caused by the low numbers and the heterogeneity of the patients investigated, by differences in statistical analyses, and by incomplete inclusion of other relevant prognostic factors in multivariate calculations. In a large study on 300 patients with advanced lung cancer, pre-therapeutic serum levels of nucleosomes demonstrated considerable prognostic impact when analyzed univariately. However, when other clinical factors and classical laboratory and oncological biomarkers with prognostic relevance were included in multivariate analyses, nucleosomes could not prove to be of independent prognostic value.[54]

Relevance of Circulating Nucleosomes for Monitoring of Cancer Therapy

In patients undergoing systemic cytotoxic therapies such as chemo- and radiotherapy, the changes in the courses of circulating nucleosomes were associated with tumor response to therapy. While strongly decreasing levels were mainly found in patients achieving remission,

constantly high or even increasing values were associated with progression in some solid and systemic tumor diseases.[29,43,49,53] In addition to these more general observations, nucleosome levels increased rapidly after the start of therapy, reaching a maximum between days 2 and 5, followed by a subsequent decrease.[29,49] This initial peak during therapy has been found for nucleosomes and EBV-DNA by other groups, too.[53,55] Various factors may contribute to these typical courses observed in diverse forms of cancer, such as lung, colorectal, pancreatic, and hematologic malignancies, during chemo-, radio-, and immunotherapy,[29,56–59] being either spontaneous and therapy-induced release of nucleosomes or the individual elimination capacity from circulation. Although nucleosomes are cell death products not specifically related to tumor cell death, lung tumor cells have shown to be more susceptible to *in vitro* radiation, as indicated by a faster and higher release of nucleosomes than from physiological bronchioepithelial cells in the same experiments.[60]

As the reduction of tumor cells is achieved by an effective induction of cell death, one would expect the initial increase of nucleosome values to be particularly pronounced in patients responsive to cancer therapy, whereas in nonresponsive patients, only minor changes in nucleosome levels might occur. This hypothesis was confirmed in patients with hematologic diseases. In a pilot study of 25 patients with acute myeloid leukemia undergoing induction chemotherapy, the area under the curve (AUC) of the first four days of therapy was significantly larger in patients achieving complete remission when compared with nonresponsive patients.[57]

While in hematologic diseases tumor cells as therapy targets are present in blood, the preconditions for solid tumors are quite different. In this context, therapeutic efficacy and release of nucleosomes may depend on (1) tissue and tumor perfusion, which regulates the accessability of the tumor for cytotoxic drugs as well as the liberation of cell death products into circulation; (2) the susceptibility or resistance of cancer cells; (3) the general tumor turnover rate; and finally (4) the efficiency of the local immune and nucleosome elimination systems.

Currently, most data about circulating nucleosomes are available on patients with lung cancer receiving cytotoxic therapy. In a prospective study, we investigated kinetics of nucleosomes and lung biomarkers in the sera of 311 patients with advanced non–small cell lung cancer patients treated by first-line chemotherapy (Fig. 2). In this homogeneous group, we observed that nonresponsive patients initially started from higher nucleosome values, had higher maximum values, and a less-complete elimination of nucleosomes from circulation at the end of the first week of therapy than did patients responsive to therapy (Fig. 3).[61,62] The lower levels of nucleosomes in responsive patients can be explained by smaller and less aggressive tumors with lower rates of cellular turnover as well as by more effective elimination of cell death products from circulation. Consistently, it is known from animal experiments that activity levels of serum DNAses, which are essential for the degradation of serum nucleosomes, are decreased in cancer, particularly in those that are nonresponsive to systemic therapies.[63] In consequence, the AUC of the nucleosome values during the first week, as well as the level on day 8 after the first application, already indicated poor therapeutic efficacy. When nucleosomes were combined with cytokeratin 19 fragments (CYFRA 21-1), the most sensitive biomarker for NSCLC, nonresponders were correctly indicated in 29% of patients with progressive disease at a specificity of 100%.[62] This information was particularly valid for patients with initially good clinical performance status (ECOG 1-2) and independent of the type of therapy applied. Thus, the information yielded by using a combination of nucleosomes and CYFRA 21-1 could help to modify the therapy earlier than can be presently done using available imaging techniques in 29% of nonresponding patients and, most importantly, without harming any of the

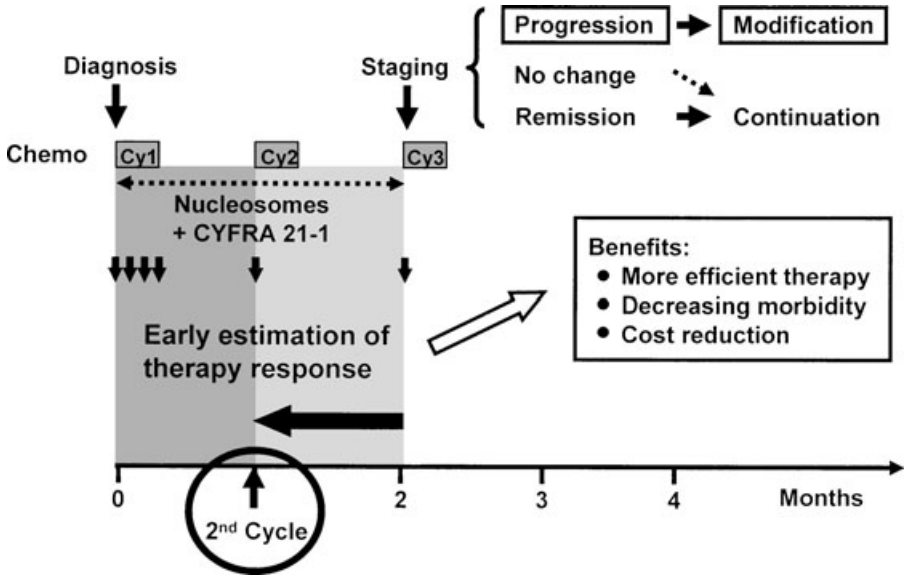

Figure 2. Study setting for the early estimation of therapeutic response in lung cancer. After diagnosis of advanced lung cancer, patients received several cycles of chemotherapy. Currently, tumor response to therapy was evaluated by staging investigations using whole-body computed tomography before start of the third cycle of therapy. In case of progression, treatment strategy was changed, while in all other patients therapy was continued. Here, the biomarkers nucleosomes and CYFRA 21-1 were determined frequently during the first cycle of therapy and, additionally, before the start of the following cycles. If they were able to indicate insufficient response to therapy with high accuracy after the first course, therapy could be then modified right away to enable a more efficient therapy and to reduce side effects and cost.

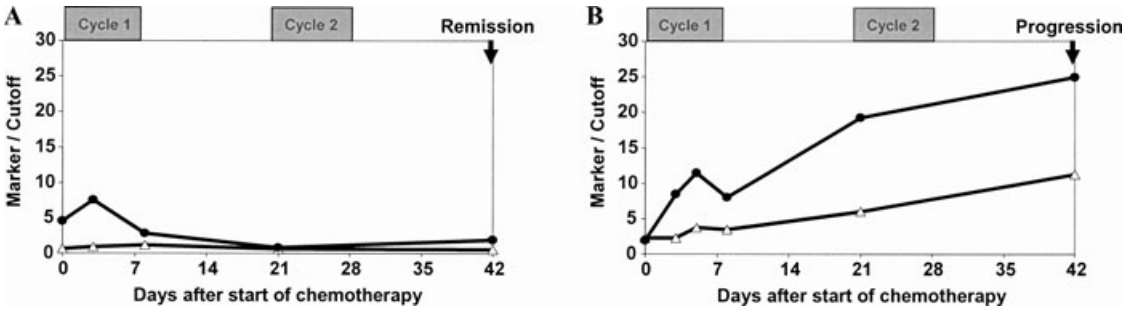

Figure 3. Characteristic courses of nucleosomes and CYFRA 21-1 in lung cancer patients during chemotherapy. Courses show circulating nucleosomes (•) and CYFRA 21-1 (△) in serum of **(A)** a 56-year-old patient with stage IIIB adeno-cell lung cancer responding well to chemotherapy, and **(B)** a 38-year-old patient with stage IV adeno-cell lung cancer with tumor progression during chemotherapy. Marker values were normalized by the 95th percentile of healthy controls, which were 56 ng/mL for nucleosomes and 2.1 ng/mL for CYFRA 21-1.

patients who *were* responsive to treatment.[62] If specificity was lowered to 90%, sensitivity for detection of disease progression rose to 55%.

Comparable results were obtained when this marker model of nucleosomes and CYFRA 21-1 was applied in 161 patients with recurrent non–small cell lung cancer during second-line therapy.[64] Moreover, nucleosomes, progastrin-releasing peptide (ProGRP), and CYFRA 21-1 indicated therapy response after the first course of treatment in 128 patients with small cell lung cancer, too.[65]

Most interestingly, though pre-therapeutic nucleosome concentrations were of limited prognostic value, the baseline values of nucleosomes the before start of the second cycle of chemotherapy had strong and independent prognostic relevance in multivariate settings, including a broad panel of clinical factors and classical laboratory and oncological biomarkers for patients with non–small cell and small cell lung cancer, respectively.[66]

Smaller pilot studies on patients with colorectal and pancreatic cancer undergoing chemo- and radiotherapy similarly demonstrated the capacity of nucleosomes to indicate early tumor response to therapy.[58,59,67]

Conclusion and Perspectives

Although serum levels of nucleosomes in cancer patients are significantly elevated when compared with healthy controls, they are not suitable for cancer diagnosis on account of nonspecific elevations in sera of patients with benign diseases. A prognostic value of pre-therapeutic nucleosome concentrations has been demonstrated in some univariate analyses; whether they will also have independent prognostic value in multivariate models has to be further elucidated. Circulating nucleosomes are most valuable for the monitoring of cancer therapy, particularly for an early estimation of the efficacy of therapy. In combination with CYFRA 21-1, nonresponse to chemotherapy was very early indicated in patients with advanced non–small cell lung cancer as a "proof of principle," and may be a new diagnostic guided step towards an individualized therapy strategy.

On the road ahead, prospective, external, multicenter validation studies will have to be performed, including the proposed marker model, comparing it with other powerful early indicators of therapeutic response such as PET or PET–CT scans.[68] Along with the development of new therapeutic options, the defined use of diagnostics mirroring the biology and metabolism of tumor disease during the first weeks of systemic therapy will be essential to improve management of cancer patients. In the future, those patients could benefit from avoiding unnecessary side effects, and saving time and expense by better and earlier adaptation of individualized treatment (Fig. 2).

Conflicts of Interest

The authors declare no conflicts of interest.

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