CNAPS in Therapy Monitoring

Stefan Holdenrieder

Abstract Monitoring of disease state and of therapy response is highly relevant for efficient patient management. Monitoring tools comprise observation of clinical signs and performing specific examinations such as imaging or blood analyses. This review focusses on the relevance of blood-based biomarker monitoring by circulating nucleic acids for diverse indications that is exemplified on patients who develop or suffer from cancer disease. These indications include (i) screening of patient groups who have a risk to develop a disease, (ii) monitoring response to local or systemic therapies in patients with a defined diagnosis and (iii) early detection of disease recurrence after the primary therapy has ended. Useful biomarkers have to fulfill the highest methodical, pre-analytical and clinical quality criteria and have to be implemented in standardized patient management procedures. The current situation of circulating nucleic acids is summarized on the levels of genetic, epigenetic, transcript, non-coding RNA and nucleosome markers and an outlook is presented as to how these markers can be integrated into a future strategy that enables a personalized management of the patients.

Keywords Circulating DNA/mRNA/microRNA • Cancer • Biomarkers • Quality criteria • Monitoring • Patient stratification • Therapy response • Nucleosomes • Predictive, preventive and personalized medicine

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1 Introduction – The Advantage of the Monitoring Approach

Risk assessment or diagnosis of a disease is often done by one-time investigations if the situation is very clear. This applies to acute pathologies such as trauma, stroke and some infections in which radiology or laboratory examinations clearly indicate the diagnosis. Further typical laboratory constellations or radiological findings suggest a specific risk of disease; for example suspicious blood lipid profiles and calcification of coronary arteries are associated with an elevated risk for myocardial infarction. In addition, a one-time judgment is required if an examination is highly invasive and the material is difficult to obtain such as organ tissue biopsies and either cerebrospinal or amniotic fluids.

In other situations that are not unambiguous, that develop chronically and that have no clear disease signs, the monitoring of the health or disease state of the individual has high importance. Serial observation of clinical signs, laboratory and radiological examinations, is performed for instance on persons with high familiar risk of developing either diabetes or cardiovascular or cancer disease. Regarding biochemical biomarkers, it has to be considered that blood levels sometimes vary greatly among healthy individuals as well as within a specific person over time. Therefore, the interpretation of serial biomarker values that were obtained under standardized conditions is often superior to one-time marker determinations.

This aspect applies also to the situation when a definitive diagnosis has been established and the course of disease during or after a therapy has to be estimated. Then, monitoring serial individual biomarker courses yields much more reliable results for a specific person than a single "snapshot"-examination. The monitoring approach is essential for the management of patients with diverse diseases. Historically it has been introduced into patient care in form of "fever curves" or "blood pressure profiles" and, nowadays, has spread beyond to blood biomarker applications. This review will focus on CNAPS and will show their relevance for monitoring purposes. To make it as practicable as possible the application of cfNAs will be exemplified on patients who develop or suffer from cancer disease. In addition, the clinical usefulness of monitoring CNAPS will also be shown for other non-cancer diseases.

2 Indications for the Use of Circulating Biomarkers

Biomarkers that circulate in the blood can be used to answer many questions that are highly relevant for the management of health and disease in a specific person. In detail they are applied for the following indications (Fig. 1):

- the screening of presumably healthy persons (without any symptoms)
- the monitoring of persons at risk for a specific disease (but without symptoms)

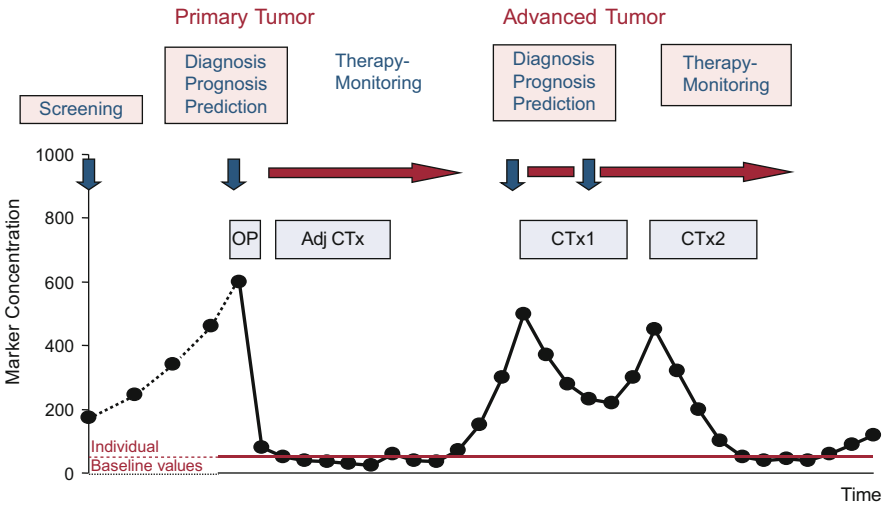

Fig. 1 During the course of cancer disease there are multiple indications for the use of circulating biomarkers: They are applied for (i) cancer detection and differential diagnosis, (ii) estimation of prognosis, (iii) prediction and monitoring of therapy response, (iv) early detection of therapy resistance and of recurrent disease. Biomarker changes in relation to individual baseline values often mirror the course of disease most sensitively. Only a few markers are suited for accurate cancer screening in "not-diseased" individuals

- the risk estimation of a person with suspicious symptoms or disease signs
- the definitive estimation of differential diagnosis in persons with specific symptoms
- the estimation of severity (and staging) of a diagnosed disease
- the estimation of prognosis in patients with a defined diagnosis
- the stratification of patients with a diagnosis for a certain therapy
- the monitoring of the response to therapy in a diseased patient
- the early estimation of therapy response as a special application
- the monitoring of a patient after the primary therapy for
- the early detection of recurrent disease.

The monitoring of serial biomarker concentrations in the blood can be made for all of these indications. At least in cancer patients, it is mostly applied for (i) the screening of patient groups who are at risk of developing a disease, (ii) the monitoring response to local or systemic therapies in patients with a defined diagnosis, (iii) the early detection of disease recurrence after the primary therapy has been finished.

For newly developed targeted cancer therapies, CNAPS are highly meaningful as companion diagnostics to stratify patients for a certain therapy and to monitor the responsiveness of this therapy as well as for the detection of drug resistance and biochemical recurrence in order to enable an early and specific therapy adaptation on an individual basis.

3 Requirements and Quality Criteria of Circulating Biomarkers

In order to give reliable and meaningful results that can be used for patient guidance, circulating biomarkers and the methods that are applied for their determination have to fulfill the highest methodical, preanalytical and clinical quality criteria it they are to be implemented into standardized patient management procedures (see also chapter "Quality Assurance").

3.1 Methodical Aspects

An assay for the detection of biomarkers has to meet many methodical preconditions [1–3]. They include a:

- high analytical sensitivity (the analyte is detected at very low concentrations)
- high analytical specificity (only the analyte is measured)
- high accuracy including a high intra- and between-run imprecision
- high recovery and dilution linearity in the given matrix
- high robustness against potentially disturbing factors.

For CNAPS methods, the sensitivity and specificity depending on primers and probes in the setting is usually high. The accuracy and also the interlaboratory comparability of quantitative measurements are strongly influenced by methods of CNAPS extraction and gene sequence chosen [4] and can be controlled by the inclusion of internal controls that undergo all of the methodical steps of the samples [5–7]. Further, the introduction of internal and external quality control systems of the laboratory are necessary if standardized CNAPS measurements are to be implemented in laboratory routine and standardized patient care (chapter "Quality Assurance").

3.2 Pre-analytical Aspects

Pre-analytical aspects may greatly influence the results of quantitative CNAPS measurements while they are less important for qualitative information on CNAPS. Nevertheless, pre-analytics should be standardized for routine diagnostics as well as for study settings. The following aspects have to be considered (Fig. 2):

- the conditions of the patient and the blood drawing (e.g. time, fasting, position of the patient, tourniquet time, type of needle)
- the conditions of the material (e.g. type of blood matrix i.e. serum or plasma, additives, tubes, volumes)

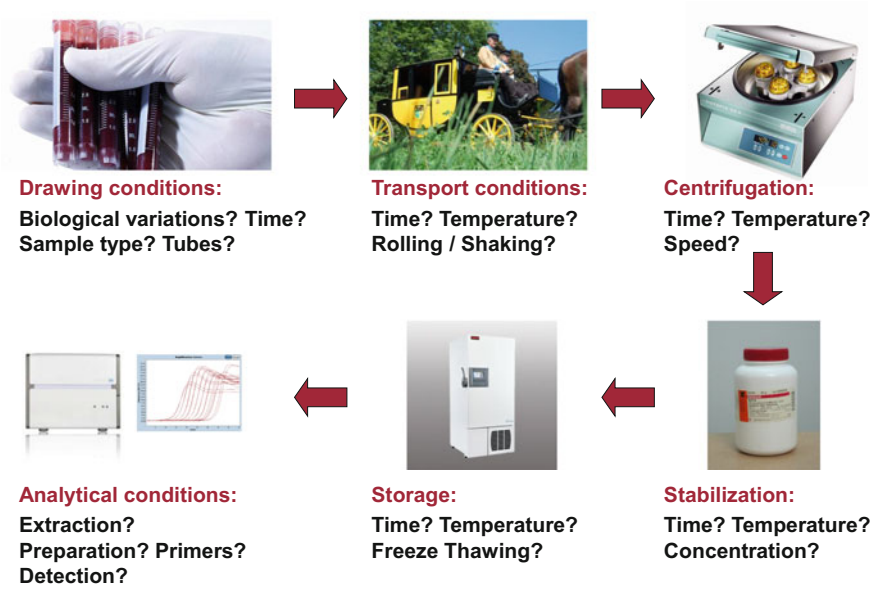

Fig. 2 Preanalytical variations of blood handling may influence the test results. They include the conditions of (i) the individual patient, (ii) the blood drawing procedure, (iii) the material used, (iv) the transport to the laboratory, (v) the handling in the laboratory or biobank facility, (vi) the analytical procedure and (vii) the instrumental setting

- the conditions of the transport to the lab (e.g. time, temperature, pneumatic delivery)
- the conditions of the centrifugation (e.g. time, temperature, speed, braking)
- the conditions of the sample handling (e.g. storage time, temperature, extraction, deep freezing, thawing frequency).

As CNAPS have different stabilities, defined pre-analytically standardized operation procedures (SOPs) including specific blood collection tubes have to be followed particularly for RNA determinations ([8]; chapter "Pre-analytical Requirements for Analyzing Nucleic Acids from Blood"). However also quantitative DNA investigations are influenced by pre-analytic variables. Therefore, pre-analytic SOPs should be particularly considered when monitoring patients by CNAPS [9–13]. cfmiRNA concentrations in plasma and serum are reported to be stable and not to be affected i.e. be freezing conditions facilitating its use for monitoring purposes [5, 7] although some recent studies identified some preanalytic influencing factors as well [14, 15].

3.3 Clinical Aspects

If biomarkers are successfully applied to the diverse clinical indications, some measures are informative as to how accurately they answer the questions posed. For differential diagnosis, the clinical sensitivity and specificity of a biomarker are very meaningful. The sensitivity indicates the percentage of positive results of the marker in the diseased patient group while the specificity is the percentage of negative results in the control group. Because, in the case of cancer disease, the value ranges of cases and controls often overlap, it is not possible to define optimal cutoffs that enable cancer detection with 100 % sensitivity and specificity. This is even more difficult if cancer patients need to be distinguished from the differential diagnostically relevant group of patients with organ-related non-malignant diseases [16].

The diagnostic performance of a biomarker can best be shown by the complete profile of sensitivity and specificity using ROC curves (Fig. 3). This figure gives the sensitivity and specificity at all possible cut-off points and it is highly informative to compare the performance of diverse biomarkers with each other. Meaningful measures are: (i) the area under the curve (AUC); ideally this should be close to 1.0 while it indicates no discriminative potential of the marker if it is close to 0.5 or (ii) the sensitivity at a defined specificity (e.g. 95 %) or (iii) an optimized sensitivity-specificity combination that is reflected in the figure by the point closest to the left upper corner. Most importantly it should be considered as to which groups are compared in this approach. Best curves will result if patients with advanced cancer disease are compared with young healthy individuals. However, it is clinically more relevant to distinguish equally aged persons with suspicious symptoms whether they suffer from an early cancer or a non-malignant pathology. Then the curves often will be less optimistic ([16, 17]; chapter "Extracellular Nucleic Acids and Cancer").

Beyond diagnostic approaches, ROC curves can also be used to illustrate the performance of a biomarker for the staging of disease (e.g. early stage cancer vs. metastatic cancer) or for the staging of therapy response (e.g. remission vs. -non-remission). In the monitoring of a disease, kinetic information (increases or decreases in marker values) also can be used as marker variables.

It has to be pointed out that for screening purposes the positive and negative predictive values (PPV and NPV) are more informative than the sensitivity and specificity. While PPV indicates the probability of disease if the value is positive, NPV gives the probability of being disease-free if the value is negative. Because, at least for cancer diseases, the frequency of cases is very low, PPV may be low even if the sensitivity and specificity are higher than 90 % [17]. Further, predictive values are important if patients should be stratified for specific therapies and responses have to be anticipated.

While prediction always relies on the response of a specific therapy, prognosis is related to the time of either disease-free (DFS) or progression-free (PFS) or overall survival (OS). Clinical and biomarker values can be obtained either before or

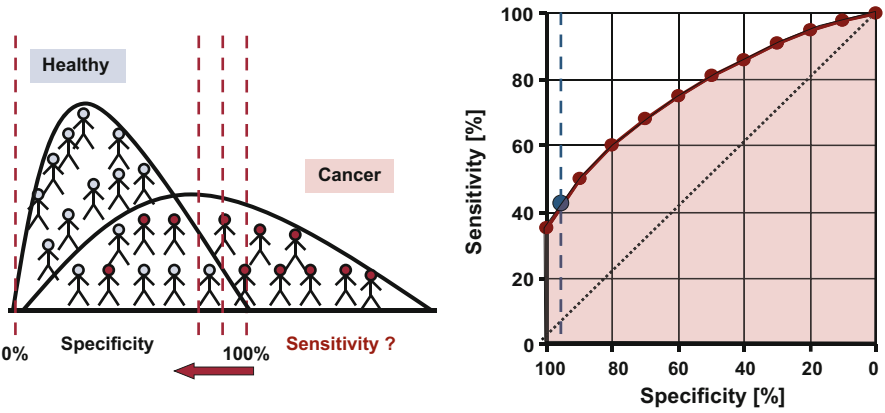

Fig. 3 The value distributions of most cancer biomarkers show an overlap of cancer patients and healthy individuals. The diagnostic performance of a biomarker is illustrated best by ROC curves. To establish this figure, the portion of correctly negative controls (specificity) and correctly positive cancer patients (sensitivity) are identified for all possible cut-off points (decreasing stepwise from 100 % specificity) and transferred to the scheme. The area under the curve (AUC) and the sensitivity at a fixed specificity (e.g. 95 %) are most informative for the comparison of diagnostic markers. As control groups, healthy individuals and patients with differential-diagnostically relevant benign diseases are considered

during a therapy. When monitoring therapy response, biomarker information that is available at the same time as the radiological staging can support the accurate estimation of the individual therapy response. If the information is available prior to the radiological staging, i.e. after one application of chemotherapy, the biomarker determination leads to a time advantage in terms of the early estimation of therapy response that would enable an early and individual adaptation of the therapy strategy (Fig. 4).

When a new cancer biomarker has to be evaluated clinically, a relevant number of patients with the target cancer disease has to be compared with healthy controls and patients with the organ-related benign diseases that are differential diagnostic relevant. To get a whole picture of the usefulness of a biomarker, further cancer diseases and benign diseases that are involved in the marker catabolism, such as renal and hepatic disorders, have to be included as well. For therapy monitoring studies, a meaningful number of patients with a certain cancer that undergo a homogeneous type of therapy with objectifiable, favorable and non-favorable outcomes have to be considered. Recently published guidelines support the professional validation of biomarkers for diagnostic and monitoring purposes [3, 18–20] as well as for the development and incorporation of biomarker studies in early clinical trials [1].

Although these aspects seem to be self-evident, many CNAPS studies show a lack of, at least, some of these points; i.e. either inappropriate controls are chosen or numbers of patients, particularly in monitoring studies, are too small to allow general and robust conclusions.

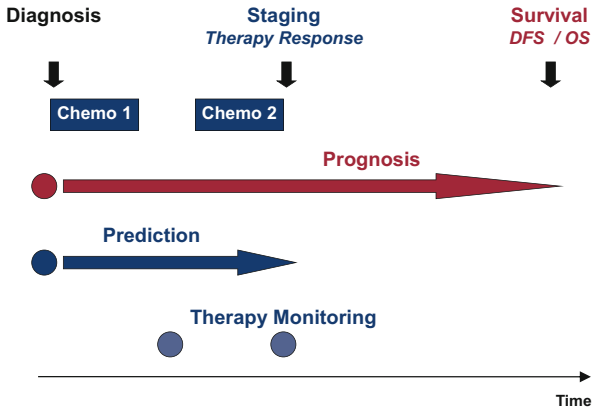

Fig. 4 Biomarkers prior to and during therapy provide prognostic information if they are related to the time of either disease-free (DFS), progression-free (PFS) or overall survival (OS). Predictive biomarkers indicate the response of a specific therapy before the start and are used for therapy stratification, while biomarkers during the course of therapy are suited to (early) estimate the (non-) response to therapy and indicate the potential need for treatment change

3.4 Multimarker Approaches

A new biomarker is only of clinical usefulness if it is superior to an existing biomarker or offers additive diagnostic, predictive or monitoring information. Therefore, new biomarkers should always be compared with those that currently are used in clinical routine [3, 18, 20, 21]. Although only very few single markers show a clear and reproducible advantage in these comparisons, the combination of multiple biomarkers could lead to a significant improvement of sensitivity and specificity. These combinations could result from a bottom-up approach that assembles biologically complementary markers – mostly based on convenient multiplex technologies – or from a top-down approach that extracts meaningful markers out of a plentitude of markers delivered from proteomic or genomic technologies. While the first approach is supported by logistic regression-, supporter vector machine- or neuronal network-models, the latter one often comprises even more complex approaches. In all cases a validation in an independent patient set is paramount to confirm the findings [1, 2, 19].

This is highly relevant for some groups of CNAPS biomarkers such as epigenetic markers and miRNAs. Studies often identify several “promising” marker candidates that may have a superior performance when combined with each other. Further, NGS approaches require sophisticated biostatistics to interpret the pattern of suspicious findings [22, 23].

4 Monitoring Disease State and Therapy Response

The easiest way of looking at circulating biomarkers in the monitoring of the disease state or therapy response is to determine the biomarkers at occasions when clear clinical correlates are present e.g. either after surgical tumor resection and at time of recurrent disease or alternatively before the start of a systemic therapy and at the time of radiological staging assuming that the marker levels ideally are only influenced by disease activity or therapy response. This approach is often sufficient to see whether or not biomarkers show a correlation with disease states in groups of patients with similar preconditions and therapies. However, this is only the starting point for developing rules as to how the markers are implemented in clinical routine and can be used for the individual interpretation of marker changes over time. Therefore, several additional aspects have to be considered:

- the biological variation of a biomarker in individual patients
- the role of influencing factors
- the disease state when the therapy is applied
- the type of therapeutic interventions
- the monitoring schedule for a biomarker and the data interpretation
- the accuracy of biomarker monitoring and its consequences for patient management.

4.1 Role of Biological Variation and Influencing Factors

For several biochemical markers, it is well known that their concentrations in blood depend on age, gender, ethnicity and can vary due to diurnal, mensal, annual or other cycles. Further influencing factors are fasting, hydration, medication, the position at blood drawing, marker-specific factors such as stress, sports and comorbidities or drug-related immune reactions. Although influencing factors cannot be ruled out completely, standardized procedures for blood collection are recommended [1]. As heterogeneity among individuals is considerable for many markers, relative marker changes on an individual basis are preferred to absolute cutoff rules orientated towards diseased patient groups.

4.2 Therapeutic Interventions

Disease states of cancer patients may be very different when a certain therapy is applied: The tumor may (i) be locally confined to the organ, (ii) be in an early, median or advanced stage, (iii) have spread to distant lymph nodes or other sites in the body, (iv) already have recurred after a successful primary treatment or (v) be

continuously progressive. Although all of these states are obviously greatly different, they have as common ground that malignant masses are present that should be reduced by the therapy.

The treatment itself can be focused on the local eradication of the tumor such as by either surgery or external or internal radiotherapy or the local application of cytotoxic therapies, e.g. in liver metastases. Alternatively, systemic approaches are applied if the cancer disease is already in an advanced stage, such as endocrine therapies, cytotoxic chemo- or radiotherapies, biological (targeted) therapies, immune, gene, vaccine or other therapies. Recently, the application of new antibody or tyrosine kinase inhibitor (TKI) drugs that target growth factor receptors or signaling pathways has gained much attention. As they are only applicable if specific pathways are altered in cancer cells, so-called "companion diagnostics" are required to check the mutation status of relevant pathway components [24]. Though all of these therapies are intended to reduce the tumor mass efficiently, they may do this with different velocities in different organs so suggesting the need for a differentiated monitoring procedure for each situation. This applies also to the different types of treatment strategy being neoadjuvant therapy before surgery, as well as primary, recurrent or palliative therapy without surgery.

In addition, there are situations of no direct evidence of cancer disease when the health/disease state of a subject has to be monitored and the (re)occurrence of a tumor has to be detected as sensitively and as early as possible. This applies to the monitoring of (i) individuals at risk of developing cancer disease and (ii) patients after successful tumor eradication. The last group is further sub-classified into patients who receive post-surgery adjuvant chemo- or radiotherapy to control potential micrometastases and patients who are purely followed without receiving any treatment. Although biomarker monitoring has not been widely established for these situations in routine patient management, a sensitive detection of micrometastases could trigger early intervention trials that lead to improved tumor control and better outcomes in recurrent or advanced tumor stages [25–27].

4.3 Biomarker Monitoring Schedule and Data Interpretation

To show the correlation with the disease state, biomarker levels are considered that are available at time points when clinical or radiological staging investigations are made. However, to guide the individual patient management by biomarkers, a prospective scenario of appropriate determination intervals has to be defined that allows the sensitive and accurate estimation of either therapy response or tumor (re)occurrence. These intervals depend very much on the efficiency of the therapy and the expected half-life of the biomarker response. It is recommended that they do not only meet the regular staging time points, but also cover the initial phase of the therapy – e.g. before every new cycle of chemotherapy and sometimes even the first hours or days after the first application of the therapy – to enable a very early estimation of the biochemical response. Then they offer a real time advantage over

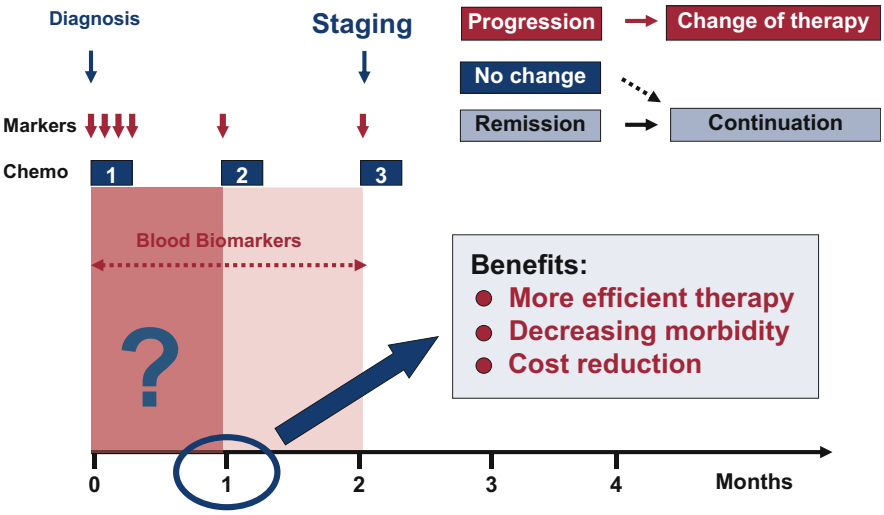

Fig. 5 Early estimation of response to cytotoxic therapies is particularly useful in patients with tumor burden undergoing systemic treatments. As macroscopic changes of tumor volume often are detected by imaging techniques only after weeks or months, the biochemical changes are frequently detected through the course of biomarkers already during the first days or weeks of therapy. Then they enable an early adaptation of the therapeutic plan that is beneficial for the patient (more efficient therapies, less toxic side effects and comorbidities) and the health care system (cost reduction)

the conventional strategy and trigger an early adaptation of the therapeutic plan. This will be beneficial for the patient in terms of more efficient therapies, less toxic side effects and co-morbidities and considerable cost reduction (Fig. 5).

However, this approach is only helpful if clear rules are defined in which relative changes of markers over time in an individual indicate a response or – even more relevant – a non-response to the therapy – with high sensitivity and specificity. Only in the latter case, will the consequence be a change in the therapeutic procedure. Therefore, the biomarker information should be highly specific for non-response because all patients, who are not clearly identified as non-responders by biomarker changes, will be followed by regular staging methods. Most cancer-related marker levels will increase in the case of insufficient response and decrease if the therapy is efficient. Thereby, it should be taken into account that (i) blood levels of some biomarkers may rise immediately after treatment as a reflection of tumor lysis, (ii) marker levels decrease with different velocity, (iii) this decrease depends on various components such as marker release, marker binding to blood constituents and marker elimination dynamics and (iv) some markers may always show a primary decrease but re-increase early in case of disease progression.

Generally, there are three major indications for the early estimation of therapy response:

- monitoring the completeness of surgical tumor eradication and potentially suggesting adjuvant therapies
- monitoring response to systemic therapies (neoadjuvant, primary, palliative) and potentially suggesting alternative or additional therapies
- monitoring resistance to a part of the (targeted) therapies and potentially suggesting an alternative approach.

For patients presenting with no direct evidence of disease who are followed for an early detection of either micrometastases or recurrence of cancer disease, the intervals will depend on the probability of the tumor in the diagnosed stage to reoccur and on the regular follow-up program. Nevertheless, the intervals should be close enough so as not to miss incidental recurrences and to offer a real time advantage as opposed to regular radiological examinations. Only if the biomarker monitoring leads to earlier therapeutic interventions and to a clear benefit for the patients in terms of better overall survival and life quality, will it be implemented in standardized patient guidance programs. Once again, clear rules with defined critical marker dynamics are needed to enable an accurate data interpretation and disease prediction on an individual level.

For example, in a prospective intervention trial, blood levels of cancer biomarkers CEA and CA 15–3 of patients with breast cancer were controlled three times after successful primary treatment to define the individual baseline values. During the further follow-up, the interpretation of the 6-weekly assessed biomarkers relied only on the changes of marker levels from this baseline value irrespective of whether or not they were either within or outside the reference range. Increases of more than 100 %, which were confirmed by an additional measurement, indicated distant metastases with a specificity of nearly 100 % and a sensitivity of 60–70 % – often with a lead-time of many months prior to the occurrence of symptoms [28]. Similar results can be assumed for CNAPS-based markers as well.

5 CNAPS in Cancer Disease Monitoring

While CNAPS has often been investigated for its role in the diagnosis and prognosis estimation in cancer disease (as reviewed by [29, 30]; chapters "CNAPS and General Medicine" and "Extracellular Nucleic Acids and Cancer"), the focus has recently shifted to the direction of the monitoring of therapy response and early detection of recurrent disease as new techniques are available that sensitively detect tumor-specific mutations in circulating cfDNA [26, 27]. In general, CNAPS is comprised of biomarkers at diverse levels:

- Genetic markers (DNA, viral DNA, nucleosomes, DNA integrity, DNA mutations)
- Epigenetic markers (DNA methylation, histone modifications)
- Transcript markers (mRNA)
- Non-coding RNA markers (e.g. miRNA, lncRNA; chapter "The Biology of CNAPS").

5.1 cfDNA

For many cancer diseases it was reported that serum or plasma levels of total cfDNA are elevated as compared with healthy controls and that levels decrease after surgical tumor resection [5, 29]. As is well known for other cancer biomarkers, the pre-therapeutic cfDNA levels and the velocity and completeness of cfDNA decrease after surgery is indicative of the further outcome for the patients [29, 31, 32]. After a potential short term increase immediately after the surgical intervention due to DNA release from operatively damaged normal and tumor tissue, the decline depends on both the biological marker half-life and residual tumor cells. Correlations of decreasing cfDNA levels and successfully performed tumor resections were reported for lung [33], breast [34], colon [31, 35], esophageal [36] and renal cancer [37]. In contrast, incomplete tumor resection or primary systemic tumor dissemination was assumed in patients with persistently increasing cfDNA values [31, 36]. In the further follow-up of patients with esophageal and colon cancer, circulating cfDNA was found to be more sensitive for tumor relapse than the established tumor marker CEA [31, 38].

While in many solid cancers, serum and plasma cfDNA can derive from malignant and non-malignant cells, a special situation is given in virus-associated cancer diseases. For example, human papilloma virus (HPV) DNA in cervical cancer or Epstein-Barr virus (EBV) in nasopharyngeal cancer and Hodgkin's lymphoma correlate with the presence and extent of disease and decrease after surgical removal of the tumor [29, 39–46]. A half-life of 3.8 days was calculated for EBV DNA after radiotherapy and of 139 min after surgery [44, 47]. During systemic therapies, decreasing values were observed in patients with remission during therapy while progressive patients had stable or increasing EBV DNA values [39, 45, 48–50]. Interestingly, a primary increase of EBV DNA was seen during the first days after the first application of therapy [44].

Similarly, this initial peak was reported in an animal model with ovarian cancer xenograft that was treated by chemotherapy. The cfDNA levels clearly correlated with tumor size and decreased strongly after tumor resection or chemotherapy [51–53]. In line with these results, cfDNA levels in human cancers decreased in patients with response to radiotherapy and showed stable or increasing values in progressive patients [54]. The results of this first study were later confirmed in patients with lung cancer undergoing chemotherapy [55–57], in patients with rectal cancer receiving neoadjuvant radiochemotherapy [58], in patients with ovarian cancer treated by chemotherapy [59, 60] and in patients with renal cancer treated by tyrosine kinase inhibitors [61]. However, it has to be mentioned that some minor studies with lower numbers of patients reported no association between cfDNA kinetics and tumor relapse or treatment response [37, 62].

Beyond the general observation that cfDNA yields in plasma and serum correlate with tumor burden and therapy response, there have not been any further

endeavours to define clinically relevant intervals of cfDNA determination, nor any rules that could be useful for the interpretation of cfDNA kinetics in the individual patient. Furthermore, it has to be pointed out that different DNA extraction and quantification methods led to greatly differing absolute cfDNA levels in various studies [4, 5]. Further, blood often was only drawn at times of staging or at the end of therapy, but not during the first week of treatment.

5.2 Nucleosomes

As a significant portion of cfDNA circulates in blood in form of nucleosomes that are complexes of 147 bp of DNA wrapped around a central protein core comprised of the double-represented histones H2A, H2B, H3 and H4 [63–65]. They are the product of DNAses that are particularly active during apoptotic cell death. The chromatin is cut into its basic elements resulting in the typical apoptotic DNA ladder with fragments of nucleosomal size and multiples thereof on agarose gel ([32, 63]; chapter "The Biology of CNAPS").

Similarly to DNA, the levels of circulating nucleosomes that were determined by ELISA technique were found to be elevated in serum and plasma of cancer patients [10, 66–68]. In patients undergoing systemic cytotoxic chemo- and radiotherapy, the changes in nucleosome levels 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 solid and systemic tumor diseases (reviewed in [69]). In addition to these general observations, nucleosome levels increased rapidly after the start of therapy, reached a maximum after 2–4 days (as with EBV DNA) followed by a subsequent decrease. Various factors may contribute to these typical courses observed in patients with lung, colorectal, pancreatic, breast and hematologic cancers who received systemic or local chemo-, radio-, and immunotherapy (reviewed in [69]):

- the spontaneous release of nucleosomes indicating the cellular turnover rate
- the therapy-induced release of nucleosomes
- the individual elimination capacity of nucleosomes from the circulation.

Though nucleosomes are not specifically related to tumor cell death, *in vitro* radiation experiments have revealed that lung cancer cells release more nucleosomes and at a faster rate than physiological bronchioepithelial cells under the same conditions [70]. Interestingly, this initial peak was found to give predictive information in cancer patients undergoing systemic therapies: In a prospective study with more than 300 patients suffering from advanced non–small cell lung cancer (NSCLC) who were treated by first-line chemotherapy, 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 [71, 72]. An explanation can be found in the presence of potentially more aggressive tumors with higher cellular turnover rates and less efficient immunological and DNA elimination capacities in patients with non-sufficient response to therapy. If nucleosome values after this first week were combined with early values of the lung-cancer-related marker CYFRA 21–1, later radiological therapy failure was anticipated in 29 % of progressive patients with 100 % specificity and in 55 % of the patients with 90 % specificity. By use of this approach, highly specific and early detection of insufficient therapy response enabled the early modification of the therapy strategy after only one application of chemotherapy while others would be followed by conventional imaging staging exams [72].

These results were confirmed in a similar set of 42 NSCLC patients with even higher sensitivities for the early estimation of non-response [73]. Data obtained from 161 patients with recurrent NSCLC were also in line with the first study [74]. 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 [75]. Furthermore, the capacity of nucleosomes to indicate early tumor response to therapy was demonstrated for patients with colorectal [76, 77], pancreatic [78, 79] and breast cancer [80] undergoing systemic chemo- and radiotherapy as well as for patients with primary and secondary liver cancer receiving local chemo- and radiotherapies such as transarterial chemoembolization (TACE) and selective internal radiotherapy (SIRT) [81, 82]. According to these approaches, the blood collection before every treatment cycle and during the first therapy week, as well as the consideration of a strictly standardized pre-analytical collection protocol, were very important in obtaining reliable data.

5.3 cfDNA Integrity

cfDNA in blood may result from apoptotic or necrotic or other forms of cell death or it is secreted actively from stimulated cells [83]. Due to more efficient DNAse activity it is assumed that apoptosis produces short, nucleosomal DNA fragments while cfDNA pieces are longer if released passively during necrosis [84]. Therefore, the ratio of long to small cfDNA fragments, also called the DNA integrity index, can be an indicator of necrosis-abundant cancer disease. Indeed, Wang et al. [85] reported a higher cfDNA integrity in cancer patients using 400 bp and 100 bp DNA amplicons. These results were confirmed with different amplicon sizes in breast [86], colorectal [87, 88], esophageal [89], prostate [90], head and neck [91], nasopharyngeal cancer [92], melanoma [93] and acute leukemias [94] while cfDNA integrity was not elevated in other studies on prostate [95], lung [96, 97] and breast cancer [98]. In addition, a few studies also found some prognostic relevance of cfDNA integrity for bladder [99], prostate [100], breast [86] and nasopharyngeal cancer [92].

Concerning monitoring therapy response, a low cfDNA integrity index in patients with nasopharyngeal cancer undergoing radiotherapy that was detected in the follow-up after the treatment was associated with better survival than higher cfDNA integrity values [92]. In breast cancer patients who received preoperative chemotherapy, dynamics of cfDNA fragments (115 and 247 bp) correlated with therapy response but not the resulting DNA integrity index [101]. In summary, the data available on DNA integrity so far are considerably heterogenous, methods are not standardized and preanalytical and analytical factors may influence the results preventing its application as biomarker in clinical routine at the moment.

5.4 Genetic Changes in cfDNA

The most limiting factor for the clinical use of the above-mentioned approaches is the lack of tumor specificity. Looking for tumor-specific markers has led to numerous studies on neoplastic alterations of the cfDNA such as microsatellite instability (MSI), loss of heterozygosity (LOH) or mutations that contribute to the cancerogenesis and can be detected in both tissue and blood of cancer patients (reviewed in [5, 29]).

Microsatellites are short nucleotide sequences that are repeated up to 60 times in tandem and are dispersed throughout the genome. The number of repeated units varies between different alleles defining a heterogenous fingerprint for a gene locus. As a consequence of (cancer-related) defective DNA mismatch repair genes, a complete or partial allelic loss can occur which is detected either as LOH or novel microsatellites appearance which is a sign of MSI. In many studies, different MSI and LOH markers were identified in the tissue and blood of cancer patients (reviewed in [29]). However, the range of detection and of concordance rates between tissue and blood showed great variations that may be attributed to the different quality of the study designs, patient numbers and methods used. As the sensitivity of single markers was only up to 30–40 %, combinations of several LOH and MSI markers were proposed. However, it appeared that the specificity of this approach is limited as positivity was found in non-cancerous conditions, too [5]. Some studies indicate also a correlation with clinical characteristics, prognosis, prediction of therapy response and appearance of tumor relapse as in breast cancer [102–104], gastrointestinal stromal tumors [105, 106], renal cancer [37] and melanoma [107]. However due to the lack of sensitivity, standardization and of large prospective studies, at present, assessment of MSI or LOH is currently not used in clinical routine.

Mutations in oncogenes and tumor suppressor genes are considered as key regulators of cancerogenesis that affect various signaling pathways in different tumor types [108, 109]. *K-Ras, N-Ras* and *TP53* mutations were the first to be detected in plasma and serum of cancer patients, mutations of the *APC, MYC, B-Raf, EGFR* and other genes following later [110–115]. Similarly to MIS and LOH, concordance rates between mutation findings in tumor tissue and plasma

varied greatly between studies and plasma positivity was found only in 10–60 % of tissue-positive patients [5]. The relevance of analytical standardization was obvious as mutations were mainly found in the small cfDNA isolation fraction [116]. In addition, sensitivity of a single mutation was limited to around 40 % while specificity was not found to be 100 % [29]. Although some studies reported associations with clinico-pathological features and prognosis, assessing the mutation status in the blood of cancer patients appeared not to be useful for patient management.

This situation has completely changed during recent years. Major advances in technical development nowadays enable a highly sensitive, qualitative and quantitative detection of mutations in circulating cfDNA in the blood. New methods such as BEAMing (beads, emulsion, amplification, and magnetics) short oligonucleotide mass analysis (SOMA), ddPCR, tagged-amplicon deep sequencing (TAm-Seq) and others have facilitated the diagnostics and improved the detection rates considerably ([117–120]; chapters "Circulating DNA and miRNA Isolation" and "Detection of Genetic Alterations by Nucleic Acid Analysis: Use of PCR and Mass Spectroscopy-Based Methods"). In addition, there is an actual clinical need for assessing the mutation status in cancer patients since it has been shown that some of new targeted antibody or tyrosine kinase inhibitor (TKI) therapies are only efficient in patients who bear or do not bear a specific mutation. Therefore, tumor tissues from patients with lung cancer, colorectal cancer and melanoma are regularly examined molecular biologically to stratify them for appropriate therapies [24, 26].

In NSCLC, the TKIs gefinitib and erlotinib directed against the intracellular part of the epidermal growth factor receptor (EGFR) showed only benefit in patients with an activating mutation (L858R or exon 19 deletion) in the *EGFR* gene, identifying *EGFR* tissue mutation analysis as stratification tool for TKI treatment of NSCLC patients [121–123]. However, although better than chemotherapy, TKI response rates in preselected patients were only around 70 % in first-line and 50 % in second-line treatment [124, 125]. Recent whole genome analyses permit the explanation that there is considerable genetic heterogeneity including spatial (either within a tumor or between primary tumor, lymph node and distant metastases) and temporal variability [126]. But also, resistance to TKI by further mutations such as *EGFR* (T790M) that prevents erlotinib binding or downstream mutations such as *K-Ras, PIK3CA, ALK* and *BRAF* may occur [26, 124]. Identification of these mutations enables the use of alternative targeted drugs such as crizotinib in case of the presence of an ALK-EML fusion gene ([24, 127]; Fig. 6) While primary resistance can be assessed in tissue by pre-therapeutic multigene analysis, secondary resistance is more difficult to identify. Even if a rebiopsy is possible, heterogeneity of mutations in diverse lesions may prevent accurate classification leading to mutation monitoring in blood as an attractive diagnostic tool.

Similarly to lung cancer, patients suffering from malignant melanoma will only benefit from inhibitor of the serine-threonine protein kinase *B-RAF* therapy (vemurafenib or dabrafenib) in the presence of an activating *B-RAF* mutation (V600E) [128, 129]. As resistance to vemurafenib will develop by activation of the MAP kinase pathway, MEK-inhibitors (trametinib) show some efficacy in these cases [130].

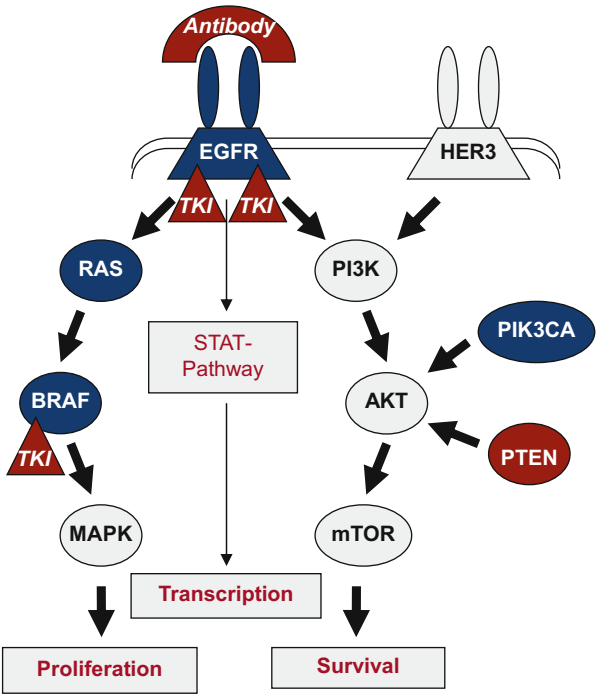

Fig. 6 The *epidermal growth factor receptor (EGFR)* pathway includes the EGFR receptor and diverse components that intracellularly transmit a growth signal to promote processes like transcription, proliferation and cell survival. Mutated parts of this pathway lead to dysregulation and may be treated by "targeted therapies" in terms of antibodies that are directed against the extracellular part of the EGFR receptor or by small molecules such as tyrosine kinase inhibitors (TKI) that are directed against intracellular components of the pathway

Finally, patients with colorectal cancer are unlikely to benefit from anti-EGFR antibody therapies (cetuximab or panitumumab) if mutations of the *K-Ras* gene are present. While pre-therapeutic mutation status assessment in tumor tissue has been established as "companion diagnostics" in routine patient management [24, 131–133], the monitoring of the therapy response and early identification of resistance development and tumor relapse detection is a new field for CNAPS diagnostics.

As source of DNA for the analysis of therapeutic targets and drug resistance-conferring genes mutations, circulating tumor cells (CTC) and circulating tumor cfDNA that are released from primary or metastatic tumor sites into the blood stream were suggested. The so-called "liquid biopsy" could outrun the limits of genetic heterogeneity as circulating cfDNA reflects a mixture of all cancerous DNA changes in the body. Because this concept is only minimally invasive it can be applied for the serial monitoring of successfully treated and newly occurring resistant cell clones at an individual level [25–27, 121].

CTCs are particularly present in the blood of patients with metastatic cancer disease. For example, CTCs were detected in 27 patients with metastatic lung cancer and expected *EGFR* mutations were identified in 11 out of 12 patients. If TKI resistance mutation T790M was detected in CTCs, progression-free survival was considerably shorter. In a follow-up, the number of CTCs correlated with the radiographic tumor response, *EGFR* T790M mutations being found in progressive patients [134]. Further CTCs were detected in 21 of 37 patients with metastatic

colorectal cancer. By use of massive parallel sequencing (MPS), striking mutations in driver genes *APC*, *K-RAS* and *PIK3CA* were observed in primary tumors, metastases and CTCs in a few index patients. Some private mutations only detected in CTCs were also confirmed in either primary tumors or metastases at subclonal levels after ultra-deep sequencing was performed supporting the highly valuable diagnostic information provided by CTC sequencing [135]. However, it has to be pointed out that these promising results were obtained in a small group of metastatic cancer patients with high CTC counts and CTCs were not detectable in a considerable portion of patients. In addition, low CTC counts could limit the interpretation of discrepancies between tissue and CTC results [136].

These limitations can possibly be overcome by circulating cfDNA diagnostics. Current techniques enable the reliable monitoring of tumor-associated mutations in circulating plasma cfDNA at frequencies as low as 0.01 % [26, 31]. Recent studies showed a concordance of *PIK3CA* and *EGFR* mutations in primary tumor tissue and plasma cfDNA between 92 and 100 % [137–139]. Diehl et al. [31] demonstrated the high accuracy of *APC, TP53* and *K-RAS* monitoring on plasma cfDNA of colorectal cancer patients by BEAMing for control of therapy efficacy and detection of tumor recurrence. This technology allows the quantification of tumor-related mutations in blood as it parallelly assesses the absolute concentration and the mutation rate of circulating cfDNA. After surgical tumor resection, tumor-related cfDNA levels decreased rapidly with a half-life of 114 min to less than 1 % of the initial value within 24 h (while total cfDNA levels could increase due to traumatic damages). Persistently high mutation values after surgery indicated residual disease. cfDNA showed more pronounced dynamics and had a higher predictive value for tumor recurrence than the conventional tumor marker CEA. If patients were monitored after successful surgery, measurable cfDNA levels after 1–2 months accurately identified patients with later tumor recurrence [31]. Similarly, promising results were obtained with metastatic breast cancer patients in whom tumor-related mutations *PIK3CA* and *TP53* were successfully found in plasma cfDNA in 97 % (29 out of 30) while CTCs and CA 15–3 were positive only in 87 % and 78 %, respectively. Circulating tumor cfDNA levels correlated better with tumor burden and indicated tumor recurrence more accurately (89 %) than either CTC (37 %) or CA 15–3 (50 %). Thereby cfDNA provided the earliest measure of treatment response in 53 % of the progressive patients with an average lead time of 5 months to recurrence detection [140].

TAm-Seq is a highly sensitive and specific method (>97 %) for entire gene sequencing as demonstrated by the screening of nearly 6000 bases for low-frequency mutations with allele frequencies as low as 2 % in plasma cfDNA of advanced ovarian cancer patients. This approach is also useful for the detection of new or unknown mutations [119]. A more simple, inexpensive and robust array-based method enabled the efficient monitoring of multiple *EGFR* mutations in plasma cfDNA during TKI therapy of NSCLC patients. However, only 56 % of patients with response had non-detectable values and only 44 % with progression had an *EGFR* increase while other resistance mechanisms were not covered by this technique [141]. By use of other approaches, *EGFR* mutations frequency on plasma

DNA was reported to decrease during chemotherapy and to correlate with response to therapy [142]. Furthermore, resistance mutation (T790M) of the *EGFR* gene was detected in plasma of 53 % and 28 % of patients with acquired resistance to TKI therapy, respectively [143, 144]. In addition, circulating *B-RAF* DNA mutations were shown to correlate with response to biochemotherapy in melanoma patients [145].

Sensitive detection of newly acquired mutations that cause resistance to anti-EGFR therapy in colorectal cancer were reported recently. Misale et al. [146] detected newly appearing *K-RAS* mutations in 6 out of 10 patients with resistance to cetuximab or panitumumab. *K-RAS* mutations occurred up to 4 months before CEA increase and up to 9 months before radiographic documentation of tumor progression. While cells were resistant to EGFR-inhibition, they remained susceptible to combined EGFR- and MEK-inhibition so opening a window of opportunity for early and individualized treatment adaptation [146]. Diaz et al. [147] found *K-RAS* mutations in 9 out of 24 patients whose tumors were initially *K-RAS* wild type and who were treated with panitumumab monotherapy. Mutations generally occurred 5–6 months after the start of therapy and were parallelled by CEA increase and treatment resistance. By mathematical modeling, the authors showed that the mutations were present in expanded subclones already before commencing the panitumumab treatment [147]. Murtaza et al. [148] monitored the acquired genomic changes of cfDNA in serial plasma of six patients with advanced cancers by massively parallel exome sequencing and identified resistance-conferring and activating mutations such as *EGFR* (T790M), *PIK3CA* and *RB1* that appeared after cytotoxic therapies were performed. Leary et al. [149] reported on a highly sensitive method with MPS for the identification of translocations in circulating cfDNA of patients with solid tumors. By so-called personalized analysis of rearranged ends (PARE) they found an average of nine rearranged sequences in four colorectal and two breast cancers. Interestingly, copy number variations on circulating cfDNA specific for the primary tumor were detected in the blood of breast cancer patients up to 12 years after diagnosis, despite no other evidence of disease, indicating dormancy of breast cancer cells [150]. Although these findings seem to be highly attractive for personalized medicine, they have to be validated in larger patient cohorts.

The current status of knowledge about circulating cfDNA as a basis of "liquid biopsy" for assessment of prognosis, recurrence detection, prediction of therapy response and acquired resistance in cancer patients is summarized by Crowley et al. [26].

5.5 *Epigenetic Changes on Cell-Free DNA*

Epigenetic markers comprise reversible changes on the chromatin that regulate transcription processes. DNA methylation, histone modifications and nucleosome remodeling processes play an important role and their patterns at specific chromatin sites are altered in diverse pathologies [151, 152]. In cancer disease, it is known that CG-rich islands in promotors of tumor suppressor genes often are hypermethylated

leading to silencing of these genes while most parts of the chromatin are hypomethylated resulting in a higher DNA instability. Modifications of histones often consist of adding specific methyl-, acetyl-, phospho-, ubiquitin-, and other groups to basic amino acids at the tails protruding from the nucleosome, that can open or close the chromatin structure and regulate the access of transcription factors (reviewed in [152–154).

As blood-based cancer biomarkers, several specific methylation markers such as *APC, DAPK, GSTP1, MGMT, p16, RASSF1A, RARß2* and *Septin 9*, have been exploited particularly for diagnostic and prognostic purposes (reviewed in [29, 151, 153]). While generally a high concordance of tissue and blood methylation markers was detected, only a portion of the studies reported on an association with clinicopathological features. The strongly varying rates of hypermethylated markers in blood points to shortcomings in (pre)-analytics and study designs [5]. Nevertheless, assessment of *Septin 9* promotor hypermethylation has reached some practical relevance for the detection of colorectal cancer [155, 156]. Furthermore, hypermethylation of the O(6)-methylguanin-DNA methyltransferase (*MGMT*) promotor in glioma tissue is considered as an indicator for the response of the tumor to alkylating antineoplastic agents [157]. In addition, plasma *SHOX2* hypermethylation was suggested as a diagnostic parameter for NSCLC [158].

Only a few studies focus on the relevance of methylation markers in monitoring the disease state or therapy efficacy. *RARß2* and *RASSF1A* were two to three times hypermethylated on plasma DNA and cell-surface-bound circulating DNA of patients with lung cancer as compared with controls and decreased significantly after neoadjuvant chemotherapy and total tumor resection. *RARß2* methylation increased again in cases of cancer relapse [159]. In breast cancer patients undergoing neoadjuvant chemotherapy, levels of *RASSF1A* hypermethylation became undetectable in serum in patients with complete remission while *RASSF1A* methylation persisted longer or throughout the treatment if partial or minimal pathological response was achieved [160]. Recently, courses of plasma *SHOX2* hypermethylation were found to correlate strongly with the response of patients with NSCLC to cytotoxic chemotherapy [161]. In a genome-scale screen, *IFFO1* methylation was identified as a meaningful marker for ovarian cancer that showed, in a subsequent validation study, similar post-resection kinetics to CA 125 [162]. Further small follow-up studies identified concordances of serum methylation markers and disease status for *RASSF1A* and ovarian cancer [163], for *ESR1* and *14-3-3*-sigma and breast cancer [164], and for *p16INK4a* and colorectal cancer [165]. Presurgery *RUNX3* methylation was predictive for the relapse of colorectal cancer [166] and serum *RASSF1A* methylation status one year after breast cancer surgery indicated the long-term outcome [167]. To establish serum or plasma methylation markers for the monitoring of cancer patients in a clinical laboratory, further endeavours are necessary to both standardize pre-analytics and analytics and define clear interpretation rules.

The same applies to blood-based histone modification markers. Though there are some studies on altered histone markers on circulating nucleosomes that detected lower levels of H3K9me9 and H4K20me3 in plasma and serum of patients with

colorectal cancer [168, 169], these modifications were found to be mainly associated with repetitive sequences on circulating nucleosomes [170]. Further trials are necessary to reveal the possible clinical usefulness of this new biomarker class.

5.6 Gene Expression Markers

Transcript markers outrun the mere genetic disposition and indicate the functionality of gene expression leading to disturbed metabolism in cancer cells. Therefore, they are highly relevant in cellular experimental approaches as well as in tissue diagnostics. Due to their lacking stability in blood, they require specific pre-analytical procedures that limit their practical use as blood-based markers to some extent.

Nevertheless, there are several studies reporting the over-expression of some genes that was partially associated with clinico-pathological features (reviewed by Fleischhacker and Schmidt [29]) such as tyrosinase mRNA in melanoma [171], mammaglobin and CK 19 mRNA in breast cancer [172], CEA and CK 19 as well as ß-catenin in colorectal cancer [173, 174], S100A4 in gastrointestinal cancers [175] and MUC-18, tyrosinase and MAGE-3 in melanoma [176]. Recently, a PBMC-based gene expression signature assay was developed and validated for the detection of NSCLC that achieved high sensitivities and specificities [177]. Furthermore, BEAMing and ddPCR analysis were applied for mutant IDH1 mRNA detection in glioma patient serum and cerebrospinal fluid extracellular vesicles [178].

Concerning therapy monitoring and prediction of treatment response, only rare data are available. In small patient cohorts it was reported that ß-catenin mRNA decreased after successful surgery of colorectal cancer [174], PSA mRNA correlated with hormonal therapy albeit that there was no correlation with PSA protein kinetics [179], and thyroglobulin mRNA kinetics may be additive to thyroglobulin protein assessment for detection of thyroid cancer relapse [180]. While the application of gene expression arrays in tissues are already established in clinical routine, such as for the stratification of patients with breast cancer for adjuvant chemotherapy [181], there is no clear clinical relevance of blood-based gene expression markers for the management of cancer patients up to now.

5.7 Non-coding RNA Markers

Beyond mRNAs that code for specific proteins there are more than a thousand non-coding RNAs that influence and post-transcriptionally regulate gene expression. This marker group comprises short, mid-size and long non-coding RNAs ([182]; chapter “The Biology of CNAPS”). Particular interest was given to the 19–24 bp small miRNAs that are involved in many regulatory functions during cancerogenesis and are remarkably stable in serum and plasma, suggesting them to

be promising cancer biomarkers ([183–186]; chapter "Extracellular Nucleic Acids and Cancer"). Similarly to other CNAPS, miRNA can be released non-specifically from lysed or necrotic cells, but also can be actively secreted packaged in exosomes or as free miRNA bound to RNA-binding carrier proteins, such as HDL and Argonaut, and then play a major role in intercellular communication [184]. While some groups have investigated miRNA in serum or plasma, others have looked for exosomal miRNA as they assumed an enrichment of cancerous miRNA therein [187–189]. In addition miRNA can also be extracted from circulating tumor cells [190].

In cancer diseases, many of them are up- or down-regulated resulting in a miRNA-pattern that can be used for diagnosis and prognosis estimation. Alternatively, most meaningful single miRNA markers, or clusters of them, can be chosen as a diagnostic tool [183, 184, 191]. Clinically relevant miRNAs are among others the let-7 and miRNA 34 families that are down-regulated in many cancers, miRNA 21, 155 and 221 that are up-regulated in many cancers and are involved in the regulation of tumor growth and invasiveness [182, 191]. Some miRNAs are up-regulated only in some cancers e.g. miR-372 and miR-373 in testicular cancer. Interestingly, the cellular and extracellular pattern of miRNAs is not identical [184]. Therefore, the levels of some circulating miRNA markers do not necessarily reflect the intracellular situation.

In the serum and plasma of cancer patients, several miRNAs were found to distinguish between cancer patients and controls, partly correlating with clinicopathological characteristics and prognosis (reviewed in [192]). For single miRNAs, specific assays have been developed such as for miRNA 21 in breast cancer [193]. However, plenty of studies also show either only a minor or no diagnostic usefulness for miRNAs e.g. in urological cancers [194–196].

Concerning monitoring disease and response to therapy, some minor studies report positive results regarding miRNA clusters 371–373 and 302 for following patients with germ cell tumors [197], miRNA 92a for non-Hodgkin's lymphoma patients [198] and a miRNA pattern in head and neck patients during radiochemotherapy [199]. In melanoma patients, a miRNA pattern was identified that predicted the recurrence-free survival and showed tumor-related dynamics in serum [200]. A recent study found small nuclear U2-1 RNA fragment (RNU2-1f) to be increased in sera of ovarian cancer patients, to correlate with the residual tumor burden after surgery and to be predictive for response to post-operative chemotherapy. Furthermore, persistently high RNU2-1f values during therapy identified a subgroup of patients with poor prognosis [201]. In addition, circulating lncRNAs have been suggested as cancer biomarkers such as in gastric cancer [202].

Obviously, non-coding RNA markers will have to be more developed, to undergo a pre-analytical and analytical standardization process and to be included into large therapeutic trials.

6 Integration Strategies

As recommended by guidelines for the use of cancer biomarkers [19, 20] and for the development and incorporation of biomarker studies in early clinical trials [1], new biomarkers have to undergo a rigorous analytical, pre-analytical and clinical validation process that finally shows their usefulness for patient management in large clinical trials and in comparison with already established markers. For CNAPS, and many other biomarkers, this process is still ongoing and more robust data are needed before they can be implemented into clinical routine laboratory diagnostics.

However, there are three approaches that seem to be particularly relevant for CNAPS and their application in disease and therapy monitoring:

- As cancerogenesis affects changes of the genetic, epigenetic, gene expression and protein levels it seems necessary to integrate all of these marker classes to see which changes are most meaningful for the clinical questions the patient and doctor face at a certain time point.
- Liquid biopsy is only one component in the diagnostic process. Diverse approaches including clinical, tissue, blood and radiographic investigations have to be integrated into a comprehensive strategy leading to the best patient management possible.
- The assessment of single markers will hardly be appropriate to answer different questions at different time points for different individuals suffering from molecularly different diseases having different preconditions (and metabolic constitutions) for a defined therapy. Marker multiplexing will be the future standard and interpretation of the resulting data will be the challenge for the individual decision-making.

6.1 Encode Project

The Encyclopedia of DNA Elements (ENCODE) project has started in 2007 with a large number of sequence-based studies to systematically map functional regions across the human genome. The elements mapped include chromatin structure (by - DNase-seq, FAIRE-seq, histone ChIP-seq and MNase-seq), DNA methylation sites (by RRBS assay), transcription-factor-binding sites (by ChIP-seq and DNase-seq), protein-coding regions (by mass spectrometry) and RNA transcribed regions (by - RNA-seq, CAGE, RNA-PET and manual annotation). Studies using 24 experimental types were performed on 180 cell lines and tumor xenografts and are still ongoing. Beyond simple mapping, ENCODE aims at investigating the interactions of the various levels and understanding the biochemical functions of the genome [22, 203–208]. Up to now, the data give insight into the function of 80 % of the genome that mainly covers the gene regulatory elements outside of the well-studied protein-coding regions. For clinical application, the new knowledge concerning the statistical correspondence of the recently discovered elements with sequence

variants linked to human disease is highly relevant. Thus, the project can – beyond its aim to provide new insights into the organization and regulation of our genome – help to integrate various marker classes into biochemical functional entities and so provide an enormous resource for future diagnostic approaches [204, 206].

6.2 Combination of Tissue and Liquid Biopsy

Monitoring health, disease and therapy response has been the mainstay of clinical and radiographic diagnostics for a long time. In the recent decade, molecular characterization of tumor tissue and cells after biopsy or surgery has successfully been implemented in the patient management process to better stratify them for targeted antibody and TKI therapies. While in some cancers the absence of a particular mutation (*K-Ras* in colorectal cancer) is the precondition for the application of an anti-EGFR antibody treatment, in other cancers the presence of a mutation (*EGFR* in lung cancer, *V600E* in melanoma) is the basis for an efficient TKI therapy [24, 26]. However, this is only a rough estimate leaving a series of open clinical questions:

- Due to the genomic heterogeneity within a tumor and between primary and secondary lesions of a tumor [126], molecular tissue analysis enables only a snapshot at a given time of a given tumor specimen and may miss relevant information of dormant or already resistant tumor cells. Liquid biopsy could provide a more comprehensive picture on the overall mutation status mirrored by circulating cfDNA in the blood deriving from different tumor sites in the body [26, 27].
- In case of an appropriate mutation state that indicates antibody or TKI therapy, only a portion of patients will respond to it (e.g. 60 % of *K-RAS* negative colorectal cancer patients to cetuximab therapy [133]). As non-responsive patients cannot be identified by pre-therapeutic tissue biopsy it would be necessary to monitor the therapy efficacy by serial assessments of biochemical markers or mutation status e.g. in circulating cfDNA. This would be helpful for the early detection of progressive or recurrent disease and enable an early modification of the treatment.
- Development of resistance to antibody or TKI treatment during or after the therapy is a frequent problem that cannot be predicted by tissue biopsy but could be addressed by monitoring the relevant mutations on circulating cfDNA. As it is known that a combination, e.g. with MEK or ALK inhibitors, is effective also in resistant cells, patient management could be considerably improved by serial liquid biopsies [24, 26].
- In many patients with either recurrent or metastatic disease or multimorbidity, invasive tissue biopsy is not supposed to be performed or does not lead to meaningful information. Either individual kinetics of mutation changes or single-time assessment of mutational status in circulating cfDNA can overcome these limitations and lead to better stratification of the patients for appropriate therapies.

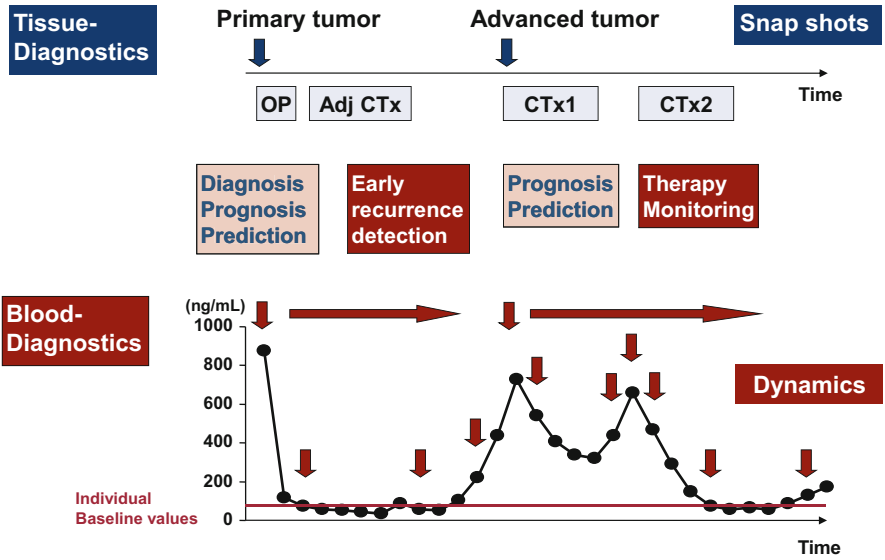

Fig. 7 The combination of pre-therapeutic tissue biopsy and serial liquid biopsies during and after therapy may improve the guidance of cancer patients considerably. The mutation status in tissue is currently required to stratify patients for certain targeted therapies although it allows only a spatially and temporally restricted "snapshot", and genetic heterogeneity, not detectable dormant and resistant cell clones and adverse patient conditions limit this approach. Liquid biopsy, however, indicates the overall mutation status in the body mirrored by cfDNA in the blood, and can be applied serially due to its non-invasive nature. It provides essential information on the dynamics of tumor biology that can be used at various time points during the course of the disease for (i) therapy stratification, (ii) assessing prognosis, (iii) monitoring therapy response, (iv) early detection of disease progression, (v) recurrence detection and (vi) identification of acquired resistances (see text)

For best patient management, the combination of pre-therapeutic tissue biopsy and serial liquid biopsies is recommended (Fig. 7). This could ideally support the clinical and radiographic estimation of disease status and help to improve (i) the therapy stratification, (ii) the assessment of prognosis, (iii) the monitoring of therapy response, (iv) the early detection of disease progression, (v) of recurrence detection and (vi) the identification of acquired resistances that leads to a more accurate individual patient guidance and be the future mainstay of personalized medicine in cancer disease.

6.3 Marker Multiplexing

New technologies such as multiplex PCRs, MPS of amplicons, exomes or the whole genome, array techniques, mass spectrometry, BEAMing and others (see ENCODE projects) enable the parallel assessment of multiple genomic, epigenomic,

transcriptomic and protein markers. Beyond the integration of many markers and biomarker classes, multiplexing will be necessary to integrate many relevant clinical questions in order to obtain a holistic view on the patient including (i) the disease characteristics, (ii) the co-morbidities, (iii) the disponibility to respond to specific drugs, (iv) the capacity and velocity of drug metabolization, (v) the disponibility to toxic reactions, (vi) the reactivity status of the immune system, (vii) the necessity of accompanying drugs, (viii) the interaction of diverse drugs, (ix) the development of resistances and (x) the probability of sustained drug response and patient outcome.

To answer these questions, the future challenge will be to bring all relevant biomarkers classes to a single platform to facilitate a quick, robust, quality-controlled and reliable determination of the markers, to integrate the resulting data in appropriate algorithms, to extract the meaningful interpretation and enabling accurate decisions for the patient management.

While technical developments should lead to continuously decreasing costs, these innovations will have to be seen as a part of monitoring and optimization of highly expensive new treatment strategies. Offering the patients more efficient therapies that will lead to better outcomes, reducing toxic side effects and complications and avoiding unnecessary risks and costs by non-effective therapies, additional invasive biopsies and radiographic examinations will compensate the higher costs of laboratory assessment by far. To document these positive effects, medico-economic evaluations that investigate the overall benefit for the patients and the health care system as a whole should accompany future therapeutic trials using companion diagnostics in the form of tissue and liquid biopsies.

7 Requirements for Individualized Diagnostics and Interventional Approaches in Cancer Disease

Essential aspects of biomarker validation and incorporation into clinical trials have been documented by several guidelines [1, 3, 19, 20]. These principally apply to the more complex and multimarker approaches and include the comprehensive evaluation of analytical preconditions, pre-analytically influencing factors and the establishment of standardized operating procedures (SOPs) to guarantee a reliable and highly quality controlled application of the methods. For monitoring purposes, the role of e.g. individual biological variation and drug-interactions also have to be considered.

Monitoring of disease by circulating biomarkers is only useful if it supports the decision making of relevant clinical questions and if it potentially leads to beneficial consequences for the patient e.g. in escalating or deescalating therapy intensities. In order to be applied in a meaningful way, biomarkers should not only correlate with the disease state and tumor burden at time points of regular staging investigations, but should contain either additional information (such as the

mutation status of cfDNA) or provide a time advantage i.e. by early indication of either therapy response or tumor progression or relapse prior to radiographic or clinical methods, and thus enable an early modification of the treatment strategy. Therefore, the biomarkers require the highest levels of sensitivity and specificity as well as positive and/or negative predictive values.

To achieve an accurate as possible prediction for the single patient, individual changes of marker levels over time may be superior in most clinical questions as compared with absolute cutoff levels orientated at patient groups with similar disease conditions. Interpretation criteria for those individual, longitudinal observations have to be defined in large-scale clinical studies in homogenous patient groups with comparable therapies or disease states (such as the follow-up situation after primary therapy) and particularly with appropriate determination intervals of the biomarkers that depend very much on the half-life or doubling time of the biomarkers. These intervals will also have to be implemented in later routine monitoring plans of regular patients. In many therapeutic situations it will be advantageous to monitor biomarkers very closely during the initial phase of the therapy, i.e. not only prior to every new cycle of chemotherapy but already during the first hours or days after the first application of the therapy, in order to enable a very early estimation of the biochemical response.

In patients with no evidence of disease who are followed for the early detection of micro-metastases or tumor recurrence, the intervals may be adjusted to the probability of a relapse for a specific tumor and the regular follow-up program. In any case, the intervals should be close enough in order not to miss incidental recurrences. The following major monitoring indications are relevant to trigger interventional approaches in cancer patients:

- monitoring the incompleteness of surgical tumor eradication may lead to additional adjuvant therapies
- early estimation of the non-response to systemic therapies (neoadjuvant, primary, palliative) may suggest therapy termination or modification
- monitoring resistance to antibody or TKI therapies may lead to modification or combination of therapies
- early detection of tumor relapse offers the possibility of earlier intervention.

The successful implementation of individualized intervention strategies into standardized patient guidance programs depends very much on a clear benefit for the patients in terms of better overall survival and quality of life, which has to be proven in large prospective intervention trials.

8 Other Areas of Disease Monitoring by CNAPS

Besides the application in monitoring disease and therapy response in cancer patients, CNAPS has shown utility in the estimation of diagnosis, prognosis, therapy monitoring and clinical follow-up of many other disease areas. Regarding

the monitoring approach, CNAPS have been applied in diverse situations (reviewed in [29, 32]; chapter "CNAPS and General Medicine") including monitoring:

- after acute events such as myocardial or cerebral stroke, burns or major trauma ([209–217]; chapter "CNAPS and General Medicine")
- the early detection of bacterial sepsis and the monitoring sepsis treatment [218–220]
- viral infections in the acute and chronic stage [221, 222]
- the monitoring of autoimmune diseases such as systemic lupus erythematosus [223–226]
- metabolic disease such as diabetes mellitus (reviewed in [227]; chapter "Circulating Nucleic Acids and Diabetes Mellitus")
- graft-versus-host disease after transplantation [228]
- the early detection of preeclampsia [229, 230]
- the detection of cffDNA in maternal plasma during pregnancy ([231, 232]; chapter "Fetal CNAPS – DNA/RNA")
- physical exhaustive exercises [233, 234].

For most applications – with the exception of infectious diseases and cffDNA in maternal plasma – CNAPS diagnostics are still in a preclinical stage. New highly sensitive technologies will presumably accelerate the implementation of CNAPS into routine diagnostics and pave the way for further clinical applications (chapters "Genomic Approaches to the Analysis of Cell Free Nucleic Acids", "CNAPS and General Medicine", "Fetal CNAPS – DNA/RNA", "Circulating Nucleic Acids and Diabetes Mellitus" and "Extracellular Nucleic Acids and Cancer").

Conflict of Interest Statement The author declares to have no conflict of interest.

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