Advances in Predictive, Preventive and Personalised Medicine
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Circulating Nucleic Acids in Early Diagnosis, Prognosis and Treatment Monitoring

An Introduction

EPMA European Association for Predictive Preventive & Personalised Medicine

Springer

Circulating Nucleic Acids in Early Diagnosis, Prognosis and Treatment Monitoring

Advances in Predictive, Preventive and Personalised Medicine

Volume 5

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Peter B. Gahan
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An Introduction

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TO
MAURICE STROUN
who overcame scientific and political resistance to lay the foundations leading to the current studies on circulating nucleic acids.

What This Book Series Is About…

Current Healthcare: What Is Behind the Issue?

For many acute and chronic disorders, the current healthcare outcomes are considered as being inadequate: global figures cry for preventive measures and personalised treatments. In fact, severe chronic pathologies such as cardiovascular disorders, diabetes and cancer are treated after onset of the disease, frequently at near end-stages. Pessimistic prognosis considers pandemic scenario for type 2 diabetes mellitus, neurodegenerative disorders and some types of cancer over the next 10–20 years followed by the economic disaster of healthcare systems in a global scale.

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Current Book Series, published by Springer in collaboration with EPMA, overview multidisciplinary aspects of advanced bio-medical approaches and innovative technologies. Integration of individual professional groups into the overall concept of PPPM is a particular advantage of this book series. Expert recommendations focus on the cost-effective management tailored to the person in health and disease. Innovative strategies are considered for standardisation of healthcare services. New guidelines are proposed for medical ethics, treatment of rare diseases, innovative approaches to early and predictive diagnostics, patient stratification and targeted prevention in healthy individuals, persons at risk, individual patient groups, subpopulations, institutions, healthcare economy and marketing.

Prof. Dr. Olga Golubnitschaja

Book Series Editor

Dr. Golubnitschaja, Department of Radiology, Medical Faculty of the University in Bonn, Germany, has studied journalism, biotechnology and medicine and has been awarded fellowships for biomedical research in Paediatrics and Neurosciences (Medical Centres in Austria, Russia, UK, Germany, the Netherlands, and Switzerland). She is well-cited in the research fields of "gene hunting" and "subtractive hybridisation" applied to predictive prenatal and postnatal diagnostics published as *O. Labudova* in years 1990–2000. Dr. Golubnitschaja is an expert in molecular diagnostics actively publishing in the fields of ophthalmic diseases, neurodegenerative pathologies, cancer, cardiovascular disease, Diabetes mellitus, hyperhomocysteinemia, etc. She is the *cofounder* of the theory of individual patient profiles, author of fundamental works in *systems medicine* (holistic approach considering molecular patterns at epi/genomic, transcriptional and post/translational levels). Dr. Golubnitschaja holds appointments, at the rank of Professor, at several European Universities and in International Programmes for Personalised Medicine, and is author of more than 300 international publications in the field. Her awards include: National and International Fellowship of the Alexander von Humboldt-Foundation, Highest Prize in Medicine and Eiselsberg-Prize in Austria. She is *Secretary-General* of the "European Association for Predictive, Preventive and Personalised Medicine" (EPMA in Brussels, www.epmanet.eu), Editor-in-Chief of *The EPMA-Journal* (BioMed Central, London); Book Editor of *Predictive Diagnostics and Personalized Treatment: Dream or Reality*, Nova Science Publishers, New York 2009; Book Co-editor *Personalisierte Medizin*, Health Academy, Dresden 2010; Book Series Editor of *Advances in Predictive, Preventive and Personalised Medicine*, Springer 2012; *European Representative* in the EDR-Network at the NIH/NCI, http://edrn.nci.nih.gov/; *Advisor and Evaluator* of projects dedicated to personalised medicine at the EU-Commission in Brussels, NIH/NCI, Washington, DC, USA, and Foundations and National Ministries of Health in several countries worldwide.

Preface

Although the first indication of the presence of DNA in blood occurred some 65 years ago, it was not until the early 1970s that there was a return to researching the DNA present in blood led by the pioneering studies of Maurice Stroun and Philippe Anker, in which they demonstrated the release of DNA in a controlled manner from living but not dead cells. They further showed that DNA found in the blood could be of tumor cell origin.

During the interim period, studies on the uptake and movement of DNA in cells and whole organisms gave rise to the idea that DNA could circulate within organisms—both plant and animal—and that a fraction of the DNA could be acting as a messenger. These new studies showed increases in blood DNA levels in cancer and trauma patients and were followed by measurements of increased DNA blood levels in patients suffering from sepsis, stroke, and acute myocardial infarction by the early 2000s. Clearly, the increased amounts of DNA found in cancer patients could not be used to identify the type of cancer present, and current studies are ongoing to identify suitable early markers for cancer-specific forms based on assays for individual sequences of cell-free DNA, mRNA and microRNAs with some successful early markers already available ranging from individual markers to panels of markers.

A major development involves the use of minimally invasive methods for identifying fetal cell-free DNA in the maternal blood, so leading to first-trimester identification of fetal sex and Rh status. The former has been incorporated in routine clinical practice in a number of countries as well as by direct-to-consumer testing. The development of techniques, including digital PCR and massively parallel sequencing, has allowed the detection of allelic imbalances and the precise quantification of sequences in the maternal plasma. In turn, this has enabled the deduction of maternally inherited fetal monogenic diseases as well as the accurate detection of fetal chromosomal aneuploidies such as Down syndrome in the first trimester. In addition, the determination of the fetal genome *in utero* through the sequencing of the fetal cell-free DNA in maternal blood has been achieved. Moreover, the sequencing of fetal cell-free RNAs found in amniotic fluid has opened up the possibility of identifying markers for fetal development and hence

potential developmental problems. This offers the possibility of initiating treatment either *in utero* or immediately after birth.

Thus, the study of circulating nucleic acids in plasma and serum (CNAPS) has yielded the first concrete steps as an additional arm to the other "liquid biopsy" methods already involved in predictive, preventive and personalized medicine (PPPM). More recently, the research has been extended to include studies on cell-free DNA and RNAs in other body fluids including saliva, urine, amniotic fluid, cerebrospinal fluid, bronchial lavages/aspirates, breast milk, colostrum, tears, seminal fluid and stools.

The study of circulating nucleic acids (CNA) is already playing an important role in PPPM, including the exploitation of early nucleic acid markers for (i) monitoring serial blood biomarker concentrations to screen patient groups at risk of developing a disease, (ii) estimating the severity (and staging) of a diagnosed disease, (iii) the stratification of patients with a diagnosis for a particular therapy, (iv) monitoring the response to local or systemic therapies and (v) the early detection of disease recurrence following completion of primary therapy.

As with other approaches, CNA has a crucial role to play in the integrative approach of PPPM, which is acknowledged as a priority by the WHO, UN General Assembly, and the European Union, among others. The European Association for Predictive, Preventive and Personalised Medicine (EPMA) (http://www.epmanet.eu) is at the forefront of PPPM-related initiatives and has provided an excellent scientific research platform through *The EPMA Journal* (BioMed Central, London). The EPMA organization of the *World Congress on PPPM* in Bonn, Germany on September 15-18, 2011 hosted participants from 44 countries worldwide, an event leading to the EPMA J publication of the ***General Report and Recommendations in PPPM 2012: White Paper of EPMA***. The subsequent release of the EPMA Book Series *Advances in PPPM* published by *Springer* has yielded a range of PPPM-related volumes. The current volume, *Circulating nucleic acids in early diagnosis, prognosis, and treatment monitoring: an introduction,* concerns the preparation of cell-free nucleic acids from peripheral blood and other body fluids, the analytical methods employed, and the application of these methods in PPPM. The book presents the current situation and is intended primarily for all researchers who would want to enter the field, be they PhD students, postdoctoral workers, current researchers, or clinicians. My special thanks go to the chapter authors for their contributions and the publisher for support during the preparation of this book.

London, UK Peter B. Gahan

Abbreviations

AF	Amniotic fluid
cfDNA	cell-free DNA
cffDNA	cell-free fetal DNA
cffRNA	cell-free fetal RNA
cfmitDNA	cell-free mitochondrial DNA
cfRNA	cell-free RNA
CGH	Comparative genomic hybridisation
CRC	Colorectal cancer
CTC	Circulating tumour cells
ddPCR	droplet digital PCR
dPCR	digital PCR
GE	Genomic equivalents
HGT	Horizontal gene transfer
lncRNA	long noncoding RNA
LOH	Loss of heterozygosity
miRNA	microRNA
MPS	Massively parallel sequencing
NGS	Next generation sequencing
NIPD	Non-invasive prenatal diagnosis
NIPT	Non-invasive prenatal testing
NOD	Non-obese diabetic
PCR	Polymerase chain reaction
QF-PCR	Quantitative fluorescence PCR
qPCR	quantitative PCR (real time PCR)
ROC	curves Receiver-operating characteristic (ROC) curves

RT-qPCR	Reverse transcription quantitative PCR
sDNA	stool DNA
SNP	Single nucleotide polymorphism
WGA	Whole genome amplification

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About the Editor

Peter B. Gahan is Emeritus Professor of Cell Biology at King's College, London, UK and a Fellow of the Society of Biology. Currently, he is Honorary Research Fellow in the Department of Anatomy and Human Sciences and has been interested in the uptake and movement of nucleic acids around organisms since the 1960s. Current research concerns the possible roles of extracellular cell-free nucleic acids as intercellular messengers and, in particular, the structure and function of virtosomes. In addition, he has performed seminal studies in a number of fields: (a) the first demonstration of the presence of phospholipids in chromatin, (b) quantitative cytochemical analyses of lysosomal activities in development and pathology, and (c) the evolution of hepatic euploidization in mammals. He has authored/edited 7 books and has over 250 scientific publications.

Part I
Background

A Brief History and the Present and Future Status of CNAPS

Peter B. Gahan

Abstract 1948 marked the identification of DNA in peripheral blood by Mendel and Métais and the beginning of modern cell biology after the 2nd world war. During the period that followed, little was heard of the peripheral blood DNA, but there was research activity concerning the movement of DNA about plants that led to parallel studies on animal systems. This resulted in the establishment of the capability of DNA to leave and enter cells as well as to circulate about plants and animals. Subsequently, together with improved technology and analytical genetics, both DNA and RNA markers have been identified that may act as early indicators of a pathological state and also have a use in the monitoring of treatment. This chapter offers some historical background to CNAPS and indicates both the current state of the art as well as the directions in which the research is developing. Attention is drawn to the ethical and legal problems arising through the development of the genetic information and the testing systems. The topics discussed are expanded in the subsequent chapters.

Keywords Circulating DNA/RNA • Brief history • Cancer • Fetal nucleic acids • Costs • Ethics • Predictive, preventive and personalized medicine

1 Introduction

Although the presence of nucleic acids in blood from healthy donors, pregnant women and clinical patients was first made by Mendel and Métais [1], this study was largely forgotten until the paper of Koeffler et al. [2] involving raised DNA levels in the blood of lupus erythematosus and rheumatoid arthritis patients. The apparent "long silence" of the period 1948–1975 was due, in part, to the fact that the

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data of Mendel and Métais were questioned because of uncertainties in the analytical methods available at the time (reviewed in [3]). Only 4 years earlier Avery et al. [4] identified DNA as genetic material and 1948 saw the start of the challenge to the idea that DNA was animal nucleic acid and RNA was plant nucleic acid. Although the presence of DNA in the cytoplasm was identified for the first time in 1949, evidence for the structure of DNA [5, 6] and the confirmation that DNA comprised the gene [7] was still awaited. 1948 was also the beginning of modern cell biology with the developments of various forms of light and electron microscopy, quantitative DNA measuring methods, isolated and in situ, as well as other biochemical and cytochemical methods for nucleic acids, proteins and lipids.

Nevertheless, the long period from 1948 to 1977 was not really one of silence but one in which a series of experiments, initially on plants and, subsequently, animal systems would ignite the studies on peripheral blood nucleic acid levels leading to a consideration of their biology and use in diagnosis, prognosis and treatment monitoring and, hence, in Predictive, Preventive & Personalized Medicine.

2 The Occurrence of Circulating DNA

Although Koeffler et al. [2] demonstrated raised DNA levels in the serum of patients with a variety of diseases, but especially in lupus erythematosus (SLE) and rheumatoid arthritis patients, these results were challenged by Steinman [8]. He claimed a lack of DNA in serum and only 2 μg ml^{-1} in plasma using 2 ml of normal blood samples though accepting that DNA may be present in serum in an unusual form that escaped detection by his methods. However, Stroun et al. [3] suggested that if only 20–30 μg DNA could be founding 200 ml of plasma [9], then Steinman would be unlikely to find only in 1 ml.

A separate study by Davis and Davis [10] using counter-immunoelectrophoresis for DNA detection, which was about 10 times more sensitive than simple immunodiffusion used by Koeffler et al. [2], showed that DNA levels from normal individuals could be as low as 1.5 μg ml^{-1} for plasma and 0.2 μg ml^{-1} for serum. Using nick-translation on purified plasma DNA, normal individuals showed 266 ± 57 ng ml^{-1} whilst two untreated SLE patients showed 4,024 and 2,437 ng ml^{-1}, respectively.

Thus, although the apparent "long silence" of 1948–1975 was due, in part, to the fact that the data of Mendel and Métais was too early in the development of the understanding of DNA, it also has to be remembered that the methods for DNA and RNA analysis were comparatively primitive when compared to the technology available today.

Nevertheless, the "silent period" was not so silent!

3 The "Silent" Period 1948–1977

The unusual beginning leading to the concept of CNAPS started with the experiments of Stroun and colleagues [11, 12]. They repeated the experiments of Glouchtchenko [13], who was based in the USSR, in which the transmission of hereditary characteristics was demonstrated through the graft between two varieties of plants – a mentor plant and a pupil plant.

Stroun et al. [11, 12] used grafting experiments with the egg-plants *Solanum nigrum* and two varieties of *Solanum melgena* e.g. *S. melongena* and *S. nigrum* that involved either the stock or the scion being deprived of all growing leaves and so subjected to the influence of the metabolism of the leaf-bearing section. They showed that the products of the pupil sometimes demonstrated genetically modified characteristics similar to those of the mentor that were very different from those seen by the sexual crossing of the two varieties. Thus, (a) some characteristics of the mentor plant were seen in the pupil plant whilst others were different to those of the mentor plant; (b) not all of the modified pupil plants acquired the same characteristics of the mentor plant, some demonstrating only one characteristic, others several characteristics whilst still others, all of the characteristics of the mentor; (c) during segregation, which occurred as early as the F1 generation, some recessive parents produced offspring with dominant features and (d) occasional linked characteristics in the mentor plant appeared individually in the pupil plant and its offspring. Similar results were obtained through grafting between S. *melongena* and *S. nigrum*. The data were interpreted as being due to the passage of DNA from the mentor to the pupil [11, 14].

During the same time period, Yagishita [15, 16] performed similar experiments using *Capsicum baccatum* and *Capsicum annuum* obtaining similar results including the non-Mendelian segregation of the new features in the progeny of the grafts. Hirata [17–19] also worked on *S. melongena* with similar results to those of Stroun [11] and Stroun et al. [12] coming to similar conclusions to these authors in that there was a movement of genetic material between the stock and the scion.

Graft-induced genetic variation was also demonstrated through the transfer of male sterility from male sterile petunia stocks to normal fertile petunia scions [20, 21]. Non-Mendelian inheritance was also reported for grafts of *Capsicum annuum* by Kasahara and co-workers (see Ohta and Choung [22]; reviewed by [23]).

Thus, these preliminary experiments led the authors to indicate a possible transfer of DNA via the graft to express itself in a subsequent generation. There was no mention of RNA transfer although this possibility could not be excluded [24].

Such plant experiments were accompanied by similar early work on animals by a number of researchers [24]. For example, Stroun et al. [25, 26] demonstrated that when blood from the gray guinea fowl was repeatedly injected into birds of the White Leghorn variety, the progeny so produced had some gray or black-flecked feathers in the second and later generations. During this time period and earlier,

many such experiments were also performed in the USSR (reviewed [23]) with similar outcomes.

These and other experiments led to the development of two approaches based upon the concept that DNA was moving in the sap between e.g. mentor and pupil plants as well as in the blood between parabiosed animals. Experiments were run to test if (a) DNA could be taken up by cells and tissues without being degraded and what changes did it cause to be made in the recipient cells/tissues, and more importantly, (b) cells can release DNA into their environment.

DNA uptake was demonstrated in both plants and animals. The introduction of ^{3}H-DNA isolated from thymine-deficient *Escherichia coli* by i.v. injection into mice showed the presence of the radio-active DNA in ovarian tissues and in particular, in the oocyte nuclei. This was confirmed by both CsCl centrifugation and autoradiography [27]. In the case of plants, uptake of similar *E. coli* ^{3}H-DNA into nuclei, mitochondria and plastids of all tissues was demonstrated by uptake into cut shoots of *Solanum esculentum*. Again, the *E. coli* ^{3}H-DNA presence was identified by both CsCl centrifugation and autoradiography [28–31].

The release of DNA from cells was even more important in the context of CNAPS. The early indications of DNA localisation, structure and mobility are given in Table 1 whilst possible mechanisms by which DNA, and RNA, could be released and hence act as sources of CNAPS are given in Table 2.

A fuller discussion of DNA/RNA release from cells and uptake by cells is given in chapter "The Biology of CNAPS" (Sects. 3 and 4).

Circulating DNA also came back into the literature through studies employing DNA isolated from serum (though plasma was shown to contain similar DNA levels) of systemic lupus erythematosus patients and using both the diphenylamine reaction and gel-electrophoresis against DNA antibody [42]. Albeit that the techniques used were less sensitive than those employed today, this DNA was compared with that from normal individuals as well as with calf thymus, salmon sperm and *E. coli* DNAs.

What is clear from the "silent" period is that the groundwork was being laid for the subsequent studies permitting the establishment of the presence of DNA/RNA in the peripheral blood and leading to the development of their possible uses in diagnosis, prognosis and monitoring of treatment.

4 Applications in the Early Studies of CNAPS

The cfDNA studies were subsequently kick-started by two approaches in particular. The first was by Leon et al. [39] using a radioimmunoassay for ng quantities of cfDNA, the levels of which were determined for serum samples from 173 patients with various types of cancer and 55 healthy individuals. cfDNA concentration in the normal controls had a range of 0–100 ng ml^{-1} with a mean value of 13 ± 3 ng ml^{-1}. However, 93 % of controls were found to be in a range of 0–50 ng ml^{-1} that was chosen for comparison. The cancer patients' cfDNA concentrations ranged from 0 to μg levels (mean 180 ± 38 ng ml^{-1}). Fifty percent of the

Table 1 Some developments in understanding DNA mobility and its cellular roles

Date	Authors	Discovery
1948	Mandel and Metais [1]	Circulating cfNAs in blood
1949	Chayen and Norris [32]	Cytoplasmic DNA localisation
1959	Gartler [33]	DNA uptake by mammalian cells
1962	Gahan et al. [34]	DNA mobility
1962	Stroun [11]	DNA mobility
1962	Pelc [35]	Metabolic DNA
1965	Gahan and Chayen [36]	Messenger DNA
1969	Stroun et al. [37]	Released bacterial DNA transcription in plants
1971	Stroun [38]	Bacterial DNA-dependent RNA polymerase released from bacteria into plants
1972	Stroun and Anker [14]	Released cfNAs with associated polymerases – a general phenomenon
1977	Stroun et al. [3]	Circulating nucleic acids
1977	Leon et al. [39]	Raised blood DNA levels in cancer
1989	Stroun et al. [40]	Cancer derived blood DNA
1997	Lo et al. [41]	Fetal DNA in maternal blood

Table 2 Possible mechanisms for the release of DNA and RNA from both animal and plant cells and tissues

Mechanism	Plant	Animal
Leucocyte breakdown	–	+
Bacteria and viruses	+	+
Cell-surface DNA	–	+
Necrosis	+	+
Apoptosis	+	+
Exosomes	+	+
Virtosomes	+	+

patients' values were found in the range of 0–50 ng ml^{-1}; the other 50 % were between 50 and 5,000 ng ml^{-1}. However, significantly higher cfDNA levels were found in the serum of patients with metastatic disease (mean 209 ± 39 ng ml^{-1}) when compared with non-metastatic patients (mean 100 ± 30 ng ml^{-1}, $p < 0.02$).

Interestingly, the levels decreased in 90 % of the patients after radiation therapy for lymphoma, lung, ovary, uterus and cervical tumours while for glioma, breast, colon and rectal tumours, the DNA levels decreased only in 16–33 % of the patients. Thus, a decreased serum cfDNA concentration correlated with an improved clinical condition. When treatment failed, cfDNA levels either increased or remained unchanged (see chapter "CNAPS in Therapy Monitoring" re monitoring of patients). Although the data derived from this study was somewhat mixed, it was one of the first studies to indicate (a) increased levels of serum cfDNA in cancer patients, (b) a decrease in serum cfDNA levels after radiation therapy and (c) a difference in cfDNA levels between tumour bearers with and without

metastases. This led to an interest in the possibilities of exploiting cfDNA concentration as a tumour marker.

The second study was by Stroun et al. [40] who employed a method based upon the decreased strand stability of cancer cell DNA. They found extractable amounts of plasma cfDNA from a range of tumour patients but none extractable from the normal control plasmas. They further demonstrated that the released cfDNA was derived from cancer cells.

Unfortunately, the increased cfDNA levels were found for a range of cancers and so could not be used to identify a single type of cancer. Moreover, similar increases were found in a range of general medical conditions (Table 3; see also chapter "CNAPS and General Medicine"). It became clear that although there was a disturbance in cfDNA levels, this alone could not form the basis of a diagnostic method although in specific cases, cfDNA amounts could prove helpful e.g. for the triage of earthquake victims [50].

This led to the search for specific cfDNA fragments that would typify a particular cancer for early diagnosis e.g. cfDNA fragments corresponding to mutant genomic sequences (see chapter "Extracellular Nucleic Acids and Cancer"). In addition, the quest for cfRNA molecules that might also be exploited either as early markers or indicators of tumour progression was launched leading to extensive analyses of cfmRNA and cfmiRNA, in particular. Similar approaches have been used in aspects of diabetes (see chapter "Circulating Nucleic Acids and Diabetes Mellitus").

Nevertheless, there has been disappointingly slow progress in the definition of routine cancer markers for the early detection of the presence of specific tumours. In contrast, the identification of the presence of cffDNA in maternal plasma and serum during pregnancy [41] was a major breakthrough, permitting the development of tests for fetal sex and Rh factor both of which are available to the general population in a number of countries. In addition, tests for trisomy disorders and ß-thalassemia are close to clinical deployment (see chapter "Fetal CNAPS: DNA/RNA"). The range of some fetal disorders identified by cffDNA/cffRNA in maternal plasma/serum are given in Table 4.

There are now many groups around the world searching for specific cfDNA and cfRNA markers for the early identification of various forms of cancer and other clinical disorders that can be applied in the clinical setting. These studies have been boosted by the development of DNA and RNA analytical methods including various PCR techniques, high throughput sequencing and microarray expression analysis (see chapters "Detection of Genetic Alterations by Nucleic Acid Analysis: Use of PCR and Mass Spectroscopy-Based Methods" and "Genomic Approaches to the Analysis of Cell Free Nucleic Acids"). The application of such methods has permitted faster analyses of genomic sequences that may be used as possible markers in searching for cfDNA/cfRNA fragments for early diagnosis including the development of a panel of cfDNA/cfRNA sequences for a particular e.g. cancer form. Moreover, this approach has permitted the sequencing of the full fetal genome [60, 61] from cffDNA fragments in the maternal blood whilst cffRNA from amniotic fluid has permitted the identification of systems development of the

Table 3 Early examples of clinical disorders with increased plasma/serum cfDNA levels

Clinical disorder	References
Injury	Tan et al. [42]
Stroke	Lam et al. [43], Rainer et al. [44] and Geifer et al. [45]
Acute myocardial infarction	Chang et al. [46], Saukkonen et al. [47] and Antonatos et al. [48]
Sepsis	Angus et al. [49]

Table 4 Some examples of fetal tests derived from cffDNA/cffRNA present in maternal plasma/serum

Test	References
Sex	Vainer et al. [51]
Blood genotyping	Lo et al. [52], Finning et al. [53], Zhang et al. [54] and Li et al. [55]
Trisomy 21	Dhallan et al. [56] and Lo et al. [57]
ß-thalassemia	Li et al. [58] and Papsavva et al. [59]

fetus as well as checking for fetal sex, trisomy 13, 18 and 21 and SNPs (see Other Body Fluids as Non-Invasive Sources of Cell-Free DNA/RNA). Systems evolution in the neonate has also been determined by analyses of cffRNA from neonate saliva (see Other Body Fluids as Non-Invasive Sources of Cell-Free DNA/RNA).

In addition to searching for early markers for clinical disorders, the use of cfDNA and cfRNAs has permitted the monitoring of treatment (see chapter "CNAPS in Therapy Monitoring"). Thus, not only can the effectiveness of treatment be checked e.g. for radio- and chemo-therapy for cancer, but the early identification of metastases after surgery, for e.g. CRC, can also be determined.

In consequence, the application of CNAPS can be seen to have an important role to play in PPPM.

4.1 Implied Costs for Such Tests

One aspect for the application of such test in PPPM concerns the cost of the test. It is clear from the example in Fig. 1 (A.R. Thierry, unpublished data) that the test costs can vary widely as a function of the particular method employed. The increased cost arises with increased genomic coverage associated with an increased data turnaround time. Thus, the methods employing small cfDNA fragments using either Intplex or dPCR or Single locus assay will be the cheapest and with the fastest turnaround together with the highest sensitivity for rare mutation detection as determined by estimating the WT/mutant copy ratio. Thus, either the need or decision for expanding the number of tested mutations outside of the conventional "hotspot" to very rare (infrequent, <1 %) mutations, will seriously impact the test cost and data turnaround time. NGS technologies could fulfil this expectation, but more development is necessary with regards to their sensitivity since mutant circulating cfDNA fragments might be present at very low frequency.

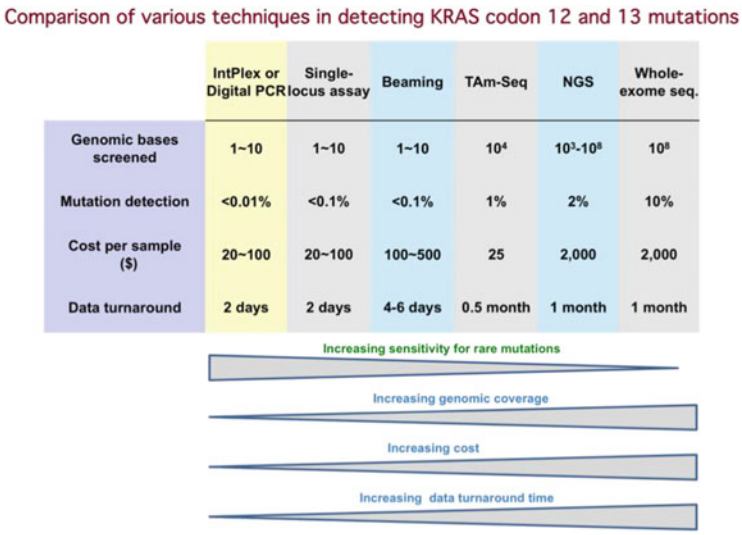

Fig. 1 A comparison of the costs of various techniques in detecting *KRAS* 12 and 13 mutations (AR Thierry, unpublished)

These figures will have an impact on the cost to the clinical application of the tests and to the cost to the individual if the test is run from a private clinic.

The costs could be further affected by the patenting of a test where the test can become restricted to being offered by a single company so resulting in (i) higher than necessary costs, (ii) no chance of second opinions, (iii) no data sharing, (iv) lack of access of the DNA sequences to other research workers leading to (v) the blocking of the possible development of other tests as well as (vi) lack of access to relevant information about the test by certain populations.

The granting of a patent for one or more genes has been challenge in the USA Supreme Court where a decision taken on 13 June 2013 ruled that synthetic genes such as complementary DNA can be patented but that naturally occurring genes cannot be patented [62]. This also formed the basis of a similar decision taken by a Federal District judge in San Francisco, USA [63].

These judgements have multiple implications including the costs of testing in PPPM.

5 Ethical and Other Implications of CNAPS Applications

The identification and application of cfDNA/cfRNAs and cffDNA/cffRNA in PPPM whilst bringing many benefits to people, also raises a number of ethical and other questions. Some of the ethical issues include the erosion of informed decision-making, pressure to test, testing for non-medical reasons or for

information only are considered in chapter "Societal Aspects: Ethics". However, there are additional questions that need to be addressed given especially that the fetal genome can be sequenced and SNPs identified. This has already occurred for the human genome with a possible SNP every 100–300 bp, i.e., 10–30 million potential SNPs per genome. Given the identification of SNPs prior to birth and subsequent genome sequencing of individuals, fetal diagnostic processes can be employed more generally in PPPM. Determining fetal gender and Rh is of less concern, but with the increased speed and sensitive methodology available and the wide selection of possible disorders identified, e.g., through SNPs, will increase the ethical problems. The similar situation arises for people of all ages who have their DNA sequenced. Although either the presence or absence of particular SNPs or DNA sequences that might lead to the development of a clinical disorder does not necessarily mean that the disorder will develop, this can lead to uncertainties that could be exploited e.g. by insurance companies with the cost of health and life insurance policies. So should an insurance company be able to demand access to such information? There should be protection for the patient concerned against additional issues such as (i) How will such medical records be stored and how safe will they be? (ii) Who will have access to such information? (iii) Who will pay for such analyses and the upkeep of such data-bases, especially in the absence of a national health service?

These topics need debating since the need for such genetic information in order to apply PPPM in society in general involves the obtention of genetic information for, eventually, each individual.

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The Biology of CNAPS

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Abstract Although nucleic acids have been known to circulate in the blood since 1948 their biology has been studied only since the 1960s. This chapter contains discussion of (a) the presence of DNA and RNA circulating in human plasma and serum from both healthy individuals and patients, (b) the amounts of DNA/RNA present together with the variables affecting these amounts, (c) possible sources of the DNA/RNA in blood and (d) the ability of the circulating nucleic acids to enter other cells and to modify the biology of the recipient cells. The relationship of the DNA from cancer patients is considered with respect to the formation of metastases.

Keywords Circulating DNA/RNA • Sources • Entry into/exit from cells • Cell modification • Metastases

1 Introduction

The background concerning the presence of cfDNA/cfRNA in blood and other body fluids has been considered in chapter "Brief History and the Present and Future Status of CNAPS". Developments in analyzing these cfNAs have permitted the study of various applications of their use in diagnosis, prognosis and monitoring of treatment of clinical disorders. Since the first identification of cfDNA and cfRNA, there has been an increase in the general identification of the members of the RNA family and in a broadening of their analysis in CNAPS. This chapter will consider the biology of cfNAs in plasma and serum as well as in urine, saliva, cerebrospinal fluid and amniotic fluid. The possible cellular origins of cfDNA/cfRNA found in

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blood, the mechanisms of release into the blood and their uptake into other cells as well as their possible biological effects in the host organism will also be considered.

2 cfNucleic Acids and Nuclease Content

Both DNA (1.8–15 ng mL^{-1}) and RNA (2.5 ng mL^{-1}) are found in plasma and serum from healthy donors [1 –3]. These levels rise in patients with various cancers, trauma, myocardial infarction and stroke with values of 3,000 μg DNA mL^{-1} and above being recorded on occasions [4]. In consequence, the amount of cfDNA and cfRNA present in plasma and serum will depend upon the health status of the individual and will also be influenced by the amount of nucleases present in blood.

The average blood plasma concentration of DNAase 1 forms 90 % of total blood DNase i.e. 41 ± 30 ng mL^{-1} for healthy men and 21 ± 21 ng mL^{-1} for healthy women yielding an activity of 0.307 ± 0.249 U mL^{-1} for men and 0.405 ± 0.509 U mL^{-1} for women. In contrast, the values for diseased individuals rise with e.g. gastrointestinal cancer patients having about 350 ng mL^{-1} [5]. The average serum RNAase value for 54 normal individuals was 104 units mL^{-1} while for those suffering from pancreatitis was 120 units ml^{-1} and pancreatic cancer was 383 units mL^{-1} [6]. Based on such measurements, it can be postulated that the relatively low levels of circulating cfDNA in healthy individuals could occur, partially, due to peripheral blood DNAase activity. However, the DNA of cancer patients could be resistant to DNAase as demonstrated by using bacterial DNAase [7]. This could be possibly due to either accessory (lipo)-protein and/or low DNAase levels. Equally, high RNA levels may also be due to RNA resistance to RNAase digestion especially when high RNAase and RNA levels co-exist. The RNA may either be protected by a glycolipid resulting from its apoptotic origin [8] or the DNA and RNA fractions are associated with the exosomes and virtosomes from living cells so protecting the cfNAs from digestion by RNAase/DNAase activity ([1, 9–13]; section "Exosomes"/"Virtosomes").

3 Nucleic Acid Sources

The presence of DNA in the blood raised the question as to its origin. The most obvious suggestion was that it must be derived from dead and dying cells. However, whilst this was a likely source, experiments by Stroun and Anker demonstrated that healthy, living cells also could release DNA and RNA [1, 11, 14–16]. Subsequent analyses have indicated that there are at least twelve possible sources of blood cfNAs (Table 1).

Table 1 Possible sources of cfDNA and cfRNA circulating in plasma and serum

DNA and RNAs[a]
1. Leukocyte breakdown
2. Bacteria breakdown
3. Viruses
4. Mitochondrial DNA
5. Cell and tissue necrosis
6. Cell apoptosis
7. Cellular release of exosomes
8. Cellular release of RNAs
9. Cellular release of virtosomes
10. Parasite nucleic acids
DNA
1. Cellular release of transposons and retrotransposons
2. Leukocyte surface DNA

[a]Including the many forms of RNA (see Sect. 3.2) though to date, the cfNA focus has been primarily on mRNAs and miRNAs

3.1 cfDNA

Leukocytes

A minimal amount of DNA is released on the death of leukocytes and is unlikely to account for the total amounts of cfDNA found in normal blood i.e. 1.8–36 ng mL^{-1} [1, 5, 17] and certainly not the levels found during clinical disorders; larger amounts have been reported for both normal individuals and those suffering from e.g. cancer [4].

However, DNA can be found on the surface of leukocytes through either trapping DNA on the outer surface prior to its internalization and destruction or secretion of a DNA network onto the surface [18–21]. Neutrophils can release sticky webs of chromatin (DNA + histones) during infection to trap invading microbes. These neutrophil extracellular traps have granules containing lytic enzymes and antimicrobial peptides exploited for the rapid killing of invading pathogens [22–24].

In addition, lipopolysaccharide from Gram-negative bacteria activates either interleukin-5 (IL-5)- or interferon-gamma primed eosinophils leading to a release of mitDNA independently of eosinophil death. The mitDNA is rapidly (<1 s) released involving a catapult-like movement. mitDNA, together with protein, forms the extracellular network that is believed to bind and kill bacteria in vitro and in vivo under inflammatory conditions [25]. However, Menegazzi et al. [26] have challenged this explanation suggesting that the DNA network was released only by dying neutrophils. This is based upon experiments in which live bacteria were released from the DNA network on treatment with DNAase.

Importantly, cell surface DNA forms the basis of a number of early diagnostic and monitoring analyses (e.g. [27]).

Bacteria and Viruses

The levels of DNA seen are unlikely to be due to the presence of bacteria found normally due to their low numbers. Some viral DNAs have been recorded in peripheral blood e.g. Epstein Barr virus (EBV) in patients with nasopharyngeal carcinoma [28] and human papilloma viral carcinoma in about 50 % of cervical carcinoma patients and hepatitis patients [21, 29]. Human herpes virus-6 (HHV-6) DNA has also been found in both plasma and serum. HHV-6 DNA in plasma was readily attacked by DNAase and so is considered to be unencapsulated. HHV-7 was also identified in plasma, but only in the P1 sub-fraction whereas HHV-6 appeared in P1, P2 and S sub-fractions, especially in P1 [30].

That bacteria and viruses are likely to form only a very small part of cfDNA/cfRNA was shown in a study by Beck et al. [31] on blood from 51 apparently healthy individuals when they obtained 4.5×10^5 DNA sequences (7.5×107 nucleotides). Of these, 87 % were attributable to known human database sequences and only 3 % were found to be xenogeneic.

Necrosis

Necrosis has also been considered as a potential source of blood cfDNA. The cfDNA derived by necrosis forms non-specifically and incompletely digested pieces in excess of 10,000 bp and, unlike apoptotic DNA fragments, forms smears when electrophoretically run on gels [32–34]. The cfDNA fragments released from necrotic cells and present in blood plasma samples have been shown, via electrophoresis and sucrose-gradient sedimentation, to contain high molecular weight DNA fragments ranging from 21 kb [35] to 80 kb in length [36]. Hence, necrosis does not appear to be a major contributor to cfDNA, the electrophoretic separation pattern showing primarily an apoptotic ladder pattern rather than the smear pattern.

Apoptosis

This would appear to present a major contributor to cfDNA. cfDNA in blood is double-stranded [1, 37] and forms a ladder pattern when separated by agarose gel electrophoresis [32, 38, 39] with fragment sizes ranging from 60 to 1,000 bp. The fragment ends are capped showing them to be present in the form of nucleosomes or apoptotic fragments. This has led to the cfDNA being considered as the apoptotic product from e.g. tumors. The typical electrophoretic ladder pattern of DNA from apoptotic cells is initially of 50–300 kb fragments that mainly fragments into multiples of nucleosomal fragments (180–200 bp). Thus, the cfDNA fragments may have their origin via apoptosis [36, 38, 40]. Apoptotic fragments are expected to be phagocytozed by macrophages and dendritic cells at the final stage [41] and so

should not be released into the blood. It has been suggested that if the release is from solid tumors there could be a breakdown in the phagocytic process [40, 42, 43].

Further evidence for the apoptotic origin of DNA is through the identification of mitDNA in CNAPS [43]. Both particle-associated and mitcfDNA are present in plasma, their respective concentrations being modified depending on the preparation of plasma from whole blood [44]. Thus, mitDNA increases were found in trauma patients [45, 46], with median plasma mitDNA concentrations having double the number of copies mL^{-1} in the severely injured subgroup compared with the minor/moderate subgroup. A 2.6-fold increase in mitDNA was found in patients dying from prostate cancer as opposed to those who survived [47]. The median mitDNA copies 100 μL^{-1} plasma for prostate cancer patients were 49,193 compared to 19,037 for benign controls.

Exosomes

These membrane-bound structures, 30–90 nm in diameter, are secreted by most cell types and may play a role in intercellular signaling. Although originally described in 1983, interest in exosomes has increased recently due to them containing both mRNA and miRNA ([48] see section "Exosomes"). DNA has also been found to be present in exosomes of both man and mouse [49–51]. DNA-containing exosomes have been linked to the initiation of both glioma and colorectal cancer under experimental conditions [49, 50]. Nevertheless, exosomes appear to be low-level contributors of DNA/RNA to cfNAs.

Virtosomes

A further contributor to CNAPS would appear to be newly synthesized DNA that has been shown to be spontaneously released, in a regulated manner, from both stimulated and non-stimulated human [1, 52, 53] and rat lymphocytes [9, 10] in vitro. This DNA is complexed with newly synthesized lipoprotein [9, 10, 54] and newly synthesized RNA [9, 10] i.e. all of the components of this complex are newly synthesized. This complex has been termed a virtosome.

The release of newly synthesized DNA from living, but not dead or damaged cells, has been shown to be of general occurrence in vitro (Table 2) as well as being released in vivo from whole chick embryos [59].

Transposons and Retrotransposons

These mobile genetic elements, or transposable elements, form a substantial part of the nuclear "c" DNA value.

Table 2 Release of newly synthesized DNA from prokaryote and eukaryote cells and tissues

Cell/tissue	References
Bacteria	Ottolenghi and Hotchkiss [55, 56] and Borenstein and Ephrati- Elizur [57]
Human stimulated and non-stimulated lymphocytes	Anker et al. [1]
Rat stimulated and non-stimulated lymphocytes	Olsen and Harris [52] and Adams and Gahan [9, 10] Adams et al. [58]
Chick embryo fibroblasts	Adams and MacIntosh [54]
Frog heart auricle pairs	Stroun and Anker [16] and Stroun et al. [11]
Frog brain	Anker and Stroun [14]

Retrotransposons copy themselves via RNA, whilst the transposons copy themselves without the intervention of RNA. The Alu repeat sequence of approximately 300 bases, being found 300,000–1,000,000 times in the human genome, is the commonest form of human transposons. ALU repeat DNA fractions have been reported to be present in CNAPS [60, 61] together with the retrotransposon LINE 1 [62]. ALU and LINE1 are distributed throughout the genome being less methylated in cancer cells as opposed to normal cells [63].

Mitochondrial DNA Release

This was found in CNAPS from trauma patients [45, 46]. Bound and mitcfDNA have both been found in plasma, the concentrations of each possibly being affected by the mode of preparation of plasma from whole blood [44]. mitcfDNA may be also derived by apoptosis [43] (see also Sect. 3.1).

Parasite DNA Release

This is a little studied area, but the possibility of parasite nucleic acids being present as cfDNA have been discussed by Gahan [64] in considering aspects of HGT.

3.2 RNA

In a similar fashion to DNA, RNA can be released from any one of ten possible sources listed in Table 1. There has been an explosion in the identification of a variety of RNAs and their various roles in cells including mRNA, tRNA, rRNA, snRNA, snoRNA, dsRNA, RNAi, siRNA, miRNA, piRNA, circRNA, ceRNA and lncRNA (see section "Exosomes"). This is reflected in the identification by deep sequencing of many of these RNAs in exosomes [65] although much of the current work on cfRNA has been essentially limited to cfmRNA and cfmiRNA.

Messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA) are all involved in transferring information from chromosomal DNA to the ribosomes where amino acids are transported for the construction of protein [66]. Involved in these processes are small nucleolar RNAs (snoRNA), a class of small RNA molecules that mainly guide chemical modifications of other RNAs including rRNAs, tRNAs and small nuclear RNAs (snRNA). The latter are a class of small RNA molecules found within the eukaryotic nucleus and having an average length of ca 150 nucleotides. Their primary function concerns the processing of pre-mRNA (hnRNA) in the nucleus.

There are a variety of small non-coding RNAs involved in cfNAs including miRNA that is important in a number of studies discussed throughout this book. It involves a single-stranded RNA of 20–25 nucleotides functioning in transcriptional and post-transcriptional regulation of gene expression through binding to the 3′-untranslated region of the target mRNAs. The human genome has over 1,000 miRNAs that function via base-pairing with complementary sequences within mRNA molecules. Their action usually results in gene silencing via either translational repression or targeted mRNA degradation.

Other small non-coding RNAs include:

(i) Long double-stranded RNA (dsRNA) is cleaved into short 21–24 nucleotide double-stranded RNAs (siRNA). Each siRNA unwinds to form two single-stranded RNAs (ssRNAs), the passenger strand and the guide strand. The former is degraded while the latter is incorporated into the RNA-induced silencing complex (RISC). A well-studied example of its function involves post-transcriptional gene silencing when the guide strand base pairs with a complementary sequence of a mRNA to induce cleavage by Argonaute, the catalytic component of the RISC complex.

(ii) Similar to the siRNAs are the piRNAs, Piwi-interacting RNAs, found in gonads, are the largest class of small non-coding RNA molecules. They are distinguished from miRNA by being of 26–31 nucleotides rather than 21–24 nucleotides, lacking sequence conservation and increased complexity. The piRNA complexes, formed by interacting piRNA with piwi proteins, have been linked to silencing of both epigenetic and post-transcriptional genes of retrotransposons in germ line cells.

(iii) Long non-coding RNAs (lncRNA) are non-protein coding transcripts longer than 200 nucleotides being much longer than the above-mentioned RNAs. There are at least four-times more lncRNA than coding RNA sequences. Their roles have not been well analyzed though it has been shown that there is involvement with the physiological aspects of cell-type determination and tissue homeostasis.

(iv) Circular RNAs (circRNA) concern a class of circular RNA molecules that may play a regulatory role in miRNA processes. Initial studies have shown that they may act as “sponges” for miRNAs since e.g. circRNA (ciRS-7) acts as a miR-7 super-sponge containing ~70 target sites from the same miR-7 at the same transcript.

(v) Salmena et al. [67] propose the presence of ceRNA – competing endogenous RNA and hypothesize that "in addition to the conventional microRNA/RNA function, a reversed RNA/microRNA logic exists in which bona fide coding and noncoding RNA targets can crosstalk through their ability to compete for microRNA binding. On the basis of this hypothesis, MREs can be viewed as the letters of an "RNA language" by which transcripts can actively communicate to each other to regulate their respective expression levels. We hypothesize that RNAs that share multiple MREs will crosstalk effectively. Importantly, we predict that this "RNA language" can be used to functionalize the entire mRNA dimension through the identification of crosstalking ceRNAs, as well as ceRNA networks." ceRNAs may be involved in cfNAs since ceRNAs for the tumor suppressor gene *PTEN* have been identified for prostate cancer [68], glioma [69] and melanoma [70].

Leukocytes

Leukocyte breakdown could release a range of RNAs into the blood so possibly accounting for the RNA amount already determined [2].

Bacteria and Viruses

The presence viral RNA has been demonstrated for hepatitis C RNA in the plasma and serum of European and African patients using an RT-qPCR and the isothermal NASBA nucleic acid amplification system encompassing a gel-based detection assay. This extraction method has allowed the detection of hepatitis C RNA equally in both serum and plasma using either heparin or EDTA [71]. Majde et al. [72] have also shown the release of dsRNA into the extra-cellular medium from influenza virus-infected MDCK epithelial cells. Little information is available of the levels of RNA released from bacteria into the blood stream.

Necrosis and Apoptosis

A spectrum of mRNAs has been identified in plasma that are presumed to have been released by either apoptosis or necrosis including those representing (i) genes overexpressed in a range of different tumors [73, 74], (ii) fetal genes in the blood plasma of pregnant women [75], (iii) genes of patients with diabetic retinopathy [76] as well as (iv) housekeeping genes detected in the plasma of healthy persons [77].

RT-qPCR detectable fragments of 18S rRNA were also found in the extracellular RNA pool circulating in blood plasma of healthy subjects and cancer patients [78]. Ribosomal 28S rRNA fragments secreted by primary and cultivated human cells into the culture medium have been demonstrated [79].

Exosomes

Early studies indicated that exosomes contained RNAse that was thought to destroy unwanted RNA [66]. In fact, exosomes contain many domains with RNAase activity. Hence, if such exosomes were to be released into the blood stream, they could contribute to the RNAse levels.

More recently, it has transpired that exosomes may act as vehicles for the transfer of a variety of RNAs and so as an inter-cellular signaling system. The RNAs include mRNAs, miRNAs, non-coding RNAs, retrotransposon elements, genomic DNA and cDNA derived from oncogenic sequences [80–82]. Exosomes from both mouse and human mast cells have been shown to contain cfRNA as have primary, bone marrow-derived, mouse mast cells [82]. The presence of cfRNA in exosomes has also been reported for those released from tracheobronchial ciliated epithelial cells [83]. Importantly, exosomes released into the blood from glioblastoma patients were shown to contain mRNA mutant/variants and miRNA characteristic of gliomas. In particular, tumour-specific EGFRvIII, containing a mutation specific for glioblastoma, was detected in the serum micro-vesicles of seven out of 25 glioblastoma patients. The glioblastoma-derived vesicles were shown to stimulate proliferation of a human glioblastoma cell line [81]. These workers also showed mRNA and miRNA to be taken up by normal host cells including brain and microvascular endothelial cells.

More recently, Huang et al. [65] characterized human plasma-derived exosomal RNAs by deep sequencing. They obtained a total of 101.8 million raw single-end reads from 14 size-selected sequencing libraries and detected significant fractions of RNA species including rRNA (9.16 % of all mappable counts), lncRNA (3.36 %), piRNA (1.31 %), tRNA (1.24 %), snRNA (0.18 %) and small nucleolar RNA (0.01 %). However, the dominant RNA fraction was miRNA that accounted for over 42.32 % of all raw reads and 76.20 % of all mappable reads. The five most common of the 593 miRNAs detected were miR-99a-5p, miR-128, miR-124-3p, miR-22-3p and miR-99b-5p. Collectively, these accounted for 48.99 % of all mappable miRNA sequences. A further 185 potential miRNA candidates were predicted (see also Sect. 11).

Virtosomes

RNA has been found to be present in virtosomes. Experimental data indicate that it is synthesized prior to the complex leaving the cell, possibly involving the DNA-dependent RNA polymerase associated with the complex [9, 12, 15, 16, 84, 85].

Mitochondria

mitRNA was also reported to be present in plasma there being no significant relation between mitRNA in plasma and patient age ($n = 69$; $r = -0.16$, $P = 0.19$ for mitRNA). However, in a study on prostate cancer patients, non-survivors were found to have a 3.8-fold increase in mitcfRNA compared with survivors ($P = 0.003$; non-survivors: median copies, 16,038; interquartile range, 5,097–48,544 copies; survivors: median copies, 4,183; interquartile range, 2,269–8,579 copies) [47].

Parasite RNA Release

As with parasite DNA, this is a little studied area, but the possibility of parasite nucleic acids being present as cfRNA exists [64].

4 Mechanisms of Exit from and Entry into Cells by cfDNA and cfRNA

4.1 Mechanisms for the Exit of cfDNA and cfRNA from Cells

It is clear that cfDNA and cfRNA are present in blood in various forms, but how they leave affected cells and enter the blood stream has not been well investigated. It is assumed that apoptotic and necrotic fragments, especially from tumors, could be released directly into the blood stream. Garcia Olmo et al. [86] considered the possible release mechanisms, but came to no specific conclusion concerning the presence of higher cfDNA levels, especially of non-mutated cfDNA significantly elevated at the early stages of tumor progression.

Release of LINE-1 from both HeLa and HUVEC cells, in vitro, could be reduced by treating the cells with inhibitors of protein secretion [87]. Thus, DNA release from HUVEC cells was reduced by 30, 35 and 19 % for monensin, glyburide and methylamine, respectively. However, monensin reduced DNA release from HeLa cells by only 15 % while glyburide actually increased cell-surface bound DNA by 50 %.

Exosome formation is a more evident process involving exocytosis of the membrane bound cfDNA and cfRNA. However, the picture for virtosomes is not so clear in that although the complex is formed with lipo-protein they do not appear to have a standard limiting membrane [88, 89]. The virtosomal release process is energy dependent [10].

4.2 *Mechanisms for the Entry of cfDNA and cfRNA into Cells*

When considering the mechanisms by which cfCNAs are taken up, two aspects need addressing: (i) entry into the cytoplasm with the avoidance of cfNA destruction and (ii) passage from the cytoplasm into the nucleus where it may act either epigenetically or be incorporated into a chromosome.

Cytoplasmatic Entry

Early studies on the uptake of DNA and RNA into cells have been reviewed by Stroun et al. [37]. However, subsequently, there have been few studies on the mechanisms of cfRNA cell uptake. Since most cfRNA release so far recorded tends to be via exosomes (Table 3), it is very likely that cytoplasmic entry will be by a form of endocytosis. This is supported by studies on plant siRNA in which the endolysosomal system is considered to be involved [93] while in Drosophila cells, dsRNA uptake from the environment requires receptor-mediated endocytosis [94]. Lee et al. [95] have linked gene silencing by miRNAs and siRNAs to endosomal trafficking. In the few RNA uptake studies available, caveoli are prominent showing no hydrolase activity (Table 3).

It should be noted that dsRNA entry into murine GEnC cells required complex formation with cationic lipids for entry via clathrin-dependent endocytosis though it was independent of endosomal acidification [90].

Although Rh110-labelled siRNA phosphorothioate (PTO)-modified ON (TM6-6) entered into ECV304 cells [96], similar results could not be obtained with either the human T-lymphoma cell line Jurkat 17 [97] or the mouse B-lymphoid cell line BJA-B [98]. In the absence of PTOs, the uptake of the siRNA was reduced and the molecules distributed throughout the cytoplasm [99].

More recently, mechanisms of cfDNA entry have been demonstrated including the entry of various sources of bacterial DNA and mitDNA through the Toll receptor system [100–105]. Nevertheless, current research indicates that cfDNA can enter cells by various routes (Table 4). The mechanisms involved in this cell entry process include endosomes, caveoli and T-tubules. However, the mechanism by which the cfDNA avoids the digestive processes of the lysosomal system is not clear. The uptake of naked plasmid DNA via endosomes has been demonstrated to block endosomal acidification resulting in the lack of activation of the hydrolases present and hence DNA digestion [108]. Such DNA was considered by these authors to remain in the endosomes and to move to the nuclear membrane where it could be transferred directly into the nucleus.

Caveoli have different endocytotic functions from those of the clathrin-coated pit pathway. Ligands bound to receptors that are internalized by caveolae can be delivered to four different locations in the cell. At least four different caveolae membrane traffic patterns are distinguishable during potocytosis so offering a

Table 3 Possible mechanisms of uptake of cfRNA for a variety of cell types

Cell	RNA	Vehicle	References
Murine GEnC	dsRNA	Endosomes	Hägele et al. [90]
ECV304	siRNA phosphorothioate	Caveoli	Fra et al. [91]
2B2318 lymphocytes	SFV-VIP21 virus (dog/simian)	Caveoli	Fra et al. [92]

Table 4 Possible mechanisms of uptake of cfDNA for a variety of cell types

Cell/tissue	DNA	Vehicle	References
Leukocytes	Bacterial DNA; mitDNA	Toll system	Chuang et al. [101], Hemmi et al. [105], Cornélie et al. [102], Barton et al. [100], Dalpke et al. [103] and El Kebir et al. [104]
Myofibres	Plasmid DNA; mRNA	Caveoli; T-tubules	Wolff et al. [106]
Human keratinocytes	Plasmid DNA	Macropinocytosis	Basner-Tschakarjan et al. [107]
J77 cells	Plasmid DNA	Endosomes	Trombone et al. [108]
Murine GEnC cells	dsRNA	Endosomes	Hägele et al. [90]
Human MCF7 breast cancer cells	Human chromatin fragments	Endosomes	Yakubov et al. [109]
NIH3T3	kras	Exosome	García-Olmo et al. [86]

mechanism for bypassing the lysosomes [110, 111]. However, there is still the possibility that the caveoli may eventually link with the lysosomal system [112, 113]. It is not clear how the RNA escapes from the either endosomes or the caveoli, but it is able to exert a biological effect upon the recipient cells [81, 90].

Thus, it is possible that cfDNA can enter primarily via either macropinocytosis into cells in vitro or via caveoli into muscle in vivo and block the acidification of the endosomes so preventing their development into lysosomes and hence degradation of the DNA. In addition, the DNA can exploit the endosomal movement to the nucleus so transporting the cfDNA prior to its release and entry into the nucleus. An example of such activity concerns exosomal mutated cfDNA from a colorectal cancer patient transforming NIH3T3 cells that were able to initiate tumors in rats [114, 115].

It has been suggested that DNA binding proteins are involved in the uptake of cfDNA. The studies of Basner-Tschakarjan et al. [107] indicated that ezrin and moesin are functionally associated with some transmembrane receptors such as the EGF, CD44 or ICAM-1 receptor. These workers considered that these binding proteins were important in the uptake of plasmid DNA into keratinocytes. Subsequently, there has been little information on such binding proteins and alternative mechanisms have been proposed.

Histones H1 [116–121], H2A [122–124], and H3 and H4 [125, 126] have been shown to be effective mediators of transfection. The postulated mechanisms by

which histone H1 increases gene transfection are through DNA condensation and DNase protection. DNA delivery activity may be mediated by two mechanisms, namely, electrostatically driven DNA binding and condensation by histone and nuclear import of these histone H2A · DNA polyplexes via nuclear localization signals in the protein [116]. It is also possible that, because histones can increase the permeability of membranes by ionic interaction, this mechanism could aid complexes such nucleosomes to enter recipient cells [127].

Virtosomes might also enter cells by one of the mechanisms described above [88]. Being comprised of DNA, RNA and glycolipoprotein and failing to either pick up or lose membrane material on either leaving or entering cells, it is possible that a mechanism similar to that exploited by histones could lead to the direct uptake of virtosomes through an ionic interaction between a part of the glycolipoprotein present and the cell membrane. This proposition is also supported by Wittrup et al. [128] who demonstrated that naked plasmid DNA uptake occurred via proteoglycan dependent macropinocytosis.

cfCNA Entry into the Nucleus

The nuclear membrane presents a considerable barrier to the entry of nucleic acids with the nuclear pores permitting a passive transport limit of 70 kDa molecular mass or ~10 nm diameter [129]. Nevertheless, DNA can be seen to enter the nucleus of chick embryo fibroblasts [54, 130], HeLa cells [130], L29 mouse fibroblasts and Krebs 2 ascites carcinoma cells [109] as well as plant nuclei (reviewed in [131]). The mechanism by which DNA enters the nucleus is not clear. It is known that, for mediated active transport through the nuclear pore complex, nuclear proteins require a nuclear localization signal that contains basic amino acids and can be recognized by cytosolic factors [132]. For this to occur, the nuclear pore can expand to approximately 30 nm [133]. This can be shown to function experimentally on coupling 100 nuclear localization signal peptides/kilobase pair of DNA for the nuclear delivery of the DNA [133, 134]. It is important to remember that the mediation of nuclear import of DNA is aided by the presence of H1 histone as seen with gene transfection.

Specific proteins appear to be involved during RNA movement from cytoplasm to nucleus. Thus, siRNAs need to be linked to an argonaut protein for transfer to the nucleus as in the case of NRDE-3 in *Caenorhabditis elegans* [135].

4.3 Conclusions

cfCNAs are present in a variety of forms that are capable of entering cells with which they come into contact. The mechanisms of entry, and in some cases exit, have still not been fully elucidated for either cfRNA or cfDNA although caveoli and pinocytosis seem to be implicated. As yet, there is no information as to the possible

rôles that connexins, innexins and pannexins might play in the intercellular movement of nucleic acids [136, 137].

Both naked DNA and virtosomes released into the blood can move to other parts of the organism and into host cells. On entering cells of a similar type no obvious effects occur.

However, if the uptake is into cells of a different type, the biological activity of the host cell may be modified [58]. The uptake of cfDNA by stem cells raises interesting possibilities [138]. The modification of a cell's biology on the receipt of tumor cell cfDNA has particular implications for the formation of metastases through both the release of cfDNA from tumor cells into the circulation and the ability of the cfDNA to move to cells in other parts of the body. In particular, and in spite of the blood brain barrier, cfDNA can move to the amphibian brain [139] and the human maternal brain [140] whilst fetal cells can move to the female mouse brain [141].

cfRNA in its various forms can behave similarly and in a few cases has been shown to modify the biology of the host cell though there are few studies currently available on this aspect of cfRNA.

In view of the fact that both cfDNA and cfRNA have been implicated in tumor induction, maybe the question should be asked "Should blood collected for blood transfusions be screened for specific forms of cfNAs prior to use?"

5 Mitochondrial Release and Uptake of cfNAs

There is not much known about the release of mitcfDNA from animal mitochondria, other than through either damage or cell death. Release of mitcfDNA from such mitochondria may be a key link between trauma, inflammation and systemic inflammatory response syndrome [142]. It has also been demonstrated that when mitochondria are damaged by external hemodynamic stress, they are degraded by the autophagy in cardiomyocytes. The mitcfDNA that escapes from the autophagic vesicles can lead to Toll-like receptor 9-mediated inflammatory responses in cardiomyocytes that is capable of inducing myocarditis and dilated cardiomyopathy [143]. In plants, a mitochondrial permeability transition pore complex exists together with the inner mitochondrial membrane so permitting the passage of molecules of $<$1,500 Da, [144]. However, currently there is no evidence for a similar passage of DNA in animal/human mitochondria. Nevertheless, studies by Ibrahim et al. [145] on isolated mitochondria from a range of organisms demonstrated that the efficiency of mitochondrial uptake depends on the sequence of the DNA to be translocated becoming sequence-selective for large DNA substrates. ATP needed to be hydrolyzed in order to enhance DNA import. The presence of ATP also allowed tight integration of the exogenous DNA into mitochondrial nucleoids [145].

6 cfNAs Can Enter and Express in Other Cells

6.1 cfDNA

In vitro studies on mammalian cells have shown cfDNA uptake by recipient cells and its subsequent expression. This may be due to either epigenetic or genetic responses.

Immune Response

An allogenic T-B lymphocyte co-operation involving lymphocyte subsets from human donors with different allotypes was studied. B lymphocytes cultured in the presence of the supernatant from the culture medium of T cells, previously exposed to inactivated herpes simplex virus, were able to synthesize an anti-herpetic antibody with some allotypic markers of the T cell donor. The same effect on B lymphocytes was found both with DNA purified from the supernatant of the T cell culture medium and the non-ultra-centrifuged supernatant [146].

Anker et al. [147] also used nude mice injected with DNA extracted from the complex released by human T lymphocytes previously exposed to inactivated herpes or polio viruses. Tested for its neutralizing activity by human anti-allotype sera, the serum from these mice showed synthesis of anti-herpetic or anti-polio antibodies depending on the antigen used to sensitize the T cells. This showed the antibodies to carry human allotypes. Moreover, on concentration, the newly synthesized complex transformed much more efficiently than did either the DNA purified from the supernatant or the crude supernatant itself.

Effects on Cell Division

The DNA released from mouse tumor cell lines J774 cells (leukemia) and P497 cells (glial tumor), as well as non-stimulated lymphocytes, was isolated from the culture medium by ultracentrifugation and agarose gel chromatography [58]. After concentration, the released DNAs were added to the culture media of each of the different cell types. Thus, the tumor cell lines were each incubated in the presence of either of the two tumor cell line DNAs or the lymphocyte DNA i.e. each cell type was incubated in the presence of either a self DNA or each of two foreign DNAs. ^{3}H-thymidine was added to the cultures of each cell type together with the particular donor DNA and the amount of nuclear incorporation of the ^{3}H-thymidine into DNA of the recipient cells was measured (the index of DNA synthesis). The levels of DNA synthesis in the tumor cell lines was the same in the presence of either of the DNAs released by the two tumor cell lines, but was reduced by about 60 % in the presence of the lymphocyte DNA [58]. Conversely, the incubation of the non-stimulated lymphocytes in the presence of either of the tumor cell line DNAs

showed an initiation of DNA synthesis in the presence of the tumor DNAs, but not in the presence of the lymphocyte DNA [58].

Viola-Magni et al. [148] demonstrated that similar reciprocal events occur between stimulated and non-stimulated lymphocytes.

cfDNA Effects in Irradiated Cells – By-Stander Effect

Exposure of Chinese hamster ovarian cells to X-rays at an adaptation dose of 10 cGys led to a transposition of the chromosomal peri-centromeric loci of homologous chromosomes from the peri-membrane sites to approach each other and an accompanying activation of the chromosomal nucleolar-forming regions [149]. The movement of the peri-centric loci appears to be associated with repair of the DNA double-strand breaks during the development of an adaptive response to radiation. Growing untreated cells in medium containing DNA fragments isolated from the medium of treated cells led to their exhibiting similar changes to those seen in the treated cells. Incubation of the untreated cells in medium containing the DNA fragments from medium of untreated cells had no such effect [149]. This is known as the radiation–induced by-stander effect and can be seen both in vitro, as described above, and in vivo [150–155].

cfDNA Effects on Myocardiocyte Contraction Rates

cfDNA was isolated from patients with myocardial infarction followed by the separation of AT-rich fragments of the human satellite 3 tandem repeat (1q12 region) and GC-rich fragments of the rDNA [156]. When fed in vitro to neonatal rat ventricular myocytes in culture, AT-rich fragments (1 ng mL^{-1}) increased the frequency of cardiomyocyte contractions whilst GC-rich fragments (0.5 ng mL^{-1}) decreased the contraction frequency. Serum cfDNA from patients with acute myocardial infarction decreased contraction frequency in proportion to the cfrDNA content so implying that the GC-rich cfrDNA circulating in the blood myocardial infarct patients might affect the contractile function of the myocardial cells [156].

Tumor Induction

The SW 480 cell line, originating from a human colon carcinoma, contains a point mutation of the K-ras gene on both alleles. These cells in culture released DNA containing the mutated K-ras gene. When NIH-3T3 cells were directly cultured in the presence of non-purified SW 480 cell culture supernatant, transformed foci appeared in similar numbers to those occurring after a transfection using a cloned K-RAS gene administered as a calcium precipitate [157].

The effects on cultured cells of plasma from healthy individuals and patients with colon cancer were also determined. NIH-3T3 cells and human adipose-derived

stem cells (hASCs) cultures were supplemented with plasma cfDNA from either patients with K-RAS-mutated colorectal tumors or from healthy subjects by either (i) direct addition of plasma to cultures in standard plates or (ii) avoiding plasma-cell contact by filtering through membranes (0.4 μm pores) placed between the plasma and the cells. No K-RAS mutated sequences were detected in hASC cells by qPCR. However, human gene transfer occurred in most cultures of NIH-3T3 cells, since they were shown to contain sequences for human K-ras, p53 and ß-globin. NIH-3T3 cells were oncogenically transformed after being cultured with plasma from colon cancer patients, confirmed by carcinoma development in NOD-SCID mice after injection with the transformed NIH-3T3 cells. The human mutated K-RAS sequence was also found free in the mouse blood. The presence of the artificial membrane between the NIH-3T3 cells and the tumor patient plasma gave similar results showing that the transforming factor had a diameter of less than 0.4 μm. A TEM study of the plasma fraction passing through the membrane pores confirmed the complete absence of cells but the presence of vesicles $<$0.4 μm diameter [114]. These were later shown to be DNA containing exosomes [50].

Trejo-Becerril et al. [115] reported a similar set of results using the same experimental system as that used by Garcia-Olmo et al. [114]. In further experiments, Garcia-Olmo et al. [158] showed that plasma K-RAS cfDNA was found during a 2-year period following surgical removal of the colorectal tumor from patients. This DNA also yielded similar results with the same experimental system involving NIH-3T3 cells and NOD-SCID mice [158].

RAR2 gene methylated cfDNA uptake into HeLa and human umbilical vein endothelial cells was twice as efficient as that of unmethylated cfDNA. Hypermethylation is a common alteration of tumor related cfDNA from cancer patients and as methylated *RAR 2* gene cfDNA is more prevalent than the unmethylated form in intracellular traffic, it is considered they pose a higher transformation potential [159].

Clearly, cfDNA released from healthy cells can move to other parts of the organism and into host cells. Entry to cells of a similar physiohistological type does not lead to a changed biological activity. However, if the uptake is into cells of a different physiohistological type, a changed biological activity in the host cell may occur. The above results have implications for (a) the formation of metastases by DNA released from tumor cells into the circulation termed "genometastasis" [114, 138, 158, 160–162] and (b) the ability of cfDNA to move to cells in other parts of the body, including the brain, in spite of the blood brain barrier, where it could be taken up and expressed [14].

Gene Replacement Therapy

Based upon data indicating that chromatin fragments possessing recombinagenic free ends were present in the plasma and serum (section "Tumor Induction"), it was possible to exploit them in gene replacement therapy. Small fragments prepared from human chromatin from non-mutant cells were added to the culture medium of

human breast cancer cells having a 47-bp deletion in the *CASP 3* gene. The restoration of caspase 3 activity occurred in 30 % of the treated cells [163].

6.2 cfRNA

Few studies have been performed on the entry of cfRNA into other cells. However, dsRNA polyriboinosinic polyribocytidylic acid (polyI:C) activated murine glomerular endothelial cells via RIG-1 in the cytosol to produce inflammatory cytokines, chemokines and type I interferons [90].

The experimental delivery of siRNA is very difficult and so it possible that the natural uptake of sicfRNA will be minimal. However, when entry of siRNA has been demonstrated, there is an apparent dose-dependent siRNA-mediated suppression of lamin A/C in primary human umbilical vein endothelial cells [96, 99]. Perhaps more importantly, Skog et al. [81] showed that mRNA and miRNA can be taken up by normal host cells including brain and microvascular endothelial cells. In addition, RNA-containing glioblastoma derived vesicles were shown to stimulate proliferation of a human glioblastoma cell line.

7 Can cfNAs Influence the F1 Generation?

This question was recently considered since cfNAs have been demonstrated to be capable of modifying cells into which they enter [64]. The continual circulation of cfNAs around the organism may result in their entry into adjacent tissues. If the cfNA is not mutated, then any entrance and expression would not necessarily be detectable. However, as has already been considered for mutated cfNAs, the likelihood of the development of metastases is a possibility [158]. Foreign cfNAs could also circulate though there has been little evidence that this is a major problem in the normal individual. Nevertheless, if such cfNA could enter the gonads, then this could offer a mechanism for horizontal gene transfer (HGT) to operate, HGT involving the movement of genes from one organism to another. Although HGT has been identified to occur in lower organisms such as bdelloid rotifers [164] or Lepidoptera [165] currently, there is no such evidence for eutherians including man. This is likely to be prevented through the presence of the distinct germ cell line being separate from the soma [166]. This would appear to act through the difficulty of the natural entry of cfNAs into mammalian sperm and ova to form a modified zygote [64, 167].

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Part II
Methodology

Pre-analytical Requirements for Analyzing Nucleic Acids from Blood

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Abstract Circulating nucleic acids have received an increasing scrutiny over the past decade with some applications, such as in prenatal diagnosis and oncology, being on the verge of use in clinical practice. It is crucial to implement optimal standardization of pre-analytical procedures. Currently, this domain has been poorly studied and there is no well-established procedure. This chapter examines the literature on the pre-analytical factors affecting nucleic acids from blood drawing to the storage of circulating cell-free DNA extracts ready for analysis and provides some elements as guidelines for a set procedure. In particular, this chapter reports on the choice between serum and plasma as the biological source but does not concern the actual nucleic acid extraction procedures (these will be dealt with in chapter "Circulating DNA and miRNA Isolation"). Currently, the lack of a standard operating procedure for the application of blood handling in a clinical setting is due to the lack of dispensing and sharing data among researchers as well as head-to-head comparative studies between techniques. This has led to in-house specific procedures that are, undoubtedly, prejudicial to the smooth translation of nucleic acid analysis into clinical practice. Hence, the proposed procedure should overcome this gap in technique.

Keywords Serum • Plasma • Blood sampling • Blood storage • DNA/RNA extraction

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1 Introduction

Despite the great interest in circulating cfNA analysis, it is not as yet transferred into clinical practice. The lack of consistency between the various procedures at each pre-analytical and analytical step constitutes one of the major hurdles for the use of cfNA analysis in routine clinical practice. In recent years, cfNA reviews have regularly highlighted the lack of standardization between the various techniques used for cfNA analysis [1–9], constituting a bias when comparing data from different laboratories.

In this chapter, we review the main factors potentially affecting circulating cfDNA analysis from blood drawing to the storage of cfDNA extracts, and finally provide an optimal guideline for the pre-analytical treatment of samples that guarantees quality analysis.

This chapter is based on data reported in the literature and our own observations [10]. Particular attention has been devoted to the study of cfDNA fragmentation considering that it is an indicator of cfDNA stability during the handling and storage of samples. Our robust and precise cfDNA quantification method enabled a precise study of both the pre-analytical handling and portability of cfDNA analysis.

2 Optimal Blood Sampling

2.1 Serum or Plasma?

State of the Art

The choice of matrix, i.e. serum or plasma, is the first parameter to define for the standardization of cfDNA analysis. Reported data comparing cfDNA concentrations in paired plasma and serum samples reveal that the cfDNA concentration is significantly higher in serum than in plasma [11–18]. Some of these data are summarized in Table 1.

It is now commonly hypothesized and shown that the increased levels of cfDNA in serum are due to the clotting process of white blood cells in the collection tube leading to their lysis [11, 13–15, 19]. As a consequence, cfDNA in serum is at least slightly contaminated by genomic DNA released from white blood cells and specific cfDNA is diluted by high concentrations of non-specific genomic DNA. This point is crucial for the accurate detection of rare cfDNA sequences. While it has been established for a few years that plasma is better than serum, many studies in the field are still based on serum samples, certainly due to the propensity of clinical laboratories to prepare sera conventionally and to perform retrospective studies.

Table 1 Non-exhaustive data focusing on differences in cfDNA concentrations in paired serum and plasma samples

Article	Clinical field	Subject numbers	Plasma cfDNA concentration	Serum cfDNA concentration	p-value
Quantification of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma [14]	Healthy subjects	18	Approximately 40 copies mL^{-1}	Median: 8,000 copies mL^{-1}	
Higher amount of r circulating cfDNA in serum is not mainly caused by contamination by extraneous DNA during separation [18]	Cancer	24	Mean ± sd: 180 ± 150 $pg{\cdot}L^{-1}$	Mean ± sd: 970 ± 730 $pg{\cdot}\mu L^{-1}$	$p = 0.0002$
Predominant hematopoietic origin of cfDNA in plasma and serum after sex-mismatched bone narrow transplantation [15]	Transplantation	22	Median: 1,195 copies mL^{-1}	Median: 16,345 copies mL^{-1}	$p < 0.0001$
Effects of pre-analytical factors on the molecular size of cfDNA in blood [11]	Fetal medicine	27	Median: 600 copies mL^{-1}	Median: 975 copies mL^{-1}	$p < 0.05$

Our Observations

We have shown unequivocally that plasma is a better source of specific tumor-derived cfDNA [20]. Using xenografted mice (n = 4) with a human colorectal cancer cell line, we showed that total murine cfDNA concentration (i.e. targeting non tumor-derived cfDNA) was higher in serum samples than in plasma samples. Conversely, when studying human cfDNA concentration (i.e. targeting specific tumor-derived cfDNA), cfDNA concentrations were higher in plasma samples (Fig. 1). This observation confirms that the increase of cfDNA in serum samples is due to the release of DNA from blood cells.

As plasma appears to be more adapted for specific cfDNA analysis, this chapter further focuses mainly on the pre-analytical factors potentially affecting cfDNA in the plasma fraction. Nevertheless, we will discuss the pre-analytical treatment of serum samples in Part 8 of this chapter.

2.2 Optimal Blood Collection Tube for Plasma Preparation

Plasma is obtained conventionally by blood drawing in either EDTA or citrate or heparin blood collection tubes. Heparin is prohibited for further PCR analysis since

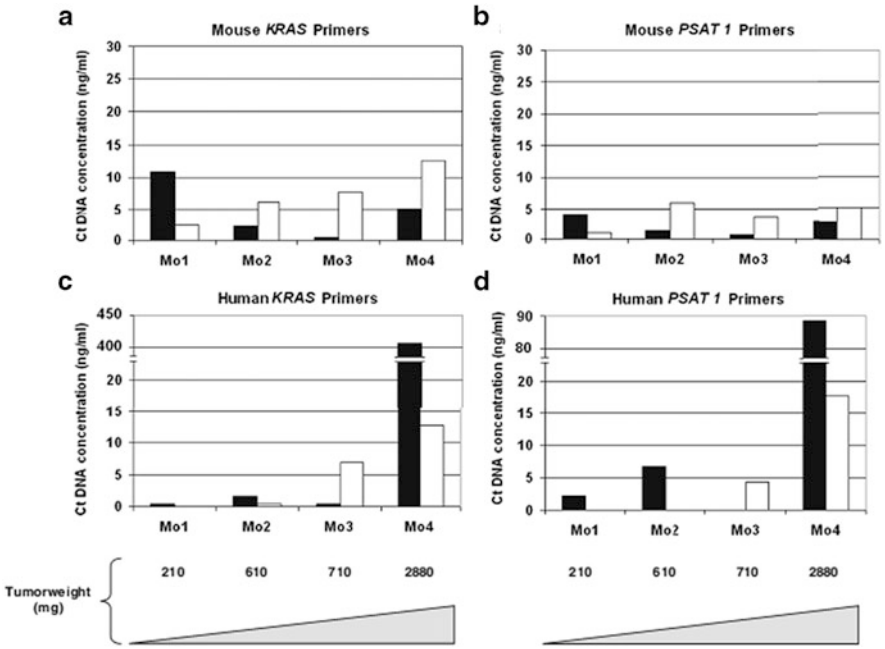

Fig. 1 Comparison of cfDNA amounts from serum (*light bars*) and plasma (*dark bars*) preparations. cfDNA concentration in plasma and serum from SW620 xenografts was determined using the mouse *KRA*S M3 (**a**), mouse *PSATI* M4 (**b**), human *KRAS* H2 (**c**) and human *PSATI* H5 (**d**) primer sets. ctDNA concentration for each mouse (Mo1, Mo2, Mo3 and Mo4) and the corresponding tumor weight (210, 610, 710 and 2,880 mg, respectively) are shown. Values were calculated from duplicate experiments each performed twice (from [20])

it is an inhibitor of the PCR [21]. K3EDTA collection tubes are commonly used for cfNA analysis, but another blood collection tube should be used, Cell-free DNA™ blood collection tubes, which are specifically dedicated for cfDNA analysis. They are composed of K3EDTA with an additive agent allowing the preservation of cfDNA in blood samples for up to 14 days at room temperature (RT) [22]. The additive agent stabilizes white blood cells, preventing the release of genomic DNA and inhibiting nuclease-mediated DNA degradation. In this chapter, we will mainly focus on blood collected in K3EDTA collection tubes and present some data published with Cell-free DNA™ blood collection tubes.

2.3 Blood Drawing Conditions

Holdenrieder et al. [23] showed that gentle hemolysis of blood samples triggered an increase in the plasma nucleosome level. They advised that blood should be drawn

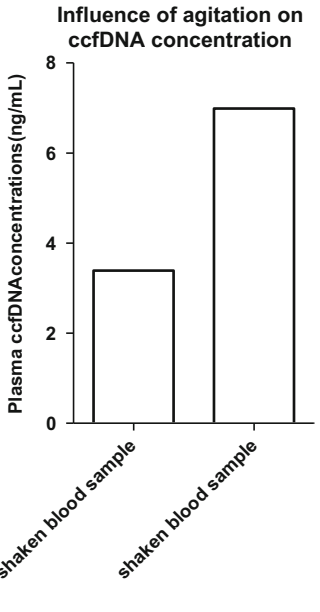

Fig. 2 Influence of agitation of K3EDTA blood samples on cfDNA concentration: samples from the same donor were incubated for 3 h at RT and one of them was submitted to constant agitation

carefully in order to avoid hemolysis. A similar observation was also reported by Norton et al. [24] where agitation of K3EDTA blood samples led to a significant increase in cfDNA concentration compared to non-shaken samples. We confirmed that agitation of blood samples was responsible for a two-fold increase in the cfDNA concentration compared to non-shaken samples (Fig. 2).

2.4 Storage Conditions of Blood Samples

As the anticoagulant effect of K3EDTA is limited over time, the main challenge in the pre-analytical treatment of blood samples is to avoid any release of genomic DNA by blood cells during storage. Generally, good practice recommends performing analysis on blood collected in EDTA collection tubes within 6 h following venipuncture as a decrease in red and white cell counts and morphology changes occur when analysis is further delayed [25]. The observed decrease in white blood cells may be due to either apoptosis or necrosis in the collection tube and lead to the release of genomic DNA from white blood cells, which may contaminate specific cfDNA. Moreover, cfDNA has also been described as being bound to the cell surface [26], assuming that nucleic acids can unbind from the cell surface and lead to an increase in cfDNA concentration with prolonged storage (see chapter "The Biology of CNAPS"). We can also hypothesize that cfDNA is actively released from blood cells in the collection tube.

Blood drawing and blood sample storage time and temperature need to be carefully defined. For these reasons, the influence of the storage time and temperature between venipuncture and plasma preparation are two parameters widely studied and reported in the literature [11, 13, 23, 27–29, 31]. It is now well known that between blood drawing and processing, cfDNA concentrations increase slightly with time, certainly due to apoptosis and necrosis of white blood cells, as described above.

2.5 *cfDNA Concentration*

State of the Art

The reported data are quite conflicting: some authors have shown a significant increase of cfDNA concentration after 2 h of storage compared to a baseline value (i.e. blood processed at $t = 0$), while other authors have reported this increase after 24 h. Conversely, all data have demonstrated that storage temperature (RT or +4 °C) has no influence on cfDNA concentration. Non-exhaustive data from various clinical fields are summarized in Table 2.

Our Observations

The influence of time delay and storage temperature on cfDNA concentrations between venipuncture and blood processing were tested in two different experiments. A slight increase in cfDNA concentration with time delay was observed and confirmed that storage temperature has no influence (Fig. 3a, b). Although a slight increase in the cfDNA concentration with time delay was observed, we confirmed that the cfDNA concentration did not vary significantly within 4 h following venipuncture at either RT or +4 °C. However, we demonstrated that 6 h of storage at RT triggered a two-fold increase in cfDNA concentration compared to samples processed soon after venipuncture.

2.6 *cfDNA Fragmentation*

Previous work on fetal cfDNA analysis reported the influence of time delay and temperature on cfDNA fragmentation [11] and demonstrated that fragmentation was not affected up to 6 h after blood sampling at both RT and +4 °C. Our group carefully examined the influence of these parameters on cfDNA fragmentation by determination of the DNA Integrity Index (DII: ratio of the mean cfDNA concentration determined using a primer set amplifying a 300-bp sequence to the mean cfDNA concentration determined using a primer set amplifying a 100-bp

Table 2 Data focusing on the influence of K3EDTA blood collection tubes storage conditions on cfDNA concentration

Article	Sample type	n	Influence of time delay	Influence of temperature storage
Predominant hematopoietic origin of cfDNA in plasma and serum after sex-mismatched bone narrow transplantation [15]	Healthy individuals	8	cfDNA concentration stable up to 6 h at RT	Not studied
Effects of pre-analytical factors on the molecular size of cell-free DNA in blood [11]	Healthy individuals	27	cfDNA concentration stable up to 6 h at RT and +4 °C. Significant increase after 24 h at RT and +4 °C	No difference between RT and +4 °C
Changes in concentration of DNA in serum and plasma during storage of blood samples [13]	Healthy individuals	10	cfDNA concentrations stable up to 8 h at RT or 24 h at +4 °C	No difference between RT and +4 °C
EDTA is a better anticoagulant than heparin or citrate for delayed blood processing for plasma DNA analysis [19]	Healthy individuals	10	cfDNA concentration stable up to 6 h at RT. Significant increase after 24 h at RT	Not studied
Implementing prenatal diagnosis based on cffDNA: Accurate identification of factors affecting fetal DNA yield [28]	Pregnant women	10	Total cffDNA concentrations stable up to 8 h at +4 °C and RT. Significant increase after 24 h at +4 °C and RT	No difference between RT and +4 °C
Nucleosomes in serum as marker for cell death [23]	Healthy individuals; solid tumors; acute infection	10	Time-dependent increase in the nucleosome values	Increase in nucleosome value is marked at +37 °C, but is less marked at RT and +4 °C
Plasma cffDNA concentrations in Maternal blood are stable 24 h after collection: Analysis of first and -trimester samples [27]	Pregnant women	29	Significant increase of maternal cffDNA after 6 h	Not studied
Isolation and extraction of circulating tumor cfDNA from patients with cell lung cancer [29]	Healthy individuals	10	No significant difference between the means of concentration up to 24 h after venipuncture	Not studied
Optimizing the yield and utility of circulating cfDNA from plasma and serum [31]	Healthy individuals	3	cfDNA concentrations stable up to 2 h at RT and 0 °C. Significant increase after 4 h at RT or 0 °C	No difference between RT and 0 °C

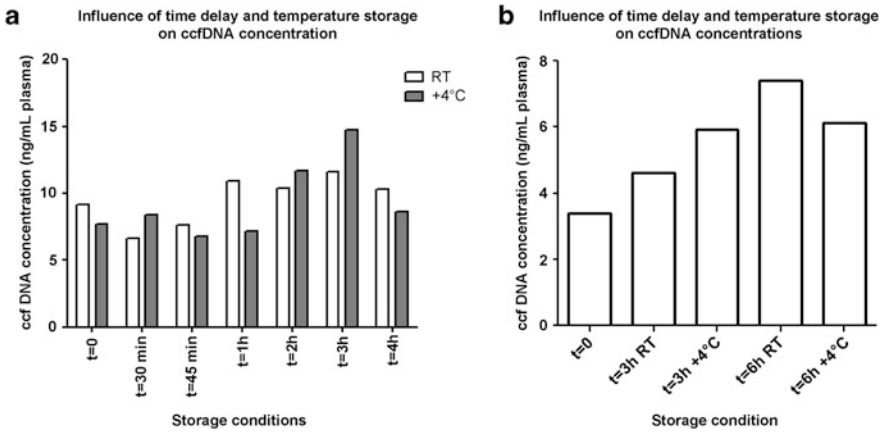

Fig. 3 (**a**). Influence of time delay between blood drawing and blood processing on total cfDNA concentration determined with a primer set amplifying a 105-bp sequence: samples from the same donor were incubated in different conditions. (**b**). Influence of time delay and temperature storage between blood drawing and blood processing on total cfDNA concentration determined with a primer set amplifying a 105-bp sequence: samples from the same donor were incubated in different conditions. Data in **a** and **b** were calculated from one either duplicate or triplicate experiment. Results were expressed as the mean (Adapted from [10])

sequence), reflecting cfDNA fragmentation. We observed that the DII was not affected for up to 3 h of storage at either +4 °C or RT. However, a long delay of 6 h and sample agitation triggered a slight decrease in the DII, indicating that cfDNA may degrade with time and agitation (Fig. 4).

2.7 Influence of Storage Conditions on Cell-Free DNA™ Blood Collection Tube-Drawn Blood Samples

The preservative agent of these tubes allows prolonged storage of blood samples at RT without any consequences on cfDNA level. Indeed, data published using these tubes report the conservation of cfDNA concentration values for up to 14 days at RT. Nevertheless, it seems that temperature variations can affect the cfDNA content and further studies need to confirm this observation. Some of the data are summarized in Table 3.

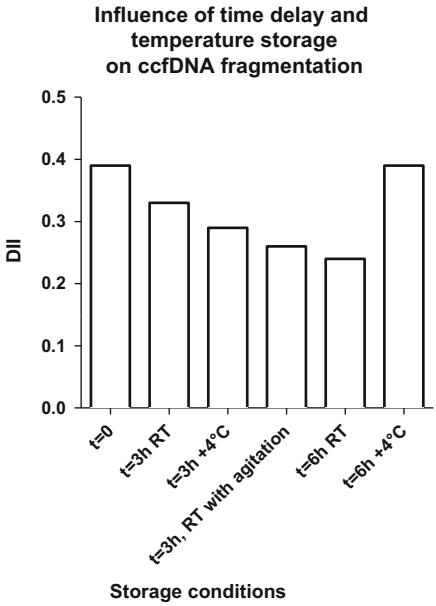

Fig. 4 Influence of storage conditions on DII: DNA integrity index (ratio of the mean cfDNA concentration determined using a primer set amplifying a 300-bp sequence to the mean cfDNA concentration determined using a primer set amplifying a 100-bp sequence). Data were calculated from one either duplicate or triplicate experiment. Results were expressed as the mean

2.8 Comparison Between K3EDTA and Cell-Free DNA™ Blood Collection Tubes

In the field of fetal cfDNA analysis, Hidestrand et al. [35] showed that there was no significant difference between cfDNA concentrations in samples shipped in EDTA collection tubes and samples shipped in cell-free DNA™ blood collection tubes when the blood was processed immediately. However, Fernando et al. [34] demonstrated that cfDNA levels in blood samples drawn in cell-free DNA™ blood collection tubes were stable for up to 14 days at RT, while they decreased when blood was drawn in K3EDTA collection tubes. Barrett et al. [28] showed that there was no significant difference for up to 3 days in the total cfDNA concentration when blood was drawn in cell-free DNA™ blood collection tubes, while it increased after 24 h when blood was drawn in K3EDTA collection tubes. In the field of oncology, there are still no comparative results when blood is drawn in either EDTA tubes or cell-free DNA™ blood collection tubes.

These data indicate that during this pre-analytical step, time delay and agitation should be treated with caution and in particular, the study protocol needs to define rigorously the time delay and the handling process. Agitation of blood samples should be prohibited.

Table 3 Data focusing on the influence of cell-free DNA™ blood collection tubes storage conditions on cfDNA concentration

Article	Sample type	n	Influence of time delay	Influence of temperature storage
A new blood collection device minimizes cellular DNA release during sample storage and shipping when compared to a standard device [24]	Healthy individuals	5	cfDNA concentration stable up to 14 days at RT	Significant changes after 7 days at +6 °C
Optimizing blood collection, transport and storage conditions for cfDNA increases access to prenatal testing [32]	Pregnant women	20	cfDNA concentration stable up to 14 days at RT	Strong temperature effect at +37 °C and +40 °C
Effects of a novel cell stabilizing reagent on DNA amplification by PCR as compared to traditional stabilizing reagents [33]	Healthy individuals	6	cfDNA concentration stable up to 14 days at RT	Not studied
Implementing prenatal diagnosis based on cffDNA: Accurate identification of factors affecting fetal DNA yield [28]	Pregnant women	9	Slight increase of cfDNA concentration after 72 h	Not studied
A new methodology to preserve the original proportion and integrity of cfDNA in maternal plasma during sample processing and storage [34]	Pregnant women	20	cfDNA concentration stable up to 14 days at RT	Not studied

2.9 Optimal Plasma Preparation

As the success of cfDNA analysis is largely dependent on the absence of contamination by genomic DNA, the main technical requirement for plasma preparation is to avoid any cell presence.

Indeed, Van Wijk et al. [36] studied the composition of plasma samples from pregnant women used to analyze cfDNA and reported the presence of fetal apoptotic cells in plasma. This was confirmed by Poon et al. [37] who found fetal cells in plasma samples from three pregnant women. This raised the following question: "Is plasma truly acellular after blood processing?"

State of the Art

Chiu et al. [38] studied different blood processing protocols on samples from pregnant women. They showed that a first blood centrifugation step at 1,600 g for 10 min. followed by a second plasma centrifugation step at 16,000 g for 10 min. was effective in producing cell-free plasma. cfDNA concentrations determined from these samples were similar to concentrations determined from plasma samples

obtained by centrifugation followed by filtration with a 0.2 μm filter (reference protocol ensuring the production of a cell-free plasma fraction). Inversely, a unique centrifugation step at 800 g was not effective to eliminate all the cells in the plasma fraction.

Swinkels et al. [39] confirmed these results and added that the second high-speed plasma sample centrifugation step at 16,000 g can be performed either before or after storage of plasma samples at −20 °C.

Another question raised is "Does centrifugation cause the ex vivo release of DNA from blood cells?" [30]. Indeed, we can assume that a high centrifugation speed destroys blood cells and leads to release of genomic DNA. Lui et al. [30] evaluated the influence of centrifugation speeds (from 400 g to 16,000 g) on cfDNA and revealed that there was no significant difference with the speed value. However, in this study, only one blood centrifugation step was performed and each plasma sample was then filtered with a 0.2 μm filter in order to ensure truly cell-free plasma.

Our Observations

We proposed the following protocol for isolating cfDNA: a first blood sample centrifugation step at 1,200 g for 10 min followed by a second plasma centrifugation at 16,000 g for 10 min at +4 °C. We checked the validity of this protocol using a third centrifugation step for 10 min. at 16,000 g: (i) DNA concentrations determined from the supernatants of samples after the second and the third centrifugation step were similar; (ii) no DNA was detected in the bottom of the tube subjected to the third centrifugation step by qPCR; (iii) microscopic observation of plasma pellets after the second and the third centrifugation step revealed the absence of blood cells.

These data confirmed that the second centrifugation step is sufficient to provide quality cell-free plasma for cfDNA analysis.

3 Pre-analytical Treatment of Plasma Samples Before Nucleic Acid Extraction

Few data on this analytical step are available in the literature, even though it is a crucial phase: indeed, we can hypothesize that cfDNA structures present in the plasma are sensitive to storage conditions. In addition to chromatin or nucleosome cfDNA complexes, cfDNA may be integrated in vesicles i.e. exosomes or apoptotic bodies, or within nucleolipoprotein complex structures, such as virtosomes [40]. These forms may disintegrate with time and, as a consequence, lead to further release of detectable cfDNA in plasma samples. Time delay before extraction,

temperature storage of plasma samples and freeze-thaw cycle numbers before nucleic acid extraction must be carefully specified.

3.1 Influence of Storage Conditions

cfDNA Concentration – State of the Art

One of the most extensive studies of this phase was by Holdenrieder et al. [41]. Even if it cannot be generalized to cfDNA analysis since the study was performed on serum and measures nucleosome levels only, this work indicates the sensitivity of nucleosomes to temperature variations: they seem to be more sensitive at +37 °C than at either +4 °C or RT. In serum samples stored at either +4 °C or RT for different lengths of time, the nucleosome level was stationary for up to 144 h of storage while at +37 °C, a significant decrease was observed after 6 h of storage. Considering that one part of cfDNA is complexed with nucleosomes, plasma samples should be stored at +4 °C or RT before nucleic acid extraction.

cfDNA Concentration – Our Observations

We showed that the cfDNA concentration slightly increased with time delay (from 0 to 4 h) before extraction at RT (Fig. 5a). We also tested different temperatures of storage and revealed that cfDNA concentrations were comparable when plasma samples were stored for 3 h at different temperatures (from −80 °C to RT) (Fig. 5b). However, we noted that the highest cfDNA concentrations were observed when samples were stored below +4 °C. The storage at RT triggered a slight decrease in cfDNA concentration.

3.2 cfDNA Fragmentation

We showed that the DII was not affected for up to 4 h of storage of plasma samples before extraction at RT. However, we observed also that the highest DII value was obtained when samples were stored below +4 °C as it seemed that storage at RT triggered a slight decrease of the DII value (Fig. 6a, b).

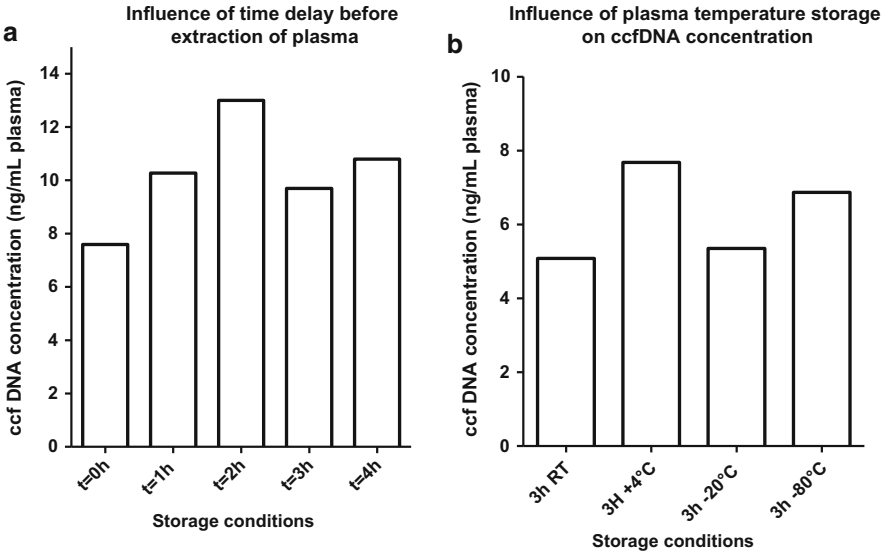

Fig. 5 (**a**). Influence of time delay before nucleic acids extraction on total cfDNA concentration determined with a primer set amplifying a 105-bp sequence: samples from the same donor were incubated in different conditions. (**b**). Influence of temperature storage between before nucleic acid extraction on total cfDNA concentration determined with a primer set amplifying a 105-bp sequence: samples from the same donor were incubated in different conditions (Adapted from [10])

3.3 Influence of Freeze-Thaw Cycles

cfDNA Concentration

Chan et al. [11] tested the influence up to three freeze-thaw cycles and showed that the cfDNA concentration was not significantly affected. We also investigated the effect of repeated freeze-thaw cycles and confirmed this observation (Fig. 7).

cfDNA Fragmentation

However, it seems that repeated freeze-thaw cycles leads to cfDNA fragmentation. Chan et al. [11] showed a significant decrease in the cfDNA DII after three freeze-thaw cycles were applied to plasma samples. Such an observation confirms the sensitivity of circulating cfDNA structures to temperature variations.

We also report that after three freeze-thaw cycles applied to plasma samples, the DII significantly decreases (Fig. 8).

These data highlight the need to carefully handle plasma samples before nucleic acid extraction.

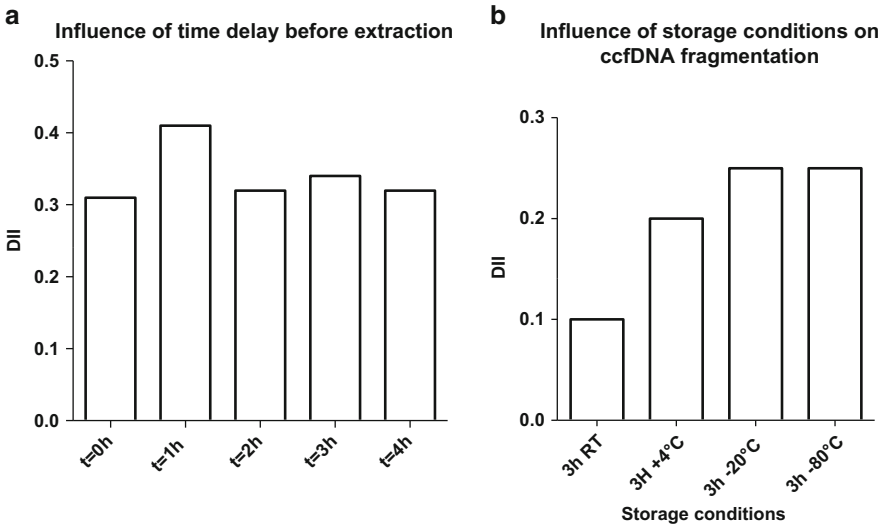

Fig. 6 (**a**). Influence of time delay before nucleic acid extraction on DII: DNA integrity index (ratio of the mean cfDNA concentration determined using a primer set amplifying a 300-bp sequence to the mean cfDNA concentration determined using a primer set amplifying a 100-bp sequence). (**b**). Influence of temperature storage between before nucleic acid extraction on DII: DNA integrity index (ratio of the mean cfDNA concentration determined using a primer set amplifying a 300-bp sequence to the mean cfDNA concentration determined using a primer set amplifying a 100-bp sequence). Data in **a** and **b** were calculated from one either duplicate or triplicate experiment. Results were expressed as the mean (Adapted from [10])

4 Pre-analytical Treatment of cfDNA Extracts Between Nucleic Acid Extraction and cfDNA Analysis

Storage of cfDNA extracts and freeze-thaw cycle number must be carefully defined.

Chan et al. [11] showed that cfDNA concentration and fragmentation were stable in cfDNA extracts stored at −20 °C for up to three freeze-thaw cycles. We also tested the influence of freeze-thaw cycles applied to cfDNA extracts stored at −20 °C. Our results did not show any modification up to the third freeze-thaw cycle, thus confirming the observations by Chan et al. (Fig. 9a, b).

5 Long-Term Storage of Plasma Samples and cfDNA Extracts

It is particularly important to define the duration of storage of plasma samples and cfDNA extracts for retrospective clinical studies.

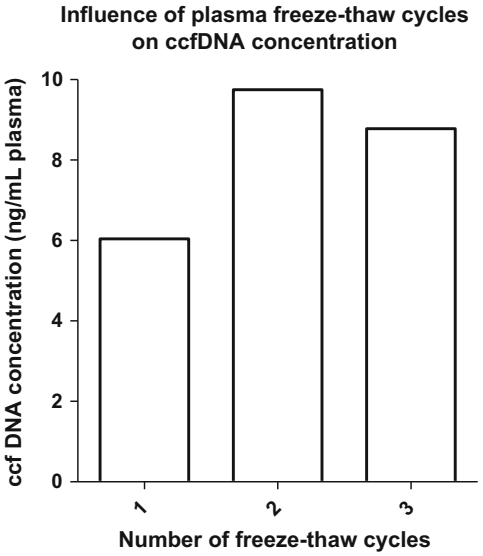

Fig. 7 Influence of repeated freeze-thaw cycles on total cfDNA concentration determined with a primer set amplifying a 105-bp sequence: samples from the same donor were submitted to either 2 or 3 freeze-thaw cycles. Data were calculated from either one duplicate or triplicate experiment. Results were expressed as the mean (Adapted from [10])

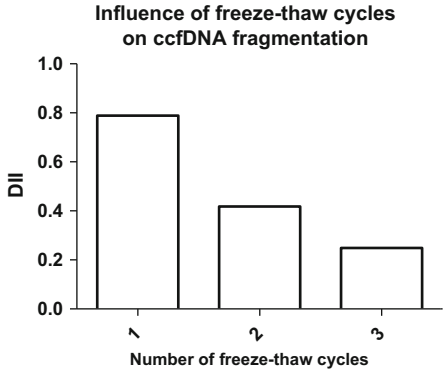

Fig. 8 Influence of repeated freeze-thaw cycles on DII: DNA integrity index (ratio of the mean cfDNA concentration determined using a primer set amplifying a 300-bp sequence to the mean cfDNA concentration determined using a primer set amplifying a 100-bp sequence): samples from the same donor were submitted to either 1 or 2 or 3 freeze-thaw cycles (Adapted from [10])

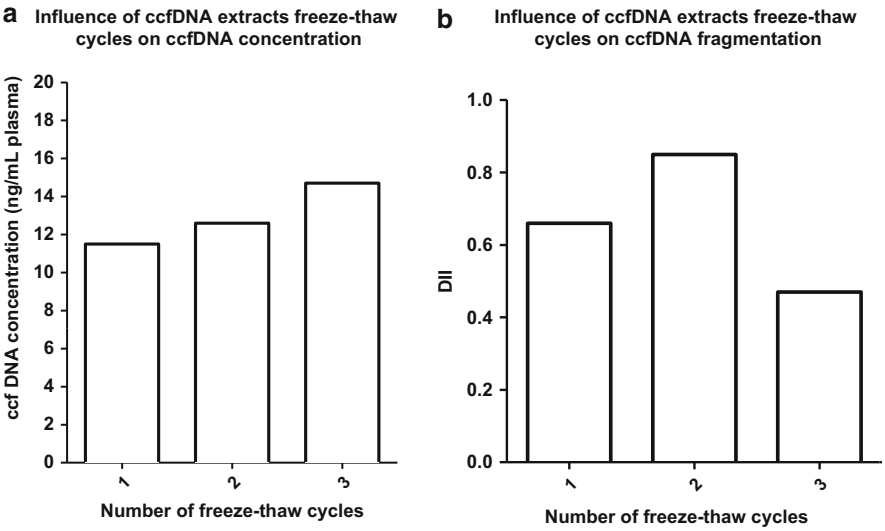

Fig. 9 (**a**). Influence of cfDNA extracts freeze-thaw cycles on cfDNA concentration on total cfDNA concentration determined with a primer set amplifying a 105-bp sequence: samples from the same donor were submitted to either 1 or 2 or 3 freeze-thaw cycles. (**b**). Influence of cfDNA extracts freeze-thaw cycles on DII: DNA integrity index (ratio of the mean cfDNA concentration determined using a primer set amplifying a 300-bp sequence to the mean cfDNA concentration determined using a primer set amplifying a 100-bp sequence): samples from the same donor were submitted to either 1 or 2 or 3 freeze-thaw cycles (Adapted from [10])

5.1 State of the Art

Table 4 summarizes the main data reported in the literature [11, 42–45] on the storage of plasma samples and cfDNA extracts. Each study compares data from two consecutive tests performed at different time intervals. The results are quite conflicting and clear conclusions cannot be drawn.

5.2 Our Observations

We performed a statistical study on the data obtained from samples used for a blinded, multicentre prospective clinical study comparing *KRAS*/*BRAF* mutational status determined from CRC plasma samples and paired CRC tumor sections (n = 106) [46]. The effects of storage at −80 °C on the cfDNA concentration were studied in 34 clinical plasma samples. Each sample was analyzed twice in the same way: extraction and immediate consecutive qPCR analysis. The time

Table 4 Data focusing on the influence of frozen storage of samples on ccfDNA concentration

Article	Sample type	Lenght of time between the two measurements (months)	n	Conclusion
Effects of preanalytical factors on the molecular size of cell free DNA in blood [6]	Healthy individuals	0.5	27	Storage of plasma at −20 °C for 2 weeks did not significantly affect the plasma DNA concentrations
Effects of prolonged storage of whole plasma or isolated plasma DNA on the results of circulating DNA quantification assays [41]	Lung cancer and healthy individuals	41	34 lung cancer 28 healthy individuals	Annual decrease of 30 % in cfDNA levels in plasma samples and cfDNA extracts
Down syndrome and cell-free fetal DNA in archived maternal serum [31]	Pregnant women	17–35	11	−0,66 GE/mL per month of storage
Long-term stability of circulating nucleosomes in serum [21]	Cancer	Median ± sd: 64.8 ± 5.5	154	Median decrease of 32 % in nucleosome levels in sera samples
Reproducibility of a semi quantitative measurement of circulating DNA in plasma from neoplastic patients [15]	Intestinal polyps or colorectal cancer	3	15	Absolute DNA content values concordant after three months of storage in cfDNA extracts

interval between the two analyses ranged from 1 to 270 days. Similarly, 25 cfDNA extracts stored at −20 °C were analyzed twice by qPCR. The time interval between the two analyses ranged from 1 to 150 days. To evaluate the effect of storage on cfDNA concentration, a variation factor, termed k, was determined (cfDNA concentration determined from the second analysis/cfDNA concentration determined from the first analysis).

The k-median values in cfDNA extracts ($n = 25$) and plasma samples ($n = 34$) were 0.88 and 1.03, respectively. No significant difference was shown between the k values in the two groups ($p = 0.293$). Alternatively, a significant difference was shown in the variance of the k values in the two groups ($p < 0.05$). The coefficient of variation of the k values in plasma samples was close to 50 %, while it was

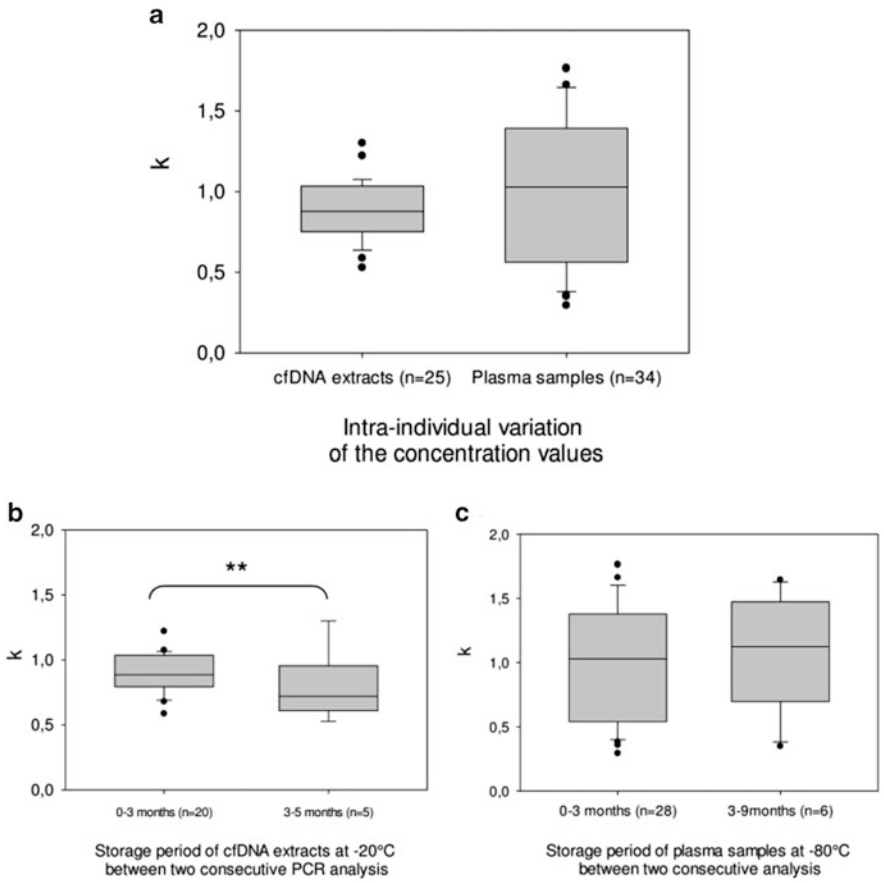

Fig. 10 Variation was evaluated using factor k: fold difference between two consecutive determinations of cfDNA concentration. (**a**). cfDNA concentration variation represented by k in 25 DNA extracts stored at −20 °C and 34 plasma samples stored at −80 °C. (**b**). cfDNA concentration variation represented by k in 20 DNA extracts stored at −20 °C for a period not exceeding 3 months and in 5 DNA extracts stored for more than 3 months. (**c**). cfDNA concentration variation represented by k in 28 plasma samples stored at −80 °C for a period not exceeding 3 months and in six plasma samples stored for more than 3 months. cfDNA concentrations were determined using the *KRAS* B1/B2 primer set (ng ml-1 plasma) (Adapted from [10])

inferior to the technical coefficient of variation (24 %) for cfDNA extracts (Fig. 10a).

Nevertheless, the k-median value in cfDNA extracts stored for less than 3 months was 0.89, while it decreased to 0.72 when cfDNA extracts were stored for more than 3 months (Fig. 10b).

In plasma samples, the k-median value was close to 1 whatever the length of the storage time, but there was a wide variation in the k values (Fig. 10c).

These data indicate that cfDNA concentration decreases with the length of the storage time; if the analysis aims to quantify and characterize cfDNA fragmentation then working on cfDNA extracts stored for up to 3 months is preferential. However, storage time has no influence on the detection of specific sequences or mutations in cfDNA as it has been shown that mutations can be detected several years after freezing plasma samples [47]; however, the sensitivity of the technique could be compromised since specific sequences may be present in smaller quantities after a long storage time.

6 Optimal Pre-analytical Treatment of Sera Samples

6.1 From Blood Drawing to Serum Preparation

Holdenrieder et al. [23] reported an optimal handling protocol for the measurement of nucleosomes in serum samples and recommended careful blood drawing in order to avoid any hemolysis. Centrifugation for serum preparation should be performed as soon as possible since it revealed an increase in nucleosome values with time delay before blood processing. This effect is more particularly marked at both RT and +37 °C. Such an observation is also reported by Jung et al. [13]: cfDNA concentrations significantly increase after 2 h of storage of blood samples and increases even more when samples are stored at RT rather than at +4 °C.

6.2 From Serum Preparation to the Analytical Process

Holdenrieder et al. [41] analyzed preanalytical parameters on serum samples such as vortexing-rolling-shaking, storage at different temperatures for different lengths of time and freeze-thaw cycles. The authors reported that vortexing-rolling-shaking serum samples had no influence on the nucleosome content. They observed that there was a slight modification of the nucleosome concentration after three freeze-thaw cycles.

The main preanalytical parameter studied was the duration and temperature of serum sample storage before analytical processing. They showed that nucleosome concentration was stable until 7 days of storage at both RT and +4 °C while it continually decreased at +37 °C and was significantly lower after 1 day of storage. This may be explained by the thermal activation of serum nucleases or nucleosome sensitivity to heating.

6.3 Long Term Storage of Sera Samples

Holdenrieder et al. [23] studied the influence of storage of serum samples at −20 °C for 0, 1, 2, 4, and 6 months and revealed no modification of the nucleosome value content. They reported an annual 7 % loss of nucleosome content value when serum samples were stored at −70 °C [43].

7 Preanalytical Treatment for cfRNA Analysis

As with cfDNA analysis, cfRNA analysis is concerned by the influence of many preanalytical parameters. Indeed, RNA is particularly known to be labile and degradable. Moreover, ribonucleases are present at high concentrations in blood and plasma. To explain the surprisingly relative stability of cfRNA in blood, it is assumed that cfRNA is protected by other structures: lipids, proteins, and nucleosomes [48].

Here the literature data on optimal treatment of samples for cfRNA analysis is summarized (see also sections "miRNA" and "Isolation Methods").

7.1 From Blood Drawing to Plasma or Serum Preparation

Tsui et al. [49] studied different protocols for optimal cfRNA analysis. Interestingly, they showed that there were two types of cfRNA: particle associated and non-particle associated cfRNA. In their study, they discriminated total cfRNA (particle associated and non-particle associated) and non-particle associated cfRNA obtained by plasma/serum 0.22 μm filtration.

Plasma Preparation from K3EDTA Blood Shipped Samples

The authors revealed that there was a significant modification of the total cfRNA yield with an increase in the time delay between venipuncture and plasma preparation at RT, while there was no significant modification when blood was stored at +4 °C. Conversely, for exclusive non-particle associated cfRNA, there was no effect of either time delay or storage temperature.

In contrast, Holford et al. [48] showed that total cfRNA concentration significantly decreased after 2 h of storage of blood samples at +4 °C.

These authors recommended processing plasma as soon as possible after venipuncture. However, in some cases, prolonged storage of blood samples is inevitable. It is clear that stringent preanalytical standardization is required when analyzing cfRNA. This is a critical issue for implementing this promising approach.

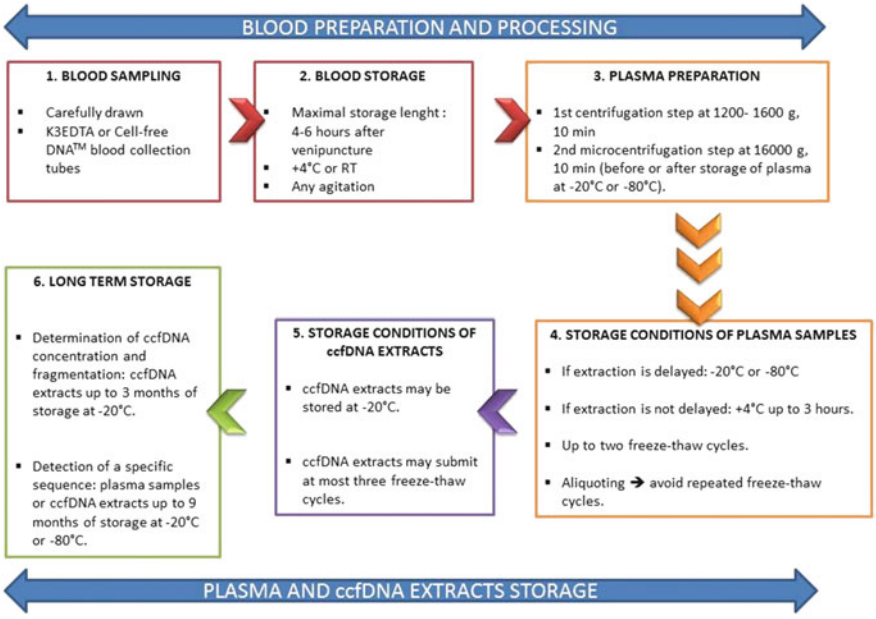

Fig. 11 Specific guidelines for plasma cfDNA analysis from peripheral blood drawing to storage

Plasma Preparation from cfRNA BCTs Blood Shipped Samples

Nevertheless, Fernando et al. [50] compared the stability of cfRNA in blood samples shipped in K3EDTA blood collection tubes and cell-free RNA BCTs. These specific tubes contain a chemical cocktail preventing RNA degradation by inhibition of RNAse. After 3 days of storage of blood samples at RT, the authors showed that cfRNA concentrations were stable in blood samples drawn in cfRNA BCTs while they significantly increased in K3EDTA blood samples.

Serum Preparation from K3EDTA Blood Shipped Samples

Tsui et al. [49] realized the same studies for serum preparation from clotted blood samples. They showed that total cfRNA concentration significantly increases with time delay before centrifugation at RT and +4 °C. Conversely, for the non-particle associated cfRNA, the time delay had no influence on its concentration.

8 Conclusions

The numerous discrepancies reported in the literature on cfDNA studies are mainly due to poor reproducibility and differences in handling procedures, thus highlighting their crucial importance. Analysis of data in the literature and our own results reveal the crucial influence of preanalytical factors on cfDNA analysis. Evaluation of all the factors potentially affecting cfDNA concentration and fragmentation leads us to describe here, for the first time, the optimal pre-analytical handling conditions for cfDNA analysis:

(i) plasma is a better matrix than serum since it avoids contamination of specific cfDNA by blood-cell genomic DNA;
(ii) EDTA or cell-free DNA^{TM} collection tubes prevent the release of genomic DNA by blood cells;
(iii) blood must be processed within 4 h following blood drawing;
(iv) high-speed centrifugation ensures the absence of any cells in the plasma and a second high-speed centrifugation step is highly recommended;
(v) plasma samples are sensitive to temperature variations and freeze-thaw cycles.
(vi) plasma must be aliquoted and may be stored at −80 °C for up to 9 months;
(vii) cfDNA extracts may sustain a maximum of three freeze-thaw cycles and storage at −20 °C for up to 3 months for cfDNA concentration and fragmentation analysis or 9 months for specific sequence detection.

The specific guidelines for plasma cfDNA analysis at each preanalytical step are represented Fig. 11.

Standardization of pre-analytical operating procedures would certainly consolidate the promising potential of cfDNA analysis as a powerful liquid biopsy [51–55] in the field of oncology and a diagnostic tool in prenatal diagnosis [56, 57].

Concerning cfRNA analysis, few data are available in the literature and further observations are needed for the standardization of handling procedures for cfRNAs.

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Circulating DNA and miRNA Isolation

Alain R. Thierry, Safia El Messaoudi, and Evelyne Lopez-Crapez

Abstract Analysis of circulating nucleic acids undoubtedly represents a breakthrough in the diagnostic field and in predictive, preventive and personalized medicine. In order to adequately and systematically study and to transfer this approach into clinical practice, standardization of the pre-analytical steps is a crucial prerequisite. Thus, during the first pre-analytical step, it is critical to achieve nucleic acid extraction from blood cell free nucleic acid with the highest purity and yields. Optimization of isolation processes will lead to a low variation of measurements and sensitive quantification of these macromolecules that are often present at low concentration and sometimes are physically tightly associated with biological constituents in the blood. Various isolation methods are used, but ready to use extraction kits appear as a good compromise with respect to routine application, especially in a clinical setting. Improvement or high specificity of the circulating nucleic acid analysis might be possible with a better knowledge of their form and structure. The choice of the biological source (serum vs. plasma) is described in the previous chapter. Circulating DNA and microRNA were recently applied in clinical practice; their isolation methods are here described and discussed.

Keywords Circulating DNA/RNA • mRNA • microRNA • Isolation • Plasma • Serum

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1 Circulating cfDNA Isolation

1.1 cfDNA Extraction Methods

Numerous methods either described in the literature or commercially available are proposed for the extraction of DNA from biological sources. They combine isolation and purification processes as DNA is a highly charged polyelectrolyte macromolecule prone to strong interaction with cationic elements, such as minerals, peptides and proteins, or supra-molecular complexes, such as histones. DNA isolation from cellular/biological components can be divided into four stages: (i) disruption, (ii) lysis, (iii) removal of proteins and contaminants and (iv) DNA recovery.

Non-commercially Available Methods

"Homemade" extraction methods have been described in the literature and many have achieved good yields, especially those using advanced procedures developed by the scientists who regularly use them. These include:

- Simple methods, such as boiling the preparations and other protocols that do not include a lysis step.
- Alcohol precipitation based on the precipitation of proteinase K-digested lysates after removal of the insoluble particles by centrifugation.
- Organic extraction with phenol/chloroform subjected to the proteinase K-digested lysate, vortexed, and centrifuged. The upper phase is then alcohol precipitated.
- The salting-out method, which consists in treatment of the proteinase K-digested lysate with a high-salt buffer. This is incubated and the proteins are precipitated by centrifugation. The supernatant is then subjected to alcohol precipitation.

Homemade extraction methods may enable correct DNA purification at a satisfactory yield using cost effective procedures.

The method based on cesium chloride density gradients, however, cannot be defined as a simple method. Cells are lysed using a detergent and the lysate is alcohol precipitated. Resuspended DNA is mixed with CsCl and ethidium bromide, and centrifuged for several hours. The DNA band, identified by ethidium bromide fluorescence, is collected from the centrifuge tube, extracted with isopropanol to remove the ethidium bromide and then precipitated with ethanol to recover the DNA. This method isolates high-purity DNA, but it is time consuming and uses toxic chemicals.

Commercially Available Methods

In commercially available DNA extraction kit procedures, protein removal is typically achieved by digestion with proteinase K followed by either salting-out or organic extraction or binding of DNA to a solid-phase support. DNA is commonly recovered by elution or precipitation using either ethanol or isopropanol. Biotechnology companies, or, more importantly, laboratory chemical and material suppliers, propose extraction kits based on either anion-exchange or silica technology or magnetic bead solid-phase support.

- The anion-exchange method provides high-molecular weight, ultrapure DNA for sensitive applications, such as cell transfection.
- Silica-membrane technology extracts high-purity DNA of medium to high-molecular weight.
- Magnetic-particle technology extracts high-purity DNA of high-molecular weight with a possibility of automation.

These three methods present the advantage of enabling standardization with relatively good repeatability and reproducibility.

1.2 Specificity of Extracted cfDNA

Only recent studies have better elucidated the forms of cfDNA with respect to the possible mechanisms of release involved. Necrosis, associated with tumor development, as well as apoptosis and phagocytosis associated with defence mechanisms, lead not only to the destruction of tumor cells, but also the adjacent, non-tumor tissues [1–3]. However, cfDNA fragmentation is higher after apoptosis than after either necrosis or phagocytosis [4]; DNA fragmentation is a hallmark of apoptosis. Specifically, cfDNA fragments longer than 10,000 bp are likely to originate from necrotic cells, whereas cfDNA fragments shorter than 1,000 bp, particularly 180 bp or multiples of this size, are reminiscent of the oligonucleosomal DNA ladder observed in apoptotic cells. cfDNA can circulate in blood by associating with histone complexes (nucleosomes) and apoptotic bodies, by binding to membrane parts, or in exosomes, or with nucleoproteolipidic complexes (virtosomes) ([5]; chapter "The Biology of CNAPS").

However, knowledge is still very poor concerning the proportion of these various mechanisms in regard to their cell origins (tumor cell-derived cfDNA vs. healthy cell-derived cfDNA), tumor types, tumor progression, patient age. It is obvious that better knowledge of this issue would favor a more specific and higher-yield extraction procedure. For instance, we have demonstrated, for the first time, the presence of a higher proportion of cfDNA fragments below 100 bp, particularly in samples from cancer patients [6]. Thus, contrary to the analysis of genomic DNA in which the concentration of quantified DNA is directly

proportional to the number of amplified copies, this proportionality varies with target sequence length in the case of cfDNA from CRC patients. Moreover, targeting 150- to 250-bp sequences (the length commonly chosen for amplification) generates a significant bias by not taking into account up to 80 % of the total cfDNA. Thus, the cfDNA size profile, as determined by amplifying targets of increasing length, reveals that optimal detection is obtained with amplicons <100 bp and that a much higher proportion of cfDNA of a size ranging from 150 to 400 bp is present in non-tumor cfDNA than in tumor cfDNA [6, 7]. Thus, higher and more accurate cfDNA quantification is now made possible, enabling a novel examination of cfDNA as a cancer biomarker. This buttresses the notion of using an isolation procedure enabling the extraction of small cfDNA fragments.

1.3 Studies on the Extraction Methods for cfDNA Extraction

Circulating cfDNA extraction is a key step in the detection process, yet very few publications exist on this subject compared to those on circulating cfDNA detection methods. According to Wang et al. [4] and Fong et al. [8], the choice of the extraction method can significantly contribute to the detection results. The main methods used for extracting circulating cfDNA are shown in Table 1 and can be distinguished as two groups: "homemade" methods that do not make use of a commercial kit (20 % of the methods listed in Table 1) and methods using ready-to-use extraction kits (80 % of the methods listed in Table 1). Chronologically, the phenol-chloroform method was the first circulating cfDNA extraction method employed followed by silica columns. Silica-coated magnetic beads are among the latest methods used.

"Homemade" methods, such as the phenol-chloroform method, are the oldest methods used for extraction and mostly use organic solvents. According to Fong et al. [8], circulating cfDNA extraction using the phenol-chloroform method with the addition of glycogen achieves higher concentrations when compared with DNA extraction kits and also extracts more small-sized fragments, but these methods take longer, are more complex and use toxic solvents. Furthermore, these methods, highly linked to the handling process, exhibit a high coefficient of variation and therefore are not suitable for quantitative clinical analysis.

On the contrary, ready-to-use extraction kits offer efficient, fast and simple extraction suitable for a clinical study. Moreover, most commercially available extraction kits are or may be automated. However, the documentary opacity of the reagents used and the lack of flexibility of these methods can be an obstacle to improving or developing new applications. For the different kits used in the cfDNA field and presented in Table 1, silica columns represent 70 % of the kits used with the QIAmp DNA blood kit alone representing 47 % of the kits using silica columns.

Methods based on silica-coated magnetic beads are more recent than the column-based methods and account for 30 % of the cfDNA extraction kits (Table 1).

Table 1 Selected reports illustrating the variety of the circulating DNA extraction methods used in the literature

cfDNA extraction methods	Extraction process	Publication title	References
Silica column	QIAmp DNA blood kit	Preferential isolation of fragmented DNA enhances the detection of circulating mutated k-ras DNA	Wang et al. [4]
		cfDNA fragments in the blood plasma of cancer patients: quantification and evidence for their origin from apoptotic and necrotic cells	Jahr et al. [1]
		Origin and quantification of circulating cfDNA in mice with human colorectal cancer xenografts	Thierry et al [9]
		Comparison of seven methods for extracting cfDNA from serum samples of colorectal cancer patients	Fong L et al. [8]
		Isolation and extraction of circulating tumor cfDNA from patients with small cell lung cancer	Board et al. [10]
		Method for extraction of high-quantity and-quality cfDNA from amniotic fluid	Lapaire et al. [11]
		Detection of circulating tumor-associated cfDNA in plasma of colorectal cancer patients and its association with prognosis	Lecomte et al. [12]
	QIAmp circulating nucleic acid kit	Analysis of circulating tumor cfDNA to monitor metastatic breast cancer	Dawson et al. [13]
		The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers	Diaz et al. [14]
		Emergence of KRAS mutations and acquired resistance to anti-EGFR therapy in colorectal cancer	Misale et al. [15]
	QIAamp MinElute virus spin kit	A modified extraction method of circulating cfDNA for epidermal growth factor receptor mutation analysis	Yuan et al. [16]
		Isolation and extraction of circulating tumor cfDNA from patients with small cell lung cancer	Board et al. [10]
		Detection of BRAF mutations in the tumor and serum of patients enrolled in the AZD6244 (ARRY-142886) advanced melanoma phase II study	Board et al. [17]
	NucleoSpin® plasma XS	Prognostic value of circulating mutant cfDNA in unresectable metastatic colorectal cancer	Lefebure et al. [18]

(continued)

Table 1 (continued)

cfDNA extraction methods	Extraction process	Publication title	References
		Quantification of cfDNA and RNA in plasma during tumor progression in rats	García-Olmo et al. [19]
	High pure PCR template preparation kit	Strategies of reducing input sample volume for extracting circulating nuclear cfDNA and mitochondrial cfDNA in plasma	Chen et al. [20]
Guanidine-resin method	Homemade method	Preferential isolation of fragmented cfDNA enhances the detection of circulating mutated k-ras cfDNA	Wang et al. [4]
		Comparison of seven methods for extracting cfDNA from serum samples of colorectal cancer patients	Fong L et al. [8]
Phenol/chloroform extraction	Homemade method	A rapid and sensitive assay for the detection of cfDNA fragmentation during early phases of apoptosis	Basnakian and James [21]
		Comparison of seven methods for extracting cfDNA from serum samples of colorectal cancer patients	Fong et al. [8]
		Strategies of reducing input sample volume for extracting circulating nuclear cfDNA and mitochondrial cfDNA in plasma	Chen et al. [20]
		A modified extraction method of circulating cfDNA for epidermal growth factor receptor mutation analysis	Yuan et al. [16]
Magnetic beads	ChargeSwitch gDNA serum kit	Comparison of seven methods for extracting cfDNA from serum samples of colorectal cancer patients	Fong et al. [8]
		Isolation and extraction of circulating tumor cfDNA from patients with small cell lung cancer.	Board et al. [10]
	ZR serum DNA kit	Comparison of seven methods for extracting cfDNA from serum samples of colorectal cancer patients	Fong et al. [8]
	Agencourt Genfind genomic DNA isolation kit	Isolation and extraction of circulating tumor cfDNA from patients with small cell lung cancer	Board et al. [10]
	Chemagic viral RNA/DNA kit	Microsystem for isolation of cffDNA from maternal plasma by preparative size separation	Hahn et al. [22]
	Chemagic viral RNA/DNA kit adapté	Circulating methylated SEPT9 cfDNA in plasma is a biomarker for colorectal cancer	De Vos et al. [23]

Based on previous work by the Thierry group and online discussions concerning extraction methods, the main problem of the technique is the variation of the extraction yield, frequently resulting in measurement repeatability problems during extraction repetitions. New automated methods (extraction in line with qPCR detection, BD MAX, Becton Dickinson) are appearing on the market and seem to be a good compromise due to the gain of time permitted by these machines. However, these automated methods may require larger initial sample volumes.

Fong et al. [8] compared the capacity of seven different extraction methods. Their unique data showed remarkable differences in the recovery of DNA from serum. The phenol-chloroform procedure, the sodium iodide method and the QIAamp DNA blood kit generated significantly higher yields of DNA, assessed by fluorescence measurement, than the four other methods (the guanidine-resin method, the ChargeSwitch serum kit, the ZR serum kit and the Puregene DNA purification system cell and tissue kit). "The higher recovery of DNA obtained with the NaI and PCI glycogen procedures was also revealed on the agarose gel, which interestingly showed recovery of substantial amounts of small DNA fragments (200–400 bp)" [8]. The phenol-chloroform and sodium iodide methods appear best suited to quantitative and qualitative DNA extraction, especially of small fragments, including cfDNA; however, they are inappropriate for routine use as part of the procedure for using cfDNA as a diagnostic tool, especially in a clinical setting. In addition, these methods use toxic chemicals and are also impossible to either standardize or automate.

In our laboratory, the commercially available methods were compared and the Qiagen DNA blood kit was found to provide the best methods for isolation and purification of cfDNA from the plasma of CRC patients [7, 9]. Under internal SOP, a coefficient of variation range of 5–10 % of the recovered amount was determined in several repeatability studies (data not shown). Also, according to Phillips and Mea [24]), this kit eliminates more PCR inhibitors compared to the homemade methods.

1.4 Criteria for Selecting an Optimal Extraction Procedure

Based on either the scientific or industrial goals, the choice of method concerns several factors: efficacy in extracting low-molecular weight DNA, the purity required for downstream applications, repeatability, reproducibility, time and the overall cost. Capacity of standardization is clearly a requirement when analyzing a biomarker potentially suitable for clinical testing. Figure 1 summarizes the characteristics of various commercially available extraction methods with respect to time, purity and small DNA fragment recovery. At present, extraction with a commercial kit, such as the DNA QiAmp blood kit (Qiagen), appears to be a good choice when analyzing cfDNA for clinical purposes.

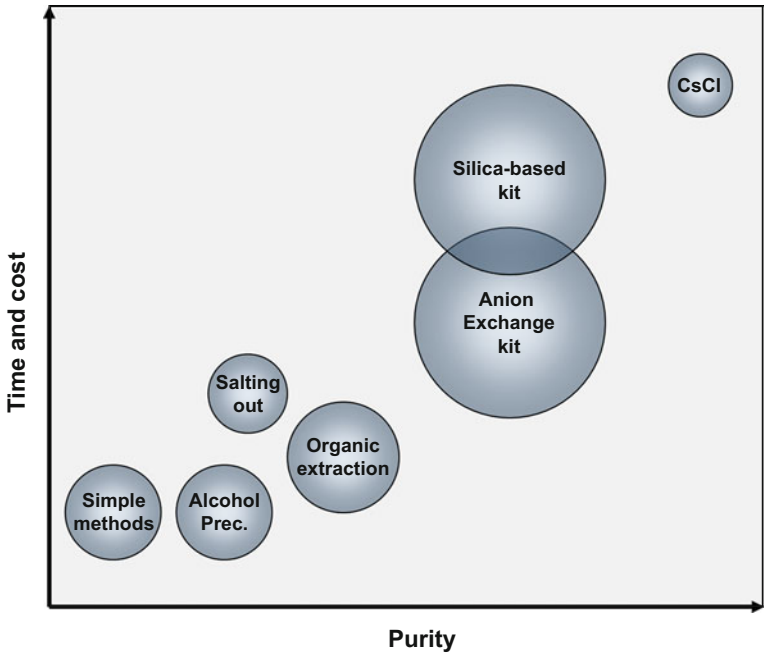

Fig. 1 Comparison of the various methods used for isolating circulating DNA from blood in regards to their time and cost, purity and standardization ability. Circles diameter is function of the standardization ability of each method

1.5 Direct Analysis of cfDNA Without an Extraction Procedure

Despite the progress in DNA tissue extraction methods, the currently available research methods for cfDNA measurement appear labor-intensive and expensive, requiring DNA extraction and qPCR amplification with specific primers. It seems worthwhile to consider investigating ways for directly analyzing cfDNA in plasma or serum. Goldshtein et al. [25] developed a convenient DNA assay applied directly to biological samples. This assay uses fluorochrome SYBR® Gold which does not require prior sample processing, i.e. DNA extraction and amplification. The assay is simply performed by adding diluted fluorochrome to the samples and measuring fluorescence. Recently, using this assay they measured cfDNA levels and followed tumor growth and rejection in mice and in CRC patients. They found that cfDNA levels are prognostic for disease progression and death Goldshtein et al. [25]. The direct SYBR® Gold assay proved to be an accurate and simple technique for measuring cfDNA in biological fluids. Current studies are ongoing to evaluate this new method for the detection and follow-up of breast cancer patients. However, this technical approach is limited to the quantification of cfDNA and does not enable the determination of other parameters such as either the presence of mutation or the cfDNA fragmentation level.

2 miRNA Isolation

Total RNA extraction is complicated by the presence of RNAse. Original extraction methods involving phase separation through centrifugation employed the addition of guanidinium thiocyanate to the organic phase to denature proteins, including RNAse, as well as separating rRNA from ribosomal proteins [26]. However, small RNAs (chapter "The Biology of CNAPS") are lost when using this approach. The better extraction methods for small RNAs employing either magnetic beads or silica columns are available as kits, as is the phase separation method of [26]. Of the small RNAs, mRNA from both serum and plasma has been exploited across a range of studies (chapters "CNAPS and General Medicine", "Fetal CNAPS: DNA/RNA", "Circulating Nucleic Acids and Diabetes Mellitus", "Extracellular Nucleic Acids and Cancer", "Other Body Fluids as Non-invasive Sources of Cell-Free DNA/RNA" and "CNAPS in Therapy Monitoring") with some success as possible early markers of various disorders though, so far, none appear to have successfully reached clinical trials for routine use. Work has only just commenced upon the other small RNAs (chapter "The Biology of CNAPS"). More recently, the presence of the more stable miRNAs in body fluids has opened up a more likely route to early markers, probably as a panel of early markers (chapters "CNAPS and General Medicine", "Fetal CNAPS: DNA/RNA", "Circulating Nucleic Acids and Diabetes Mellitus", "Extracellular Nucleic Acids and Can cer", "Other Body Fluids as Non-invasive Sources of Cell-Free DNA/RNA" and "CNAPS in Therapy Monitoring"). Therefore, this section will be specifically concerned with isolation of cfmiRNAs.

miRNAs are small non-coding RNAs that regulate RNA stability and gene expression ([27]; chapter "The Biology of CNAPS"). These ubiquitous molecules are involved in many key cellular processes, such as development, proliferation, differentiation and apoptosis [28–29] and are differentially expressed in various diseases. In addition to their presence in cells and tissues, miRNAs have been detected also in several biological fluids ([30]; chapter "Extracellular Nucleic Acids and Cancer"). Particularly, significant amounts of miRNAs are present in the human circulation [20, 31]. This finding has two major implications: (i) miRNAs may be considered as mediators of cell–cell communication [32] and (ii) circulating miRNAs hold great promise as potential non-invasive biomarkers for a broad spectrum of clinical conditions, such as cancer, heart disease and pregnancy (chapters "CNAPS and General Medicine", "Fetal CNAPS: DNA/RNA", "Circulating Nucleic Acids and Diabetes Mellitus", "Extracellular Nucleic Acids and Cancer", "Other Body Fluids as Non-invasive Sources of Cell-Free DNA/RNA" and "CNAPS in Therapy Monitoring").

However, accurate profiling and quantification of cfmiRNAs in blood samples are challenging and depend on proper miRNA isolation, choice of a sensitive detection technique and appropriate data analysis/normalization.

The main variables that may affect miRNA isolation, such as sample collection, storage and processing have been characterized [33]; however, there is no consensus on the optimal starting biological material and the techniques to extract cfmiRNAs vary from report to report. Moreover, to exploit the information obtained by miRNA profiling/quantification for clinical use two major considerations must be taken into account before pre-analytical choices: (i) the physical forms of circulating cfmiRNAs in blood samples and (ii) the presence of significant amounts of detectable cfmiRNAs in blood cells.

2.1 miRNA Stability in Blood and Plasma/Serum Samples

Several studies have demonstrated that, overall, miRNAs are stable in blood-derived samples for several hours or even days [31, 34, 35]. Successful miRNA profiling was performed using plasma samples that had been stored for more than 12 years [36]. Nevertheless, both the length and the temperature of storage before blood sample processing and before miRNA plasma/serum extraction affect significantly the quality of the circulating cfmiRNA profiles. Particularly, the stability of specific miRNAs in serum samples can differ. For instance, it has been reported that the levels of some miRNAs (e.g. miR-1 and miR-122) can decrease, while others (e.g. miR-16, miR-21 and miR-142-3p) remain stable upon prolonged serum incubation before extraction [37]. Moreover, digestion by the abundant human pancreatic RNase is the major cause of cfmiRNA degradation. Therefore, to prevent cfmiRNA degradation it is suggested to centrifuge blood samples at 4 °C within 2 h of sampling [36] and to add RNase inhibitors [37]. In all cases, it is important to follow serum and plasma standard sampling procedures (e.g. the standard operating protocols from The National Cancer Institute Early Detection Research Network – EDRN). Moreover, plasma/serum samples that are not analyzed straightaway should be stored at −70 °C where miRNAs can remain stable for up to 1 year [38], but freeze-thawing cycles should be avoided.

Another element that can influence the stability of circulating cfmiRNAs and their isolation is the existence of different forms of extracellular circulating miRNAs. miRNAs can be encapsulated in various types of vesicles (exosomes, microvesicles) that are secreted from cells [39, 40] or apoptotic bodies. Moreover, in the circulation, high-density lipoproteins transport and deliver miRNAs to recipient cells [41]. Then, miRNAs form complexes with proteins, such as Argonaute2 [42], or the RNA-binding protein nucleophosmin 1 [43]. As each miRNA form may have a different clinical impact [44] and because encapsulated miRNAs appear to be more stable than non-vesicle associated miRNAs [37], it is important to: (i) preserve all complexed miRNAs during the entire pre-analytical process and (ii) choose the adequate isolation technique corresponding to the specific form of circulating miRNA of interest.

2.2 Sample Collection: Plasma vs. Serum

As serum is the clinical sample more frequently stored in biobanks, a large number of studies have investigated miRNA quantification in serum. Nevertheless, the use of serum instead of plasma for the quantification of circulating miRNAs is debatable. In the first study on circulating miRNAs [31] the measurement of different miRNAs (miR-15b, miR-16, miR-19b, and miR-24) in matched plasma and serum samples from the same patient were strongly correlated, suggesting that both sample types are suitable for cfmiRNA analysis. However, further studies have highlighted differences in the types of miRNAs and/or their concentration in matched serum and plasma samples. For instance, Wang et al. [4] observed that, in healthy individuals, serum samples contain more detectable cfmiRNAs than do plasma samples. In addition, higher cfmiRNA expression levels were detected in serum compared to plasma samples. These authors concluded that plasma samples should be preferred for cfmiRNA profiling to avoid bias induced by the release of cellular cfmiRNAs during the coagulation process. In contrast, other studies reported lower amounts of circulating cfmiRNAs in serum than in plasma and suggested to use serum samples as starting material [35, 45]. These discrepancies are largely related to the coagulation process that modifies the true spectrum of circulating cfmiRNAs and to the contamination of plasma and serum samples by blood cells containing significant miRNA amounts [38]. Indeed, profiling of 365 miRNAs demonstrated a clear association between circulating cfmiRNA expression and residual platelet contamination of plasma specimens from patients and healthy controls [46]. Particularly, the subset of miRNAs that were most affected by plasma processing corresponded to those that are highly expressed in platelets (e.g., miR-142-3p, let-7a and miR-223) [47]. As a general rule, a platelet count in plasma samples is recommended before sample processing and addition of a high speed centrifugation (10,000 g) or a 0.22 μm filtration step for serum or plasma samples (even when stored for a long period) is advisable to avoid the confounding effects of platelet miRNA contamination. Similarly, circulating cfmiRNA quantification is affected by miRNAs coming from erythrocytes and hemolysis is associated with an increase in the copy number of red blood cell-associated miRNAs, such as miR-16, miR-15b and miR-451 [35, 48].

In conclusion, plasma is frequently considered as the sample of choice for cfmiRNA studies. Nevertheless, even when the plasma sampling and preparation methods are well controlled, the choice of the blood collection tubes can also influence cfmiRNA isolation and subsequent analyses. As heparin inhibits PCR assays, plasma for cfmiRNA profiling studies should be collected preferably in EDTA vacutainers and post-collection sample treatment with sodium fluoride and potassium oxalate (NaF/KOx) appears to be an attractive solution to increase cfmiRNA recovery [49].

In addition to serum and plasma, some authors assessed the potential of whole blood-derived miRNA profiles as a new tool for either cancer screening [50] or acute myocardial infarction detection [51]. Analyses performed using whole blood

samples are attractive because no further handling is required and snRNAs, such as RNU6, may be used for data normalization. Although cell lysis is prevented by using whole blood, the high cell proportion in such samples requires accurate, complete blood cell counts for adequate data interpretation.

2.3 miRNA Extraction Methods

Extraction of miRNAs from blood samples is challenging. Indeed (i) their abundance is low compared to proteins and lipids, (ii) they circulate predominantly associated with proteins or packaged in vesicles and (iii) they are small-sized single-strand entities (17–23 nt). In the case of packaged extracellular miRNAs, vesicles can be isolated using various techniques such as centrifugation/ultracentrifugation, filtration, column chromatography, immunoaffinity, polymeric precipitation and microfluidic devices [52].

Independently from the chosen starting blood material, the general principles for miRNA isolation are similar to those used for RNA extraction and combine chemical extraction and silica column-based purification. While some approaches allow the isolation of total RNA including small RNAs, others focus on miRNA enrichment. In addition, direct analysis of serum samples based on qPCR, without the need for miRNA isolation, has been performed to detect circulating cfmiR-21 in samples from patients with breast cancer [53]. This straightforward approach using as serum treatment a simple incubation step with 2.5 % Tween 20 followed by centrifugation at 9,000 g to eliminate proteins is both effective and reproducible.

The use of TRIzol® for solubilization/denaturation of biological material and proteins followed by a phase separation in the presence of chloroform and then alcohol precipitation is effective for RNA extraction. Its application for the isolation of circulating cfmiRNAs has given robust and reproducible results [54] and according to some authors could surpass the performance of column-based approaches. However, as TRIzol® fails to isolate miRNAs with low GC-content or secondary structure (e.g. miR-141 and miR-21) from small numbers of cells [51], its efficiency for blood-derived samples is arguable.

Several commercially available kits have been optimized for the retention of small RNAs along with many methodological modifications (Table 2). Their extraction efficiency, yield and reproducibility for circulating cfmiRNAs have been recently investigated and compared [36, 38, 55, 56]. The miRNeasy® serum/plasma kit has been reported [10] to give the highest miRNA yields from plasma. McAlexander et al. [56] compared four miRNA extraction methods and concluded that the Exiqon miRCURY™ RNA isolation – Biofluids kit was better than the frequently used miRNeasy® serum/plasma and *mir*Vana™ kits. A recent study compared seven different RNA extraction methods (phenol-based, column-based and combined phenol/column-based) starting from isolated exosomes and concluded that the miRNeasy® and miRCURY™ kits gave the best results in terms of relative amount of extracted cfmiRNAs [55]. Despite their convenience and

Table 2 List of commercially available miRNA extraction kits frequently used for miRNA profiling in blood-derived samples

Kit name	Manufacturer
miRNeasy® serum/plasma	Qiagen
mirVana™ PARIS	Life Technologies
miRCURY™ RNA isolation – biofluids	Exiqon
NucleoSpin® miRNA plasma	Macherey-Nagel
MicroRNA isolation	BioChain®
mirPremier® microRNA	Sigma-Aldrich®
miRNA isolation	Geneaid
PureLink®miRNA isolation	Life Technologies

reproducibility, one major drawback of column-based kits is the limited amounts of extracted miRNA, due to column saturation, when more than 200 μl of plasma are used [57]. Moreover, the use of a carrier (preferentially glycogen, otherwise bacteriophage MS2 RNA) is recommended to enhance miRNA recovery and for reproducible and robust isolation [41].

2.4 Quality Control of the Extracted miRNAs

Assessment of the quality and quantity of the miRNAs extracted from blood-derived samples is important for the reproducibility and accuracy of miRNA studies. Traditional methods for checking the quality/quantity of extracted miRNA from tissues, including spectrophotometry or automated capillary electrophoresis, are inappropriate for circulating cfmiRNAs due to low cfmiRNA yields obtained from serum and plasma samples. An alternative method based on spiked-in synthetic miRNA oligonucleotides is currently used [31]. These synthetic miRNA oligonucleotides, which are not naturally present in biological samples, are added before the RNA isolation step and are quantified to normalize for variations in RNA extraction efficiency and the presence of reverse transcription or PCR inhibitors [58]. Recently, two quality control procedures to identify samples with potential pre-analytical problems have been developed [59]. Specifically, the normal reference ranges for 119 circulating cfmiRNAs in serum and plasma samples are provided as well as a hemolysis indicator based on the difference of expression between miR-451 (erythrocyte-specific) and miR-23a (stable).

In conclusion, sample storage/processing and the RNA extraction method have a major impact on the results of cfmiRNA profiling and around 70 % of detectable circulating miRNAs show expression variations related to processing alone. To obtain accurate and reproducible data, one must keep in mind that serum and plasma samples, as well as the specific pre-analytical conditions related to the forms of circulating cfmiRNA, are not interchangeable and result in different circulating cfmiRNA profiles and concentrations. Therefore, a standardized list of details concerning the sample collection and processing should always be reported in publications and the same miRNA extraction protocol should be used for all

tested samples. Finally, whenever possible, cfmiRNA isolation from a given specimen should be performed at least in duplicate.

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Detection of Genetic Alterations by Nucleic Acid Analysis: Use of PCR and Mass Spectroscopy-Based Methods

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Abstract Cell free circulating DNA, isolated from blood has emerged as a potential biomarker in oncology. There has been also considerable progress towards theranostic application of circulating DNA. These applications were enabled by the increased use of the PCR technique and its derivates. PCR assays have become a widely used method for the quantification of circulating DNA in either plasma or serum samples. Moreover, PCR amplification is the basic method implicated in the majority of the circulating DNA analytical methods. This review focuses on the PCR and Mass-spectrometry methods developed to detect circulating DNA alteration from blood, in evolving applications such as cancer diagnostic tools. This review also gives advices and guidelines for designing PCR experiments with the specific requirements of circulating DNA. The concentration of circulating DNA, especially mutant circulating DNA, fragments can be too low for accurate measurements with other spectrophotometric methods. However, the accuracy, the high through-put and multiplexing capabilities of mass spectrometry becomes an interesting tool for the quantification as well as for the characterization of circulating DNA.

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1 PCR-Based Methods

PCR assays have become a widely used method for quantification of circulating cfDNA in plasma or serum samples as the concentration of circulating cfDNA can be too low for accurate measurements by other spectrophotometric methods. The process involves amplification of a single or a few copies of a piece of DNA to generate thousands to millions of copies of a particular DNA sequence. The method employs a pair of primers that span a target region in template DNA to polymerize partner strands in each direction via a thermostable polymerase in the presence of nucleotides. The process is repeated through multiple cycles to produce amplification of the targeted DNA region. In each cycle, the template and primers heated to separate the newly synthesized molecule and template and on cooling, the molecules become the template for the next synthesis round - and so on. There is a doubling of the number of copies with each round of synthesis. The PCR revealed itself to be a very sensitive method; however, this sensitive property is counterbalanced by either a high risk of false-positivity or prone to nonspecific amplifications of exogenous contaminants. Good laboratory practice and specific strategies to avoid contamination need to be settled to decrease the risk of contamination. Moreover, PCR amplification is the basic method involved in the majority of the circulating cfDNA analytical methods and in order to optimize its efficiency, we will consider the particular characteristics of the cfDNA in this chapter.

1.1 First PCR Application in CNAPS

In the story of CNAPS research, there a clear gap between the research performed before and after the implementation of PCR and subsequently, of its derivates. There was a sharp increase in the number of publications at the time when PCR was widespread in the research community and also at the time of quantitative PCR (qPCR) diffusion. The PCR methods, with their ability to detect and amplify unique fragments of DNA, are one of the key methodologies for the analysis of CNAPS.

PCR amplification is being used in a growing number of applications including gene expression quantification, expression profiling, SNP analysis, allelic discrimination and the detection and monitoring of genetic alterations.

The quantification of the template DNA during qPCR cycles is based on the re-emission of a fluorescent signal during the exponential phase of the amplification [1]. qPCR enables the sensitive and specific measurement of the fluorescence level at each cycle of the amplification process. The ability to read fluorescence at an early phase of the amplification, enables an acute quantification before the limiting

reagents, the inhibitors inactivate the polymerase so leading to a decrease in the efficiency of the amplification. The fluorescence intensity proportionally increases with each amplification cycle in response to the increase in the amplicon concentration i.e. the concentration of the piece of either DNA or RNA that is the product of the amplification.

The first cycle at which the instrument can distinguish the fluorescence generated by the amplification is termed the threshold cycle, or more recently, the quantification cycle (Cq) in the MIQE guidelines [2]. The MIQE guidelines were set up to enable researchers who are willing to work with qPCR to publish all the elements necessary for a precise reproducibility of their experiments.

It is also important to note that conscientiously performed good sample preparation is one of the key to unlocking qPCR's true potential. By preparing all nucleic acid samples in a highly defined and careful manner, the enzymatic reactions on which PCR, qPCR, RT qPCR depend are allowed to occur as intended [3]. A better knowledge of the DNA template structure and composition is also a key for getting optimal and accurate results in qPCR.

In 1997, Lo et al. [4] first described PCR detection of circulating cffDNA in maternal plasma and serum. Since then, circulating cffDNA amplification by PCR has emerged as an important method for non-invasive prenatal diagnosis. The advent of qPCR soon made it possible to measure circulating cffDNA concentrations in maternal plasma and soon after that, in the plasma of cancer patients ([5]; chapter "Extracellular Nucleic Acids and Cancer").

The first utilizations of qPCR systems with circulating tumor cfDNA focused mainly on the quantification of the total level of cfDNA and to estimate the diagnostic utility of the observed increased in cfDNA in the blood of cancerous patients.

One form of PCR, reverse transcription quantitative PCR (RT-qPCR), is mentioned here briefly. It is the method for the amplification of cfRNAs (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", "Extracellular Nucleic Acids and Cancer", "Other Body Fluids as Non-invasive Sources of Cell-Free DNA/RNA" and "CNAPS in Therapy Mon itoring") and most of the following general comments on PCR are also applicable to RT-qPCR.

1.2 Importance of a Good Knowledge of Structure and Size of cfNAs Prior to PCR Analysis

The specific detection of circulating tumor DNA within a mix of cfDNA fragments in plasma requires the use of molecular methods and is based on the genetic, epigenetic or structural differences between normal and tumor- derived DNA. Given the small fragment size of cfDNA and the varying proportions of tumor-

derived DNA in the pool of circulating plasma cfDNA, cfDNA detection initially proved to be technically challenging.

As PCR amplification is closely dependant on the size of the targeted DNA, a good knowledge of the size and structure of circulating cfDNA, is firmly recommended in order to optimize and rationalize experiments using PCR methods. qPCR is widely used to characterize and quantify circulating DNA (Table 1). qPCR methods exhibit an apparently lower sensitivity than other sophisticated methods for studying cfDNA e.g. droplet digital PCR or BEAming (Table 2). But this limitation is not only attributable to the technique itself and a better knowledge of cfDNA structure enables the reaching of equivalent levels of sensitivity with a modified qPCR technique [23, 37].

Also, measurements of local variations in gene copy numbers can be performed in circulating cfDNA and the correct measurement of a copy number variation relative to a reference region by qPCR hinges on the use of a non-biased quantitative PCR assay. A reliable determination of the quantities of different DNA targets is important as well in the analysis of circulating cffDNA in the plasma of a pregnant woman. In a situation where a duplex qPCR assay or two separate singleplex assays are used to compare abundances of two different target regions in a sample of fragmented DNA, it is crucial that the quantitative detection of the two targets is not influenced by different degrees of degradation of the target DNA sequences so leading to incorrect quantities of the respective target.

1.3 Methods Based on an Increased Sensitivity for the Detection of Genetic Alterations with CNAPS

Standard PCR–based assays have a relatively limited sensitivity for detecting infrequent genetic alterations and cannot detect mutations that represent less than 5–10 % of the total pool of alleles. Identification of somatic genetic and epigenetic aberrations now has been facilitated by the advent of highly sensitive techniques [38]. Moreover, high sensitivity polymerase is known to induce less error during the amplification process. Tailored PCR focusing on specific structural genomic variants—translocations, insertions and deletions—known to be present in the primary tumor allows for high sensitivity analysis (0.001 %; which represents the detection of one mutant allele among 100,000 wild-type alleles).

Other qPCR-derived methods permit a high sensitivity of detection of the mutational profile. Nested PCR, ARMS, ASB, Intplex, Bi-PAP, Allele specific ligation PCR (Table 2) are methods already used on clinical cohorts and validated for the detection of genetic alterations in plasma samples. In addition new digital based approaches have been developed, including BEAMing, digital PCR and digital droplet PCR [35, 39]. These PCR-based methods with a high sensitivity are limited by the fact that the exact genomic aberrations to be investigated must be known *a priori* or because the methods are either labor intensive or challenging to

Table 1 Bibliographic study of the main publications analyzing the integrity of cfDNA

Publications	Cancer type	Targeted amplicons size (bp)	Observed DII (Long amplicon concentration/ short amplicon concentration)	DII modification in the corresponding study	Sample origin	Extraction method	Isolation method	Analysis method	Targeted area
Wang et al. [6]	Breast	100/400	0.66 (cancer) 0.14 (healthy)	DII increased for cancer samples	Plasma	Qiagen blood kit	1 centri 2,000 g 10 min	qPCRSYBR green	Beta actin
Umetani et al. [7]	Breast	115/247	0.35 (cancer) 0.13 (healthy)	DII increased for cancer samples	Serum	No extraction method detailed	1 centri 1,000 g, 15 min, process in 6 h, use of a CORVAC separation tube	qPCRSYBR gold	ALU
Deligezer et al. [8]	Pancreas	115/247	0.4 (cancer) 0.19 (healthy)	DII increased for cancer samples	Serum	Roche kit	Non detailed	qPCR	ALU
Schmidt et al. [9]	Lung	135/419/ 618	2.6 (cancer) 1.9 (non tumoral) 1.9 (cancer) 2.3 (non tumoral)	DII stable for cancer samples DII stable for cancer samples	Plasma serum	NucleoSpin Plasma XS kit (MN)	1 centri 11,000 g, 10 min	qPCR	ERV
Ellinger et al. [10]	Testicular	79/220	0.760 (cancer) 0.560 (healthy)	DII stable for cancer samples	Serum	ChargeSwitch DNA Serum Kit (Invitrogen)	Coagulation, 1 centri 1,800 g, 10 min	qPCR	16s-rRNA (circulating mitochondrial DNA)

(continued)

Table 1 (continued)

Publications	Cancer type	Targeted amplicons size (bp)	Observed Dll (Long amplicon concentration/ short amplicon concentration)	Dll modification in the corresponding study	Sample origin	Extraction method	Isolation method	Analysis method	Targeted area
Ellinger et al. [11]	Spleen, Kidney, Prostate	79/220	0.852 (cancer) .662 (sain)	Dll increased for cancer samples	Serum	ChargeSwitch DNA Serum Kit (Invitrogen)	Coagulation, 1 centri 1,800 g, 10 min	qPCR	16s-rRNA (circulating mitochondrial DNA)
Ellinger et al. [12, 13]	Testicular	106/193/ 384	0.384 (cancer) 0.681 (healthy)	Dll decreased for cancer samples	Serum	ChargeSwitch DNA Serum Kit (Invitrogen)	Coagulation, 1 centri 1,800 g, 10 min	qPCR	ALU
Holdenrieder et al. [14]	Gastrointestinal urological gynecological	137/347	0,33 (cancer) 0.38 (healthy)	DII stable for caner samples	Plasma & serum	Nucleic acid large volume kit & MagNA pure	Non precise	qPCR	Reference gene of light cycler kit
Chan et al. [15]	Non cancerous	105/145/ 201/ 280/ 356/ 449/ 576/ 697/ 798	fetal Dll smaller than maternal Dll	99 % of fetal DNA have a size smaller than 300 pb	Plasma	Qiagen blood kit elution in 200 lil water	2 centri 1,600 g 10 min, 16,000 g 10 min EDTA tubes	qPCR Taqman probe	Leptin & SRY
Mouliere et al. [16]	Colorectal	73/145/300	0.122 (cancer) 0.565 (healthy)	Dll decreased for cancer samples	Plasma	Qiagen blood mini kit	2 centri 1,200 g 10 min, 16,000 g 10 min	qPCR	*KRAS*, *BRAF*, *ACTB*

The type of cancer, the presence of a particular physiological state, the extraction method, the targeted gene, the size of PCR amplicons are detailed in this table

Table 2 Brief explanation of the variety of PCR developments

Method	Description	References and/or application to CNA
Quantitative PCR (Q-PCR)	Classical quantification of fluorescent intercalating molecules or probes during PCR amplification	Lo et al. [17] and Jahr et al. [18]
Amplification Refractory Mutation System (ARMS)	Sequence-specific PCR primers that allow amplification of test DNA only when the target allele is contained within the sample. In general the 3′ nucleotide of one of the PCR primers matches the targeted gene mutation.	Little et al. [19] and Board et al. [20]
Allele Specific Blocker PCR (ASB-PCR)	Terminal 3′ nucleotide of one of the PCR primers matches the targeted gene, low Tm primer, and oligo-blocking nucleotide (primer with phosphate group for blocking WT non-specific amplification).	Morlan et al. [21]
Intplex (Q-PCR based method)	Multi-marker analysis of short fragments cell-free DNA concentration, mutation and fragmentation.	Mouliere et al. [22] and Thierry et al. [23]
Pyrophosphorolysis-activated polymerization (PAP/bi-PAP)	The 3′ extremity of the PCR primer is blocked. This base can be removed (which leads to extension and amplification) only if the template DNA is mutated.	Liu and Sommer [24, 25] and Madic et al. [26]
Peptide Nucleic Acid/Locked Nucleic Acid (PNA/LNA)	PNA/LNA probes block primer binding to nonmutated DNA.	Däbritz et al. [27]
Digital PCR (dPCR)	Microfluidic system separate template DNA in partition cells and enables individual copy amplification and detection	Vogelstein and Kinzler [28];
Digital droplet PCR (ddPCR)	Each DNA template is amplified separately in emulsion droplets	Hindson et al. [29] and Taly et al. [30]
Co-amplification at lower denaturation temperature PCR (COLD PCR)	Enrichment of mutated fragments of DNA in a mix of mutated and wild type DNA fragments, by preferential amplification of heteroduplexes DNA produced during PCR thermal cycling.	Milbury et al. [31] and Makrigiorgos et al. [32]
DISSECT (Differential Strand Separation at Critical Temperature)	A method that enriches unknown mutations of targeted DNA sequences purely based on thermal denaturation of DNA heteroduplexes without the need for enzymatic reactions	Guha et al. [33]
BEAMing (Beads, Emulsion, Amplification and Magnetics)	This method combines emulsion PCR with magnetic beads and flow cytometry for the detection and quantification of mutant tumor DNA.	Diehl et al. [34, 35] and Higgins et al. [36]

design. The DISSECT seems to be a nice method to overcome the limitations of locus specific assay [33], otherwise NGS methods are also enabling non-locus specific analysis of cfDNA [40, 41].

With its ability to perform absolute quantification of cfDNA, the dPCR technology presents a conceptual advantage compared to classical qPCR, especially for the determination of copy number variations [42]. Whenever cfDNA is analyzed, copy numbers derived from PCR assay with fragmented DNA as a template cannot easily be interpreted as haploid whole genome equivalents because the qPCR assay counts only the copies of the available full length target. This implies that the detected copy number is, in fact, dependent on the either the qPCR or dPCR assay performed. Therefore, target copy numbers obtained for the same sample using different assays should only be compared after performing appropriate tests that check for quantification bias via a proper normalization. Digital droplet PCR is an evolution of dPCR where the partitioning consists in an emulsion generation. This method presents a conceptual advantage that the number of emulsion might be increased, which will also increase the sensitivity of this approach to very high level (0.001% of mutant DNA detected [30, 43]).

1.4 Other Approaches

Common PCR will amplify both the major (wildtype) and minor (mutant) alleles with the same efficiency, excluding the ability to easily detect the presence of low-level mutations. Another strategy for detecting low frequency mutations is based on the specific enrichment of the mutated fraction of the cfDNA. COLD-PCR and its derivatives are able to efficiently enhance the proportion of mutated cfDNA ([44]; Table 2). The underlying principle of COLD-PCR is that single nucleotide mismatches will slightly alter the melting temperature (Tm) of the double-stranded DNA. Depending on the sequence context and position of the mismatch, Tm changes of 0.2–1.5 °C are common for sequences up to 200 bp or higher. Each double-stranded DNA has a 'critical temperature' (Tc) lower than its Tm. The PCR amplification efficiency drops measurably below the Tc, the latter being dependent on the DNA sequence. Two template DNA fragments differing by only one or two nucleotide mismatches will have different amplification efficiencies if the denaturation step of the PCR is set to the Tc. This is achieved by using preferential denaturation of mismatch-forming mutations at critical denaturation temperature [32]. Using two consecutive COLD-PCRs, mutations can be enriched by 100-fold or more [33, 45].

A multi-marker approach, and beyond a multiparametric method, would lead to an increased sensitivity and performance in answering current diagnosis questions [16, 37, 46]. In previous work, the concentration of cfDNA, the fragmentation of this DNA, the presence of mutation, the allelic frequencies of the detected mutations and the epigenetic alterations were used with a multiparametric analysis for discriminating between cancer and healthy patients [22, 46]. The relative

contributions of each of these parameters in this analysis are not yet investigated in order to answer other biological questions, but this could increase either the discrimination of early stage cancer or the prognosis of patients that are difficult to investigate by other methods.

NGS technologies have allowed the development of new approaches for cfDNA analysis. NGS analysis of plasma cfDNA can be used to (i) determine the presence of a given mutation and estimate its allelic frequency within a sample [40, 47] and (ii) perform whole-exome or whole-genome characterization of the mutational profile in a cancer ([41, 48]; chapter "Genomic Approaches to the Analysis of Cell Free Nucleic Acids"). These latter sequencing methods would enable the identification, and follow-up of new mutations linked to the emergence of treatment resistance.

1.5 General Guidelines Applicable to All PCR Applications for CNAPS Analysis

DNA Preparation

It is important to note that the lack of reproducibility between the different studies has already been identified for a long time as one of the most important drawback in cfDNA analysis (van der Vaart [49]; chapter "Extracellular Nucleic Acids and Cancer"). The preparation of the DNA corresponds to the isolation and extraction steps (chapters "Pre-analytical Requirements for Analyzing Nucleic Acids from Blood" and "Circulating DNA and miRNA Isolation"). These two steps, as well as the storage of the extracted DNA, require a better standardization to increase the reproducibility of cfDNA analysis. In order to avoid variability induced by the quantification method and strategy, it is also recommend to set up a MIQE or a dMIQE guideline as soon as possible [2, 50].

Anti-contaminations Procedures

As for the classical PCR protocol for genomic DNA [51], the preparation of each of the analytical steps, namely blood isolation, DNA extraction, PCR preparation, preparation of the controls and PCR assay, should be performed in separate and designated working areas. A one way workflow is also required to avoid PCR contaminants for the later steps of the process [52]. The preparation of the PCR reagents should also be made in a dedicated laminar flow cabinet. In previous work, it has been reported that the inclusion of uracil N-Glycosylase would prevent the unwanted amplification of PCR products from previous reactions.

Choice of the Target Size

The choice of the size of the template DNA to amplify is an essential information, especially when it is intended to work with highly fragmented DNA. The majority of the studies using PCR systems for quantifying cfDNA were targeting sequences ranging from 120 to 150 bp. In addition to the high PCR efficiency of amplifying sequences of this range, this choice was based on agarose gel electrophoresis size analysis of cfDNA indicating a typical ladder at 180 bp and multiples. This size would correspond to the fragmentation induced by the apoptosis phenomenon. However, more recent work seems to indicate that circulating cfDNA would be more fragmented than was previously thought, especially in samples from cancer patients, with a peak of fragmentation comprised of between 80 and 145 bp, depending on the study [16, 53, 54]. Mouliere et al. found that qPCR primer pair amplifying DNA fragment ranging from 60 to 100 bp are optimal to quantify circulating cfDNA from metastatic colorectal cancer patients. This apparent greater fragmentation could be induced by a subsequent degradation of the cfDNA by phagocytosis in the bloodstream. Thus, different levels of fragmentation are also observed depending on the cancer type, the targeted gene and the analytical method (Table 1). However, the smaller the amplicon targeted, the greater are the potential number of amplified products. Nevertheless, as the efficiency of the PCR decreases when targeting amplicons of low size, the normalization process of the PCR efficiency is essential to ensure a precise quantification. Table 1 summarizes the different sizes observed depending on the study.

Thermal Cycling

cfDNA analysis with PCR methods does not required a particular thermal cycling. It is, however, recommended so as to avoid long steps of annealing and extension (as cfDNA is highly fragmented into short size fragments, longer steps are not required if it is considered that an error rate of incorporation of 1 per 10,000 nt with classical Taq Polymerase). The fidelity of amplification by PCR is dependent on several factors such as: annealing and extension time, annealing temperature, dNTP concentration, salt concentrations and the type of polymerase used. In general, the rate of misincorporation may be reduced by minimizing the annealing/extension time, maximizing the annealing temperature and minimizing the salt concentrations.

By default, a classical thermal cycle would be composed of a first step of denaturation of 5–10 min at 92–94 °C for initiating a first denaturation and/or activate hot start Polymerase when used. During cycle succession, a denaturation at 92–94 °C for 1–2 min is generally used. The temperature and annealing duration is dependant on the base composition, the length and concentration of the primers. An annealing step at 55–60 °C for 30 s is generally accepted. This step is crucial for a difficult primer-template pair and requires optimization to avoid non-specific

product formation. The primer extension step is generally performed at 72 °C for 1 min. In fast-PCR protocols, annealing-extension steps are grouped together with a temperature of >60 °C only. The recently developed microfluidics system for qPCR or dPCR would require specific uncommon thermal cycling.

Normalization

qPCR is widely used to characterize and quantify circulating cfDNA [3]. qPCR methods exhibit an apparently lower sensitivity than other sophisticated methods for studying cfDNA such as BEAming or dPCR. This limitation is not only attributable to the technique itself and a better knowledge of cfDNA structure enables nearly equivalent levels of sensitivity with a modified qPCR technique to be reached [23]. However, the normalization procedures used in such qPCR experiments are, in general, identical to qPCR realized with a genomic template DNA (Table 3) even if the cfDNA is structurally different. In general, the amplification of either one or a few target sequences in a quantitative PCR assay is used to quantify the target DNA molecules by comparing the amplification signal of the unknown sample to a standard curve with a known DNA concentration. In the case of circulatory cfDNA, the concentration of the sample is usually determined by comparing the sample to a standard that is made up of high-quality genomic DNA. The concentration of this quantification standard is, in general, determined by measuring UV absorbance with a spectrophotometer (for example the nanodrop system), or fluorescence with a spectrofluorimeter (for example, the Qubit system).

In most cases a standard curve with genomic DNA from human healthy lymphocytes is used to determine the DNA quantification. However, this template is not the better way to mimic circulating tumor cfDNA as genomic DNA is not fragmented and tumor cfDNA seems to be highly fragmented with more than 80 % of the fragments being less than 145 bp [16, 70]. This raises the question as to whether or not circulatory, fragmented DNA can be accurately quantified by qPCR when high-quality genomic DNA is used as a quantification reference. When analyzing circulating tumor cfDNA it is necessary to modify the classic procedures of normalization to ensure an accurate DNA quantification with qPCR. More precisely, taking into account the size of the DNA template and its structure and complexity would be important for the accurate determination of DNA concentrations. DNA fragmentation leads to a lower availability of intact target sequences when compared to high quality genomic DNA such that, in a circulatory cfDNA sample, it may no longer be possible to determine the number of diploid or haploid genome equivalents from the detected number of target sequences.

In this sense, digital dPCR represents a time-saving and more reproducible method as no standard curve is required for normalizing the data and for determining the absolute quantification of the PCR products. The main technologies for dPCR are those based on a physical partitioning of the DNA molecules [40] and those based on an emulsification process for separating the DNA molecules [30, 43].

Table 3 Analysis of the normalization procedures used in qPCR and qPCR derived methodological studies related to circulating cfDNA and cfRNA quantification

Publications	Objectives of the study	Template	Normalization method	Positive controls	Type of PCR	Normalization gene	Disease
Thierry et al. [23]	KRAS, BRAF mutation determination	cfDNA	Standard curve on genomic DNA	Mutated cell-lines	Intplex	None	Colorectal cancer
Spindler et al. [55]	KRAS, BRAF mutation determination	cfDNA	Standard curve on PCR products (381 bp)	Directed mutagenesis	ARMS	*gCYC*	Colorectal cancer
Ellinger et al. [12]	Discriminate apoptotic and necrotic DNA fragments	cfDNA	Not described	Not described	Q-PCR	*PTGS2, Reprimo*	Bladder cancer
Madic et al. [26]	GNAQ, GNA11 mutation determination	cfDNA	Peripheral blood cells DNA	Mutated cell-lines	PAP qPCR	PBMC	Uveal melanoma
Perkins et al. [56]	KRAS, BRAF, PIK3CA mutation determination	cfDNA & FFPE	OncoCarta (?)	OncoCarta (?)	ARMS	OncoCarta (?)	Different cancer types
Schwarzenbach et al. [57]	8 LOH & μsatellite markers	cfDNA	leukocyte DNA of each patient serve as reference	N/A	qPCR	Leukocyte DNA	Breast cancer
Shaw et al. [58]	Confirm amplification identified on SNP array	cfDNA	ΔΔCT mean relative concentration	Not described	qPCR	Normal genomic DNA	Breast cancer
Musolin et al. [59]	Determine levels of cfDNA in pediatric lymphomas	cfDNA	Standard curve on genomic cell line DNA	none	qPCR	None	Lymphoma
Garcia-Olmo et al. [60]	Quantify cfDNA & cfRNA during tumor progression	cfDNA & cfRNA	Standard curve of differents size for cfDNA & RNA	DHD/K12 cell-line	qPCR clamp	None	Colon cancer
Gao et al. [61],	Monitoring of personalized chemotherapy	cfDNA	Relative/control	N/A	qPCR	Linear recombinant plasmid	Lung cancer

Flamini [62]	Comparison CEA & cfDNA as biomarker	cfDNA	Standard curve on DNA extracted from peripheral blood of healthy donor	N/A	qPCR	N/A	Colon cancer
Gal et al. [63]	Quantify level of cfDNA in serum of breast cancer	cfDNA	Standard curve on genomic DNA	N/A	qPCR	N/A	Breast cancer
Pinzani. et al. [64]	cfDNA integrity in the blood of cancer patients	cfDNA	Standard curve on genomic DNA + utilization of DNA from healthy donor blood (unclear ?)	N/A	qPCR	N/A	Melanoma
Rago et al. [65]	Tumor burden on xenografted mice	cfDNA	Standard curve on normal DNA purified from human lymphocytes	N/A	qPCR	N/A	Colon cancer
Schmidt et al. [9]	Integrity of cfDNA in lung disease	cfDNA	Not described, relative tumor/non tumor deduced from data	Not described	qPCR	Not described	Lung cancer
Umetani et al. [7]	Prediction of tumor progression with cFDNA integrity	cfDNA	Standard curve on genomic DNA from healthy donor peripheral blood	N/A	qPCR	N/A	Breast cancer
Gorges et al. [66]	Cancer therapy monitoring	cfDNA	Standard row (with short amplicons ?)	Cancer cell lines	qPCR	N/A	Breast and colon cancer
Saukkonen et al. [67]	Predictor of outcome in severe sepsis	cfDNA	Standard curve with human genomic DNA (provided by Roche)	None	qPCR	None	Sepsis
Soh et al. [68]	MASI	cfDNA	Relative/LINE1	Cell line	qPCR	LINE1	Lung, Panc, Colon cancer
Zhou et al. [69]	Rare allele enrichment	Mutated DNA	Second derivative for determining Cq	Cell line	AS qPCR	Not given	Thyroid biopsy

Melt Curve Analysis

The introduction of melt-curve analysis is a plus in the detection strategy of low frequent mutations in a mix of WT samples. However, this step would be time consuming e.g. if the fluorescence level is monitored from 50 to 90 °C with an increment of 0.2 °C, this represents approximately 1 h of machine time. Surprisingly, the High Resolution Melt strategy has not been heavily explored for the discrimination of mutated tumor cfDNA and healthy cfDNA [31, 71]. For downstream mutation screening, high resolution melting (HRM) curve analysis is simple, rapid and inexpensive to perform yet exhibits a high sensitivity when scanning for unknown, low-abundance mutations and variants. The reported sensitivity of HRM is largely determined by fragment length, sequence composition, mutation identity, PCR quality and equipment. Although recent publications report the ability to detect <1 % mutant in wild-type DNA, most applications of HRM-based assays exhibit a detection capability of approximately 5–10 % mutant among wild-type alleles. Although HRM mutation scanning is highly sensitive and efficient, HRM lacks the ability to identify the specific nucleotide change; this is a particularly important issue when mutations or variants are not known *a priori* and are likely to occur at any position within the amplicon sequence.

Melt-curve analysis is not automatically available in recent dPCR technologies and in particular, in ddPCR. In this case, the specificity of the primer amplification would need to be investigated before the experiment or based on the droplet repartition. With ddPCR, specific strategies should be employed in particular in case of multiplexing [30, 43].

1.6 Conclusion

PCR and its derivates have been extensively used since the beginning of the 1990s for detecting and quantifying circulating cfDNA in both cancer and prenatal diagnosis research. qPCR and dPCR have found wide spread applicability in the analysis of gene alteration measurement and cfNAs in body fluids (chapter "Other Body Fluids as Non-invasive Sources of Cell-Free DNA/RNA"). The assay is readily amenable to automation by making use of either the current real-time PCR 96–384 well formats or microfluidics system. PCR amplification is the basic method involved in the majority of the circulating cfDNA analytical methods. In order to optimize its analysis, the consideration of the structural particularities of cfDNA, as well as their pre-analytical parameters, are recommended. In spite of these technical challenges, the quantitative analysis of circulating cfDNA is gaining increased importance as a tool of molecular diagnostics, addressing various pathological or physiological conditions. Circulating cfDNA derived from tumors can be detected in either plasma or serum and the concentration of a tumor-specific target region may reveal details about the malignant condition which might be explore with other technological approaches.

2 Mass Spectrometry-Based Methods

The mass spectrometer has been used for the analysis of biological complex structures such as DNA and peptides since the development of soft ionization techniques known as ElectroSpray Ionization or ESI [72] and Matrix Assisted Laser Desorption/Ionization or MALDI [73, 74]. In the electrospray ion (ESI) source of the mass spectrometer, the DNA solution, either directly infused or injected through a liquid chromatography device, is converted into a plume of fine droplets and submitted to an intense electric field. DNA molecules are thus ionized, accelerated and resolved with respect to their mass to charge ratio (m/z) in a time of flight analyzer (TOF). The MALDI ionization mode requires the use of a matrix that, under LASER illumination, transfers to DNA molecules the electric charges necessary for their extraction and analysis as described for the ESI mode.

2.1 Mass Spectrometry in Nucleic Acids Sequencing

In the 1990s, the first attempts to use either ESI or Maldi mass spectrometry to sequence DNA fragments [75] were quickly surpassed by the rapid development of large scale sequencing technologies. One of the major pitfalls of this approach was the necessary purification of the DNA fragments before their mass spectrometry analysis and the propensity of DNA molecules to be associated with one or more Na+ or K+ ions. Several groups have then developed sample purification procedures compatible with mass spectrometry [76–79] in return for which mass spectrometry had some advantages over electrophoretic techniques in the detection and resolution of frameshift mutations [80]. Recently, taking advantage of the information-rich spectra obtained from high resolution ESI-MS/MS QqTof apparatus, the use of mass spectrometry in fast DNA sequencing was reinvestigated. The interpretation of fragmentation ion mass spectra is accomplished using specific comparative sequencing algorithms (COMPAS) [81]. By applying these algorithms, the sequencing of DNA fragments of up to 80 nucleotides could be verified in a few seconds. However, the most popular application of mass spectrometry in DNA analysis concerns the detection of single-nucleotide polymorphisms.

2.2 SNP Genotyping Using Mass Spectrometry

As the full sequence of the human genome is now uncovered, there is an increasing demand for the precise knowledge of individual variation in the sequence. Some of these variations known as SNPs only affecting one per 1,000 bases, represent 90 % of the genome modifications. Mutations induced by these SNPs must be synonymous (i.e. no change in the amino acid composition if the mutation occurs within an

exon), mis-sense (changing one amino acid) or nonsense (introducing a defect in the resulting protein). As the number of SNPs identified increases, their impact on population genetics, genetic disease identification and disease susceptibility becomes essential. The SNP-disease association is now a parameter that could not be ignored and many of them are indexed in specific databases (geneticassociationdb.nih.gov).

Whereas sequencing is the standard method for the characterization of a SNP, MALDI mass spectrometry methods for SNP genotyping have been designed. Methods are based on allele specific hybridization with either PNA-probes [82] or allele specific ligation [83] or allele specific cleavage of oligonucleotides [84]. However, only the primer extension method followed by mass spectrometric analyses was implemented for high throughput genotyping. For the assay, a primer is annealed immediately upstream of a SNP and a combination of dNTP and ddNTP is added with the aid of a DNA polymerase.

The single extension primer protocol utilizes a single primer per SNP and generates allele specific products with distinct masses. The Sequenom MassARRAY platform utilizes this method that was demonstrated to be attractive for accurate custom genotyping assay. Many detailed protocols describing the primer extension method are accessible [85, 86]. This approach was chosen to profile critical cancer gene mutations in clinical tumor samples of mutations in patients and then to predict patient outcome and/or inform on treatment options [87]. In this study, 396 mutations on 33 cancer genes were analyzed by the primer extension mass spectrometry approach establishing an OncoMap sequence for each patient.

Today, this technology is widely used and has become a standard in specific gene mutation detection in large cohorts of patients [88], in pharmacogenomics response prediction of the side effects in chemotherapy in cancer treatment [89, 90] and in the discovery of disease susceptibility due to gene mutations [91].

2.3 Application of Mass Spectrometry to the Studies of Circulating Cell-Free Nucleic Acids

cfDNA, and more particularly, circulating cfDNA is now demonstrated to become an important tool for either clinical diagnostics or disease follow-up. Since the discovery that patients with various types of cancers have high level of circulating cfDNA in their plasma, a new era opens where possible mutations could be detected after a minimally invasive act on the patient [37, 41, 92–94].

In this area, the accuracy, the high throughput and multiplexing capabilities of mass spectrometry becomes an interesting tool for the quantification as well as for the characterization of circulating cfDNAs. Each of them requires an initial step of PCR amplification of the genomic region of interest. Specific methods were developed for the analysis of amplified fragments. Both quantitative and qualitative

information could be drawn from the short oligonucleotide mass analysis (SOMA) based on the ESI-MS assay on short fragments generated by use during the PCR of two Bmp1 containing primers and the accurate determination of the mass of each amplified allele fragment [95]. This approach was validated by comparison with alternative methods [96]. Some refinements of the mass spectrometry characterization of circulating cfDNA, but coupled to a MALDI-MS analysis were proposed such as the single allele base extension reaction (SABER) [97] and the allele specific base extension reaction (ASBER) [98]. These two last cited methods are refinements of the most used MassEXTEND (hME) reaction but using specific (SABER) or competitive (ASBER) ddNTP as elongation reaction inhibitors [99].

2.4 Conclusion

Coupled with either existing PCR methods or directly on DNA extracts, mass spectrometry becomes the adequate technology in this domain due to its flexibility, high sensitivity and adaptability to high throughput analysis at affordable costs. The complementarity of MALDI and ESI ionization modes together with the development of new software will make mass spectrometry a method of choice for analyzing circulating cfDNA. The past decade has seen the increasing use of circulating cfDNA from various origins in the early diagnosis of several diseases. As interest increased, the methods of characterization needed to be more robust, sensitive and accurate. The technological improvement of PCR, mass-spectrometry and NGS enable now to probe ctDNA at different levels of resolution, and would increase the clinical utilization of cfDNA as a biomarker.

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Genomic Approaches to the Analysis of Cell Free Nucleic Acids

Wenying Pan and Stephen R. Quake

Abstract Recent advances in high throughput DNA sequencing and microarray technologies have revolutionized the field of genomics and also opened up many opportunities for the analysis of cell-free nucleic acids. These genomic approaches have not only provided a more comprehensive portrait of the landscape of cell-free nucleic acids, but also enabled a number of non-invasive genome-wide diagnostic methods. In this chapter, we introduce the basic mechanism of high throughput DNA sequencing and discuss some unique characteristics of cell-free nucleic acids that make their experimental procedure for high throughput analysis different from ordinary cellular nucleic acids. We describe different DNA sequencing protocols that have been used for cell-free DNA, including whole genome sequencing, exome sequencing and targeted amplicon sequencing. We explain the statistical model underlying the detection of copy number variation and point mutation from cell-free DNA. We also review recent clinical applications of sequencing cell-free DNA, from the non-invasive diagnosis of fetal genetic defects, to detection of tumor mutations from plasma and monitoring rejection of organ transplantation. In addition, we outline the perspective of profiling cell-free mRNA and cell-free microRNA using RNA-seq and microarray, and their potential applications. Finally, we conclude with discussions of the current challenges and possible future advances for genomic analysis of cell-free nucleic acids.

Keywords High through-put sequencing • Sample preparation • Counting principle • Cancer • RNA profiling

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1 Introduction of High Throughput Sequencing

High throughput sequencing methods are a variety of the DNA sequencing methods that can read the sequence of thousands to millions of DNA molecules in parallel. To differentiate these methods from Sanger sequencing, these methods are referred to as next generation sequencing. High throughput sequencing usually can be divided into two steps: DNA template preparation and sequencing [1]. There are two strategies used in preparing DNA templates: (1) clonally amplified templates originated from a single DNA molecule that could enhance the fluorescence signal during imaging; (2) a single DNA molecule template that could reduce the errors introduced during amplification. There are two common methods for clonal amplification: solid-phase amplification and emulsion PCR and two popular strategies for sequencing: sequencing-by-synthesis and sequencing-by-ligation. For example, the most widely used high throughput sequencing platform – Illumina sequencer, is based on solid-phase amplification and sequencing-by-synthesis. The mechanism of Illumina sequencing technology involves: hundreds of millions of DNA templates are immobilized on the surface of a glass slide. For each single template molecule, up to 1,000 identical copies are created in close proximity by solid-phase amplification. The hundreds of millions of DNA templates are then sequenced using the sequencing-by-synthesis method. A single labeled dNTP is added to the nucleic acid chain during each sequencing cycle. The nucleotide label serves as a terminator for polymerization, so after each dNTP incorporation, the fluorescent dye is imaged to identify the base and then enzymatically cleaved to allow incorporation of the next nucleotide (Fig. 1). Besides Illumina, other commercially available high throughput sequencing platforms include 454 Roche that incorporates emulsion PCR amplification and sequencing-by-synthesis technology, SOLiD that uses emulsion PCR amplification and sequencing-by-ligation technology and PacBio that provides single molecule sequencing.

2 Overview of the High Throughput Analysis Methods for cfNAs

Generally, the high throughput sequencing or microarray profiling of cfNAs follows similar protocols to those used for bulk tissue or cultured cell lines (Fig. 2). Sequencing analysis can be divided into two major categories: one is shotgun sequencing, which means globally sequencing an unbiased sample of the whole genome or transcriptome from the sample. The advantage of shotgun sequencing is that it does not require assumptions about which molecules may be present, but obtaining such comprehensive coverage can be expensive. The other category is targeted sequencing which can achieve much greater coverage at locations of interest. The targeted region could be all of the exons in the genome, termed exome sequencing; it could be also for specific genes, such as the genes related to

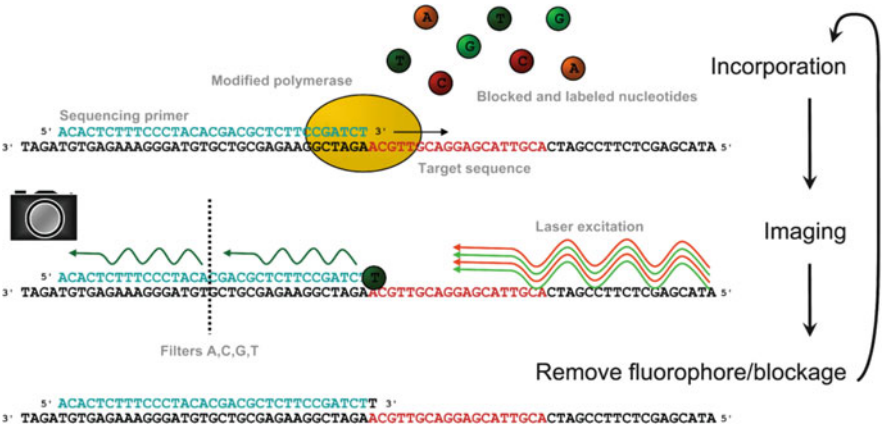

Fig. 1 Scheme of Illumina sequencing method. Sequencing primers are annealed to the adapters of the sequences to be determined. Polymerases are used to extend the sequencing primers by incorporation of fluorescently labeled and terminated nucleotides. The incorporation stops immediately after the first nucleotide due to the terminators. The polymerases and free nucleotides are washed away and the label of the bases incorporated for each sequence is read with four images taken through different filters. Subsequently, the fluorophores and terminators are removed and the sequencing continued with the incorporation of the next base [2]

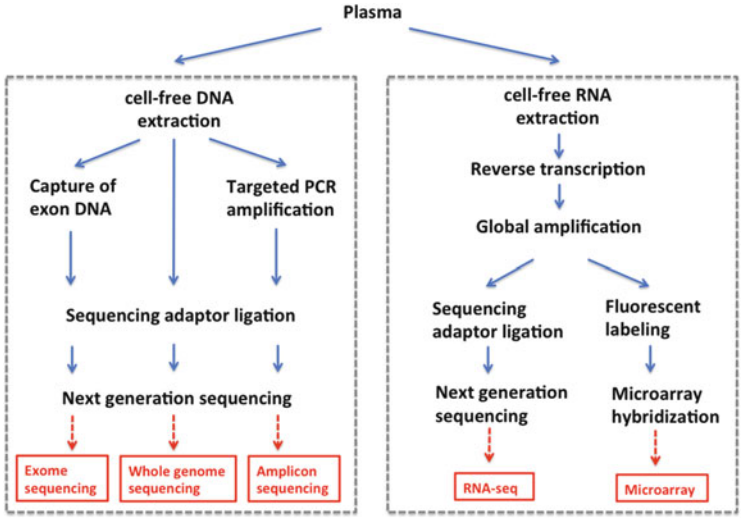

Fig. 2 The workflow of high throughput methods for cfNAs

cancer. Two methods are widely used to enrich the targeted sequences: the first one is through PCR amplification (see chapter "Detection of Genetic Alterations by Nucleic Acid Analysis: Use of PCR and Mass Spectroscopy-Based Methods")

followed by the sequencing of the PCR product, which is named amplicon sequencing. Amplicon sequencing is a cost-effective and time-efficient choice for a target region of relatively small size. The larger the targeted region, the more primers are needed. A common pitfall for a mixture of a large number of primers is the generation of a large amount of primer-dimers. The second enrichment method is to capture the target sequences with magnetic beads linked with oligonucleotide probes. This method can be applied to a target region of large size, such as the whole exome. However, it normally requires more starting DNA/cDNA molecules when compared with PCR-based enrichment.

Microarray analysis has been the first gold standard for the quantification of the transcriptome. Since the invention of RNA sequencing (RNA-seq) by Nagalakshmi et al. [3], global characterization of RNA has begun to shift from microarray to RNA-seq. Besides the measurement of the expression level of RNA transcripts, RNA-seq can also reveal splicing information and allow the discovery of novel RNA species, such as non-coding RNAs and circRNA (see chapter "The Biology of CNAPS").

3 Sample Preparation for High Throughput Analysis of cfNAs

There are some unique characteristics of cfNAs that make the sample preparation procedure for high throughput analysis different from that for ordinary cellular nucleic acids.

cfNAs are highly fragmented. For nucleic acids extracted from intact cells, the size of genomic DNA is more than 20 kbp and the majority of mRNA is in the range of 1–5 kb. In comparison, the average size of cfDNA is approximately 200 bp. Similarly, the size distribution of the degraded cfmRNA has the peak around 160 bp (Fig. 3).

The DNA/mRNA isolated from intact cells is generally too long for direct downstream sequencing or microarray analysis and so the isolated DNA/mRNA normally needs to be fragmented to a smaller size (200–500 bp) before adaptor ligation in sequencing or fluorescent labeling in microarray. One benefit of the fragmented nature of cfNAs is that this fragmentation step can be omitted. However, another characteristic of cfNAs, its ultralow concentration, can make its sample preparation technically challenging.

Normally, only 0.5–50 ng of cfDNA (equivalent to 100–10,000 copies of the genome) and 1–100 ng of cfRNA are isolated from 1 ml of plasma. However, for standard Illumina sequencing library preparation, more than 1 μg of DNA is recommended. For conventional samples with ultralow DNA amounts, WGA, which is based on random priming, is usually employed before library preparation. Because of its significantly shorter size, this approach generally does not work well with cfDNA molecules. As a result, it is better to avoid random-priming based

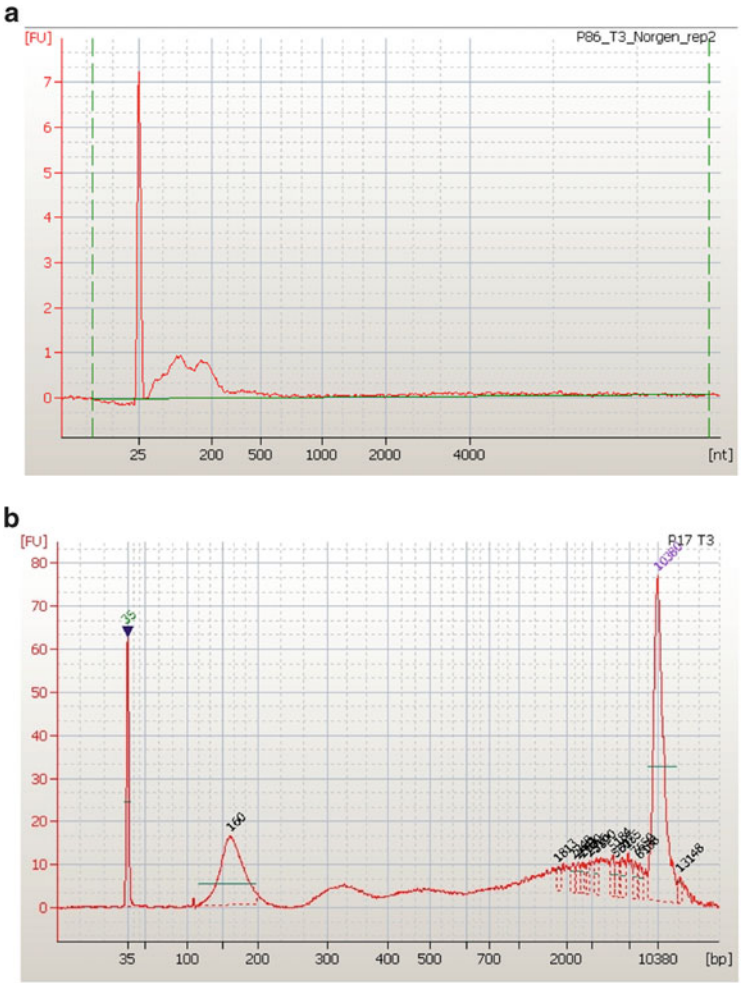

Fig. 3 The size distribution of cfRNA and cfDNA. (**a**) size distribution of extracted cfRNA by Bioanalyzer measurement; (**b**) size distribution of cfDNA by Bioanalyzer measurement

amplification for cfDNA. Rather, the tendency is to ligate known primers (such as Illumina sequencing primers) directly to the cfDNA and then amplify the fragments via PCR. We have also had success in library preparation with automated platforms such as NuGen's Mondrian system.

One technical issue that comes into play is that PCR amplification efficiency depends upon the size of the fragments being amplified: smaller fragments are generally amplified more efficiently. In the context of high throughput sequencing, this results in an over-representation of short fragments and an under-representation of longer fragments. Fan and Quake characterized this distortion and corrected for it, so enabling them to study the true length distribution of cfDNA (Fig. 4) [4].

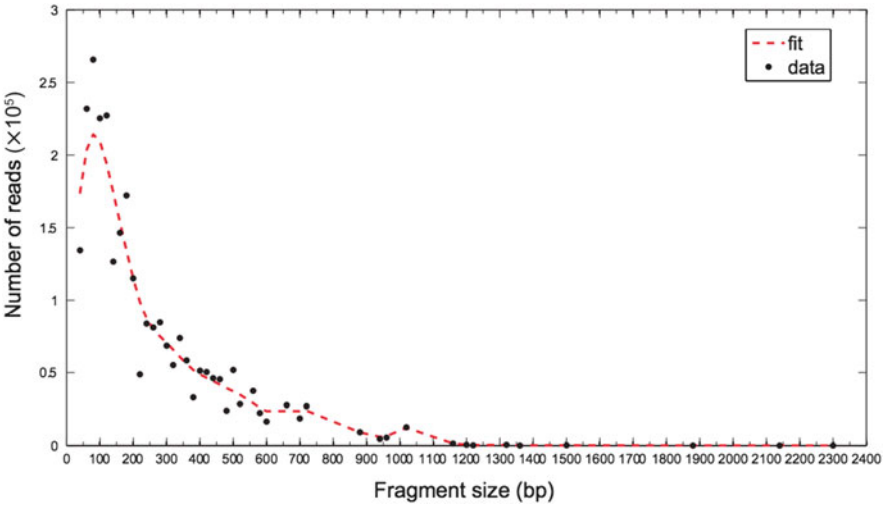

Fig. 4 The effects of library preparation and amplification on the size distribution of DNA. DNA was digested with AluI and then paired-end sequenced. The number of sequenced fragments is plotted against length. Each *black dot* represents the mean number of reads in every 20-bp bin. The *red line* is a locally weighted logistic regression fit [4]

Another important systematic error that must be reckoned with is GC bias. GC bias describes the dependence between GC content in a region and the count of sequencing reads mapped to it [5]. Both the PCR amplifications and the sequencing process itself lead to significant GC bias. This was recognized and partially corrected for in the first non-invasive measurements of fetal aneuploidy [6]. More sophisticated methods to correct for GC bias were subsequently developed and their application has reduced these systematic errors below the level stochastic error due to counting statistics [7].

cfRNA faces similar challenges. Because one normally requires ~5 μg of cDNA for the hybridization of microarray, and ~1 μg of cDNA for RNA-seq, whole transcriptome amplification is necessary. The most widely used whole transcriptome amplification methods take advantage of the poly-A tail of mRNA for the reverse transcription and amplification, which is not applicable to cfmRNA to its fragmented state. As an alternative, random primers can be used to convert mRNA to cDNA, but with the cost of introducing amplification bias and uneven coverage of the transcripts. In principle, universal adaptors could first be ligated to the 3′ and 5′ end of the mRNA and the following reverse transcription and amplification could be primed by a sequence complementary to the adaptors (Fig. 5). This method has been used for the library preparation of small RNA, but so far it has not been tested on cfRNA. However, the sensitivity of this method would rely on the adaptor ligation efficiency.

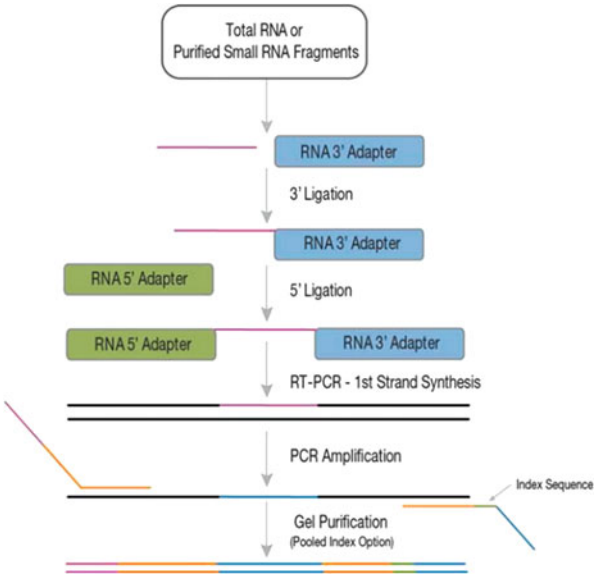

Fig. 5 Scheme of adaptor-ligation based method for library preparation of cell-free RNA (Source: TruSeq™ Small RNA Sample Preparation Guide)

4 High Throughput Sequencing and Analysis

4.1 Whole Genome Sequencing

The extracted DNA can be sequenced globally or selectively. For the whole genome sequencing, the DNA molecules are ligated with sequencing adaptors and subsequently put onto a sequencer for sequencing. The whole genome sequencing of cfDNA has been applied to the diagnosis of fetal aneuploidy from maternal plasma [6], the detection of tumor-associated copy number aberrations [8, 9] and the non-invasive monitoring of organ transplant rejection [10].

4.2 Exome Sequencing

The global sequencing of either the DNA or mRNA could capture the whole genomic landscape. However, its widespread coverage could also make its sequencing not deep enough to reveal the genomic variation in the minor DNA population. For example, one lane of Illumina HiSeq sequencing generates 200 million of 2×100 bp pair-end reads, which only equate to $7\times$ coverage of the whole genome. As 99 % of the human genome is comprised of e.g. repetitive elements, the introns, ribosomal RNA genes which are not informative for disease diagnosis. Therefore, one strategy to enhance the sequencing depth with the same budget is to enrich and sequence only the exome DNA. The exome DNA can be selectively

hybridized by biotin labeled oligonucleotides with complementary sequences and captured by streptavidin coupled magnetic beads. DNA sequences with more specific annotations than exome could also be enriched by customized oligonucleotides, such as the capture of a set of cancer related genes. Exome sequencing has demonstrated its application for cfDNA in the inference of the fetal exome directly from the maternal plasma DNA [11], and the non-invasive analysis of cancer therapy by tracking the fraction of tumor DNA in plasma over time [12].

4.3 Targeted Amplicon Sequencing

If the interested genomic region is more focused (for example, only several genes or within 50 kbp), targeted amplicon sequencing is a more sensitive and cost-effective method. If the concentration of the input DNA is low, which is normally the case for cfDNA, a pre-amplification step is required. During the pre-amplification, a cocktail primer set is used to amplify all of the targeted DNA fragments simultaneously. To exclude the primer-dimers and non-specific products generated during the pre-amplification step, a second round of PCR amplification with only one pair of primers in each reaction is performed in parallel format. The amplified products from each reaction are then pulled together for sequencing (Fig. 6). Targeted amplicon sequencing of plasma cfDNA has recently been employed to identify and monitor cancer mutations [13, 14].

5 Characterization of cfNAs Using High Throughput Sequencing

High throughput sequencing has opened up a new portrait of the landscape of cfDNA. Previous work had suggested that cffDNA is shorter than maternal cfDNA. Using pair-end sequencing, Fan et al. [4] directly measured the size distribution of both cffDNA and maternal cfDNA. Their results showed that cfDNA had a dominant peak at approximately 162 bp and a minor peak at approximately 340 bp that corresponded to the size of monochromatosome and dichromatosome, respectively, implying the apoptotic origin of the cfDNA. In addition, their sequencing measurements also agreed with previous findings that cffDNA is mostly shorter than 300 bp, whereas a portion of maternal cfDNA is $>$300 bp in size (Fig. 7).

Interestingly, one can directly observe an over-representation of cfDNA that appears to correspond to nucleosome protection. When representation is plotted as a function of distance from the transcription start site, oscillations can be seen, the period of which corresponds precisely with the nucleosome frequency (Fig. 8) [6]. These results are concordant with other studies that directly map nucleosome position in the genome.

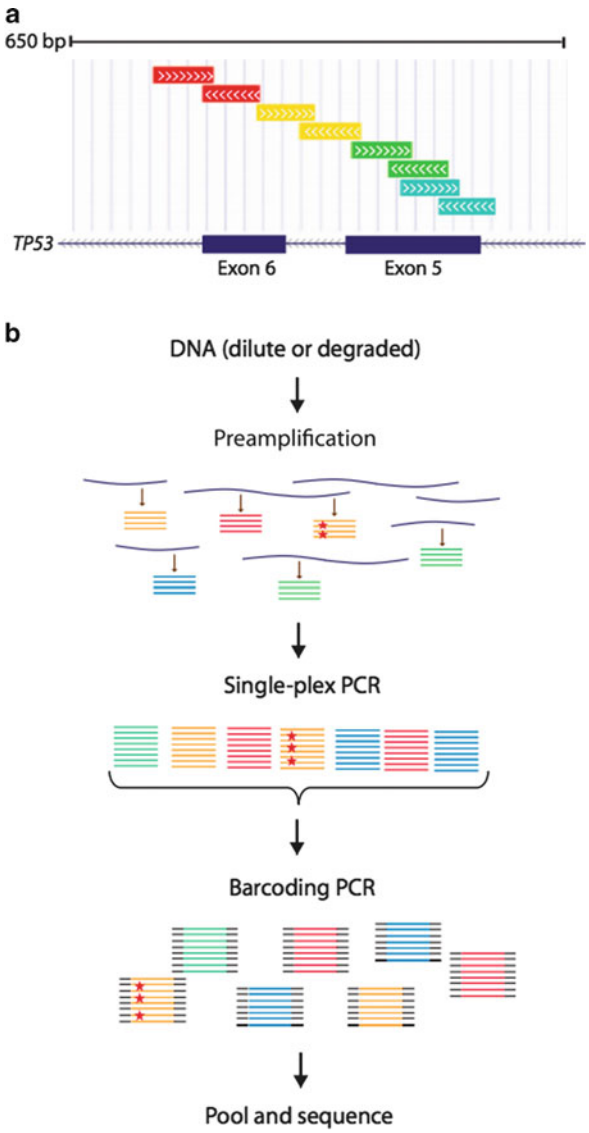

Fig. 6 Overview of tagged amplicon sequencing. (**a**) Illustration of amplicon sequencing. Primers were designed to amplify regions of interest in overlapping short amplicons. Amplicon design is illustrated for a region covering exons 5–6 of TP53. Colored bars, segmented into forward and reverse reads, show regions covered by different amplicons (excluding primer regions). Sequencing adaptors are attached at either end such that a single-end read generates separate sets of forward and reverse reads. (**b**) Workflow overview. Multiple regions were amplified in parallel. An initial preamplification step was performed for 15 cycles using a pool of the target-specific primer pairs to preserve representation of all alleles in the template material. The schematic diagram shows DNA molecules that carry mutations (*red stars*) being amplified alongside wild-type molecules. Regions of interest in the preamplified material were then selectively amplified in individual (single-plex) PCR, thus excluding nonspecific products. Finally, sequencing adaptors and sample-specific barcodes were attached to the harvested amplicons in a further PCR [13]

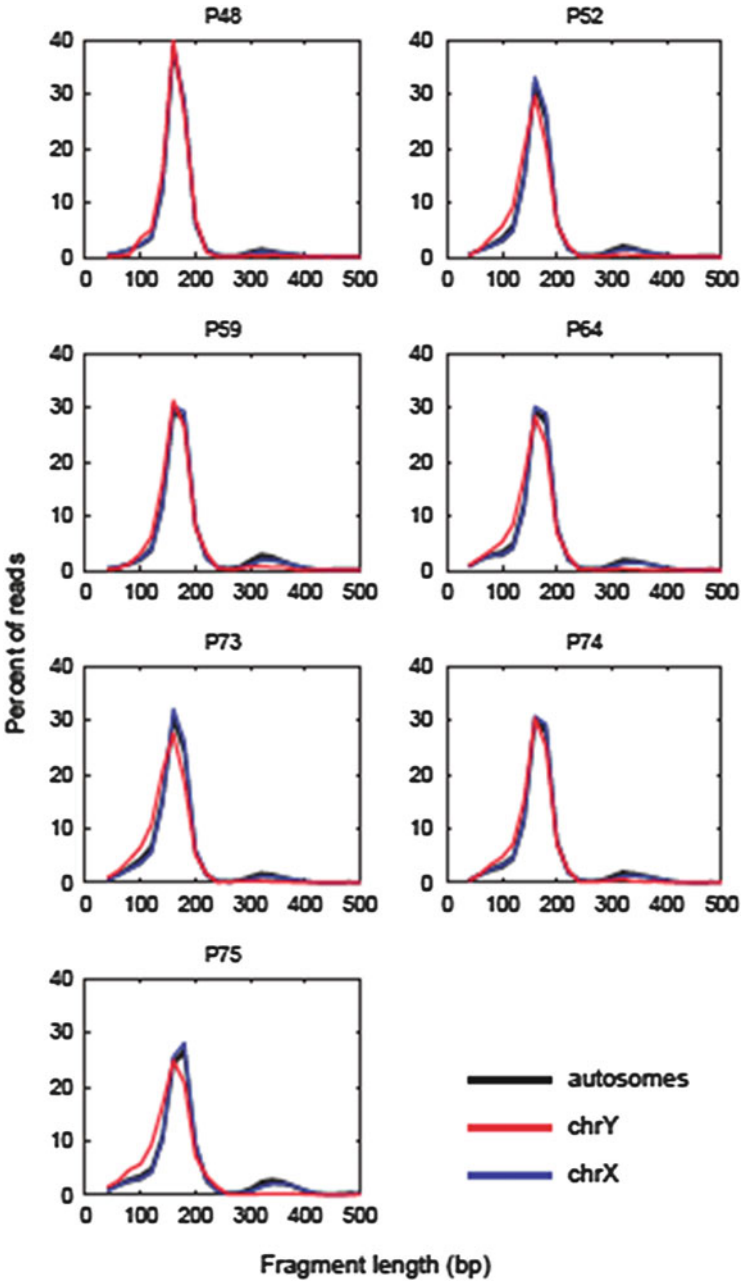

Fig. 7 Length distributions of fetal (chromosome Y) and total DNA sequenced from 7 samples of maternal plasma. (chrY: chromosome Y) [4]

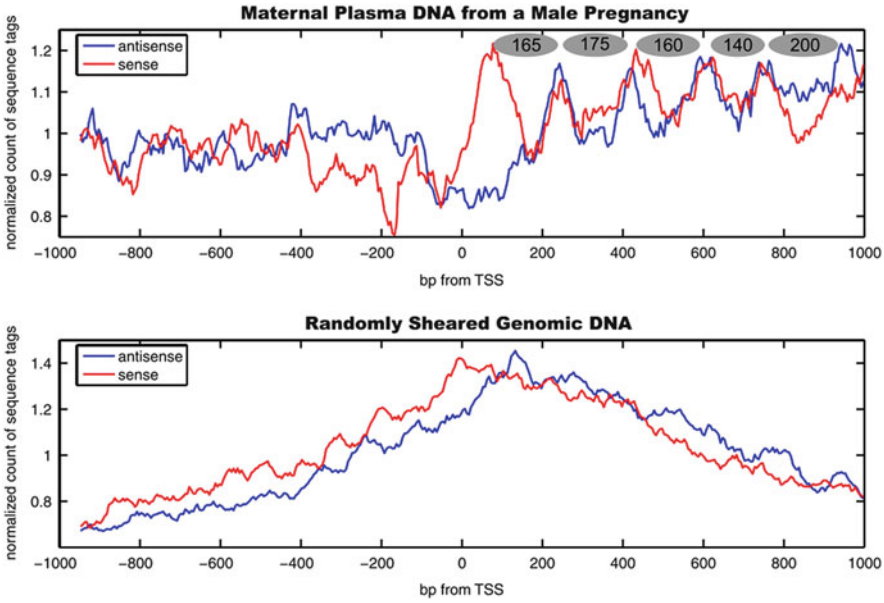

Fig. 8 Distribution of sequence tags around transcription start sites (TSS) of ReSeq genes on all autosomes and chromosome X from plasma DNA sample of a normal male pregnancy (*Upper*) and randomly sheared genomic DNA control (*Lower*). The number of tags within each 5-bp window was counted within 1,000-bp region around each TSS, taking into account the strand to which each sequence tag mapped. The counts from all TSS for each 5-bp window were summed and normalized to the median count among the 400 windows. A moving average was used to smooth the data. A peak in the sense strand represents the beginning of a nucleosome whereas a peak in the antisense strand represents the end of a nucleosome. In the plasma DNA sample shown here, five well-positioned nucleosomes are observed downstream of the TSS and are represented as gray ovals. The number within each oval represents the distance in base pairs between adjacent peaks in the sense and antisense strands, corresponding to the size of the inferred nucleosome. No obvious pattern is observed for the genomic DNA control [6]

6 The Counting Principle

One of the most powerful ideas to be used in the analysis of cfDNA is the counting principle. This enables the teasing apart of mixtures of genomes without the need to physically separate them. It was first applied for the detection of fetal aneuploidy in maternal cfDNA, containing mostly maternal DNA and a small component of cffDNA, but it has also been used to detect copy number variation, measure point mutations and to perform exome and genome analysis in similar contexts (see chapter "Fetal CNAPS – DNA/RNA"). It has parallel applications in cancer to determine the difference between tumor genomes and the somatic genomes of the affected individuals, as well as in organ transplant diagnostics to measure the health of the transplanted organ. The basic idea is to detect over-representation of a particular part of the genome – whether it be a chromosome or a gene region or

an individual SNP location – by comparing the relative amount to the deviations expected by counting statistics.

6.1 Detection of Copy Number Variation (CNV)

The basic idea is that the number of the sequencing reads mapped to a targeted genomic region is correlated to the copy number of that region. In normal human diploid cells, the copy number of most genome regions should be two. So the abnormal copy number could indicate diseases in which genetic aberrations are involved. The genomic region could be either a whole chromosome (e.g.: fetal aneuploidy, tumor-associated aneuploidy), or part of a chromosome (e.g.: Digeorge syndrome, CNV in tumor cells).

There are several factors that affect the detection limit of copy number variation: the number of sequencing reads M, the fraction of foreign DNA ϵ, the proportion of targeted region in terms of size relative to the entire genome ρ and the times of copy number variation n. At false positive rate α and false negative rate β, their relationship can be summarized by the following formula:

$$(n-1)^2\varepsilon^2\rho M = (z(\alpha) + z(\beta))^2 \tag{1}$$

The following case is used to illustrate the derivation of formula (1). Assume that we already have the cfDNA sequencing data from a group of normal subjects, now we have the cell-free DNA sequencing data from a new subject. The objective is to determine whether or not there is tumor DNA with CNV in the plasma of this subject.

Using basic statistical principles, the number of reads x mapped to target region should follow a hypergeometric distribution, which can be approximated by the Poisson distribution in this circumstance.

$$\textit{For a normal sample}:$$

$$\rho_{nor} = \rho$$

$$\lambda_{nor} = \rho M$$

$$E(x_{nor}) = \rho M$$

$$Var(x_{nor}) = \rho M$$

$$\textit{For a tumor sample}:$$

$$\rho_{tum} = \frac{(1 + (n-1)\varepsilon)\rho}{1 + (n-1)\varepsilon\rho} \approx (1 + (n-1)\varepsilon)\rho$$

$$\lambda_{tum} = (1 + (n-1)\varepsilon)\rho M$$

$$E(x_{tum}) = (1 + (n-1)\varepsilon)\rho M$$

$$Var(x_{tum}) = (1 + (n-1)\varepsilon)\rho M$$

$$Denote\ y = \frac{x}{M}$$

$$u_{nor} = E(y_{nor}) = \frac{E(x_{nor})}{M} = \rho$$

$${\sigma_{nor}}^2 = Var(y_{nor}) = \frac{Var(x_{nor})}{M^2} = \frac{\rho}{M}$$

$$u_{tum} = E(y_{tum}) = \frac{E(x_{tum})}{M} = (1 + (n-1)\varepsilon)\rho$$

$${\sigma_{tum}}^2 = Var(y_{tum}) = \frac{Var(x_{tum})}{M^2} = \frac{(1 + (n-1)\varepsilon)\rho}{M}$$

Distribution of y could be approximated to normal distribution

$${\sigma_{tum}}^2 \approx {\sigma_{nor}}^2 = \sigma^2 = \frac{\rho}{M}$$

$$P_{nor}: \quad y_{nor} \tilde{} N(u_{nor}, \sigma^2)$$

$$P_{tum}: \quad y_{tum} \tilde{} N(u_{tum}, \sigma^2)$$

Hypothesis test :

$$H_0 : u_y = u_{nor}\ (\textit{this is a normal sample})$$

$$H_A : u_y \neq u_{nor}\ (\textit{this is a tumor sample with CNV})$$

The test statistic is the z statistic :

$$z = \frac{y - u_{nor}}{\sigma}$$

For a test at significance α level ($\textit{false positive rate}, FP = \alpha$) :

Consider the case of amplification ($n > 1$)

Rejection region :

$$z = \frac{y - u_{nor}}{\sigma} > z(\alpha)$$

$$y > u_{nor} + z(\alpha)\sigma$$

$$\textit{False negative rate}, FN = \beta$$

$$\beta = P_{tum}(y \leq u_{nor} + \ z(\alpha)\sigma)$$

$$\beta = \phi\left(\frac{u_{nor} - u_{tum} + z(\alpha)\sigma}{\sigma}\right)$$

$$z(\beta) = \frac{-u_{nor} + u_{tum} - z(\alpha)\sigma}{\sigma}$$

$$z(\alpha) + z(\beta) = \frac{u_{tum} - u_{nor}}{\sigma}$$
$$(n-1)^2 \varepsilon^2 \rho M = (z(\alpha) + z(\beta))^2 \quad (1)$$

One more thing to mention about formula (1): there appears to be no limit for the fraction of foreign DNA ε and the size of copy number variation, as long as there is no limit for the number of sequencing reads M. However, in reality, there is an upper limit of M, which cannot exceed the total number of original DNA molecules in the sample. When more reads than the number of original DNA molecules in the sample are sequenced, essentially there are more PCR duplicates being sequenced and the formula does not apply.

Returning to the experimental data, and it is possible to test if the prediction from formula (1) matches the experimental observations.

In the case of detecting a certain type of fetal aneuploidy from maternal plasma, the n and ρ are fixed. It is easy to conclude from formula (1) that the smaller is the fetal fraction ε, the larger the number of sequencing reads, M, is required. In the case of detecting the tumor CNV from plasma, if the tumor fractions ε and n are fixed, the smaller the size of CNV, the larger the number of sequencing reads M required.

For example, when detecting fetal Down syndrome, $n = 1.5$, $\rho = \frac{50\text{ Mbp}}{3000\text{ Mbp}} 0.017$ (the size of chromosome 21 is 50 Mbp, the size of human genome is 3,000 Mbp). If the total number of sequencing reads is ten million and we set $\alpha \leq 0.01$ *and* $\beta \leq 0.01$, then according to formula (1), the detection limit of the cffDNA fraction is 2.3 %. This theoretical prediction matches very well with the experimental result. In 2008, the Quake group detected fetal aneuploidy from maternal plasma [6]. Their data shows that the minimum fraction of cffDNA that would be detected is when the sequencing reads are 10 million. In the subsequent paper, they also comprehensively discussed the relationship between the requirement of sequencing depth and the fetal DNA fraction (Fig. 9) [7]. In another study of the detection of chromosomal alterations in the circulation of cancer patients [8], Leary et al. could detect the loss or gain of the whole chromosome arm with the tumor DNA concentration as low as 0.75 % at a sensitivity >90 % and specificity >99 %, using one lane of Illumina HiSeq (200 million reads). They also made a simulation to show the relationship between sensitivity and specificity in a ROC curve (Fig. 10).

6.2 Detection of Point Mutation

The principle behind this scenario is straightforward: count the allele frequency (AF) of the locus of interest and see if there is either an appearance of novel alleles or if the AF is beyond the threshold of background noise. However, it could turn into a competition between the sequencing error and the true signal when the fraction of foreign DNA becomes low, which is the case for the early detection of

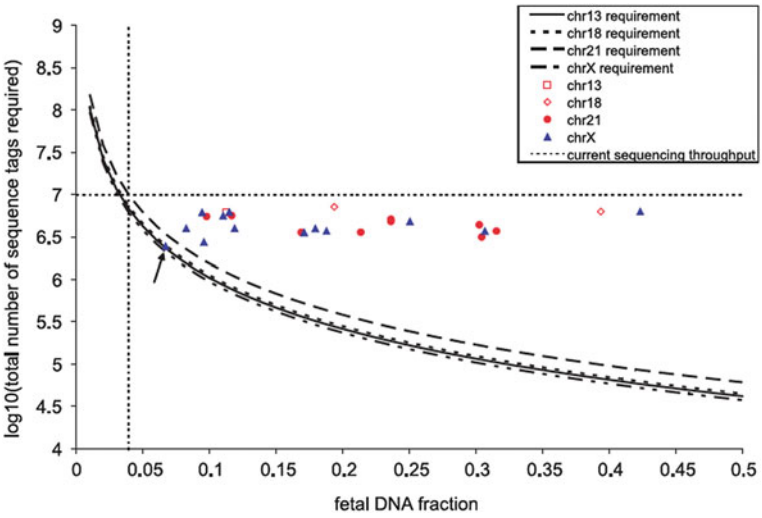

Fig. 9 Estimation of the requirement of sequencing depth for the detection of fetal aneuploidy in cell-free plasma as a function of fetal DNA fraction. The estimates are based on level of confidence a, 0.001 for chromosomes 13, 18, 21 and X, each having different length. As cffDNA fraction decreases, the total number of shotgun sequences required increases. With a sequencing throughput of ten million sequence reads per channel on the flow cell, trisomy 21 can be detected if 3.9 % of the cfDNA is fetal (*dashed lines*). The total number of sequence tags and the estimated fetal DNA fraction from our set of 19 patient samples are also plotted. For one of the normal male samples (P19, indicated by the *solid arrow*), chromosome X was not detected as underrepresented. This was probably due to insufficient sampling, as the total number of sequence obtained for this sample was close to the limit of detection given its fetal DNA fraction [4]

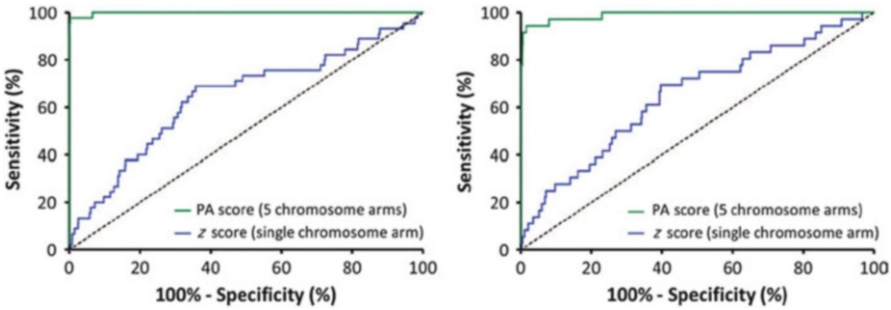

Fig. 10 Detection of circulating tumor DNA in breast and colon cancers using simulated copy number analyses. ROC analyses of simulated mixtures of breast cancer DNA (*left*) or colorectal cancer DNA (*right*) with normal plasma DNA using the PA score derived from the five chromosomal arm copy number alterations with the highest absolute z scores in each sample. Detection of 0.75 % circulating tumor DNA could be achieved with a sensitivity of >90 % and specificity of >99 % using the equivalent of one HiSeq lane of sequencing and a fixed PA score threshold in both tumor types. ROC analyses of a z score from a single chromosome arm, 17p, were similar to chance alone at this simulated tumor DNA concentration in the plasma [8]

cancer or fetal single-gene disorders. The following mathematical derivation and simulation is to demonstrate the relationship between sequencing error, sequencing depth and the detection limit of the fraction of foreign DNA. The principle is illustrated using the case of the detection of tumor point mutations in plasma.

The reference genome sequence of one individual can be measured by sequencing the white blood cells of the same person. At any given locus, it is either homozygous or heterozygous. Thus the reference genome is relatively easy to determine even when sequencing errors exist. However, it becomes more ambiguous when determining the rare point mutation among the dominant normal background signals. When there are non-reference bases present in the mapped reads, a criterion is needed to decide whether it is a real mutation signal from the tumor DNA or it is just a sequencing error. One strategy is to analyze the distribution of allele frequency (AF) from both mutation signal and sequencing error and to apply a statistic test to differentiate them.

The following analysis is a statistical point of view of this problem. At one locus, the number of a non-reference base caused by sequencing error is denoted by x_{err}, and the number of non-reference bases caused by mutation is denoted by x_{mut}. We assume that C is the sequencing coverage at that locus, e is the sequencing error rate and ϵ is the fraction of tumor DNA (assume all the tumor DNA has this point mutation homogeneously).

Based on basic statistic principle, x_{err} *follows Poisson distribution with* $\lambda_{err} = eC$.

$$\lambda_{err} = eC$$
$$E(x_{err}) = eC$$
$$Var(x_{err}) = eC$$

Similarly, x_{mut} *also follows Possion distribution with* $\lambda_{mut} = \epsilon C$.

$$\lambda_{mut} = \epsilon C$$
$$E(x_{mut}) = \epsilon C$$
$$Var(x_{mut}) = \epsilon C$$

$$\textit{Let AF denote allele frequency} : AF = \frac{x}{C}$$

$$u_{err} = E(AF_{err}) = e$$
$${\sigma_{err}}^2 = Var(AF_{err}) = \frac{e}{C}$$
$$u_{mut} = E(AF_{mut}) = \epsilon$$
$${\sigma_{mut}}^2 = Var(AF_{mut}) = \frac{\epsilon}{C}$$

Differentiate tumor mutation signal from sequencing error using Hypothesis test :

$H_0 : u_y = u_{err}$ (*The non − reference base at AF originated from sequencing error*)

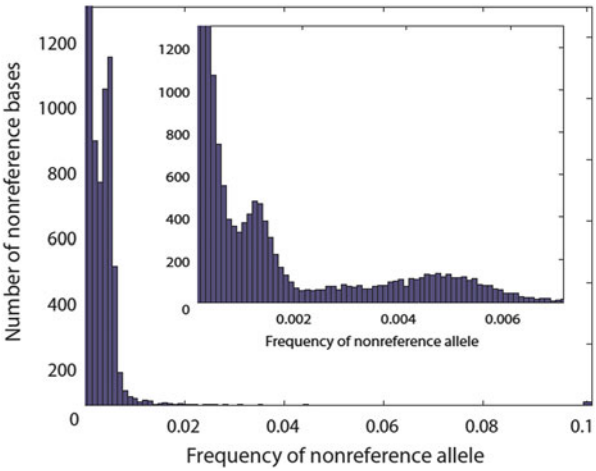

Fig. 11 Distribution of observed non-reference read frequencies, averaged over 47 FFPE samples, across all loci and all non-reference bases. Inset expands the low-frequency range [13]

$$H_A : u_y > u_{err} \ (The\ non - reference\ base\ at\ AF\ originated\ from\ tumor\ mutation)$$

$$Rejection\ region : AF > AF_c$$

$$\sigma = P_{err}(AF > AF_c)$$

$$\beta = P_{mut}(AF \leq AF_c)$$

AF_c is the threshold for background noise caused by sequencing error. σ is the false positive rate and β is the false negative rate of this hypothesis test.

There are three factors that determine the detection limit: the sequencing error e, the fraction of tumor DNA ϵ and the sequencing coverage C. When sensitivity, specificity and sequencing error are fixed, the requirement of the tumor DNA fraction decreases as the sequencing coverage increases. On the other hand, when the sensitivity, specificity and coverage are fixed, the requirement of the tumor DNA fraction decreases when the sequencing error rate decreases.

The current sequencing error rate of Illumina sequencing is around 0.1 %. Based on experimental data, Forshew et al. [13] have demonstrated the distribution of the non-reference allele frequency originated from sequencing error (Fig. 11).

In theory, there should be no lower boundary for the fraction of tumor DNA that could be detected, as long as there is no upper boundary of the sequencing depth. However, once the sequencing depth exceeds the number of genomic copies in the original sample, more reads only correspond to more PCR duplicates that have been sequenced. After a certain point, an increase of sequencing depth will not reduce the variance of allele frequency (AF) or the detection limit.

On average, there are approximately 5,000 genomic copies of cfDNA in 1 ml of plasma. This means 5,000 is the maximal sequencing coverage for 1 ml of plasma sample. Figure 12 shows the distribution of the allele frequency from point mutation and sequencing error when the sequencing coverage is 5,000. Given the maximal sequencing coverage and current sequencing rate, the point mutations

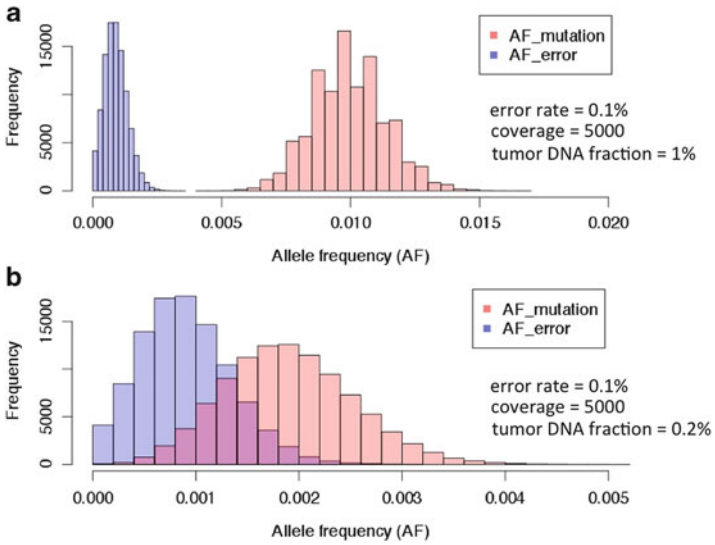

Fig. 12 Distribution of allele frequency from simulation data. (**a**) Error rate = 0.001, fraction of tumor DNA = 0.01, sequencing coverage = 5,000. (**b**) Error rate = 0.001, fraction of tumor DNA = 0.002, sequencing coverage = 5,000

could apparently be differentiated from the sequencing error when the tumor fraction is 1 % (Fig. 12a). However, the point mutations are not separable from the sequencing error when the tumor fraction is as low as 0.2 % (Fig. 12b). It is not infrequent for the tumor DNA fraction to be below 1 % in the plasma of a cancer patient. Vogelstein's group has characterized the tumor cfDNA in the plasma of colorectal cancer patients with the fraction of tumor DNA ranging from 0.01 to 1.7 % [15]. In order to detect tumor DNA with an extremely low fraction, one strategy is to reduce the sequencing error. Kinde et al. [16] have developed a method called "Safe-SeqS" to correct PCR and sequencing error by assigning a unique ID (random barcode) to each original DNA molecule (Fig. 13). They have applied this Safe-SeqS method to the detection of ovarian and endometrial cancer from the Papanicolaou test [17]. This method could potentially be used to increase the sensitivity of cfNAs analysis as well.

7 Applications for Human Biology and Diagnostics

In 2008, Fan et al. reported the invention of non-invasive diagnosis of fetal aneuploidy from maternal plasma using high throughput sequencing. The basic idea is to use the counting principle to analyze the number of sequencing reads mapped to each chromosome when either the over- or under- representation of any chromosome in maternal plasma DNA contributed by an aneuploid fetus can be

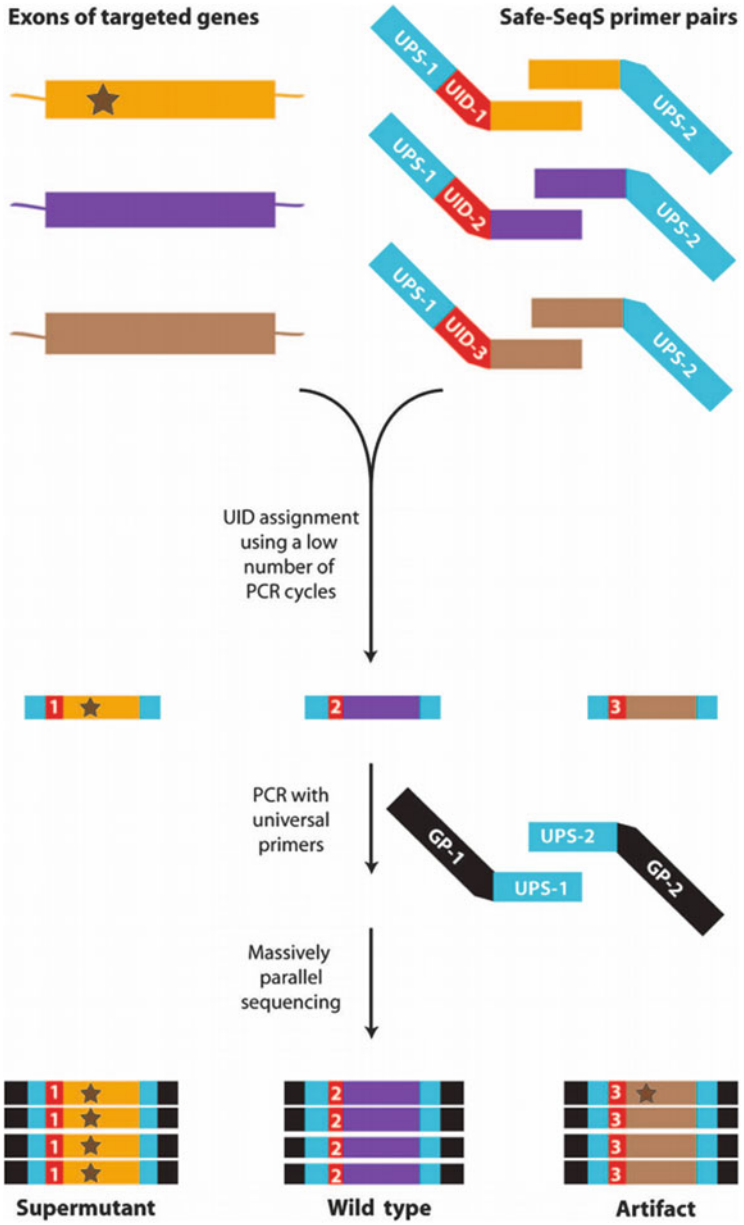

Fig. 13 Scheme of Safe-SeqS assay. *Top left*: DNA templates from three exons of different genes (*yellow*, *purple*, and *brown rectangles*) to be queried for mutations. Note that only one of the templates contains a mutation (*star*) that exists before any sample preparatory steps or sequencing. *Top right*: Safe-SeqS primer pairs contain binding sites for universal primers ("UPS," *blue*), a unique identifier ("UID," *red*) and gene-specific sequences (colors match the targeted exon). Next, the templates and primers are combined into a single PCR compartment and a UID is attached to each targeted template, along with UPS binding sites, after a low number of PCR cycles ("UID assignment"). The Safe-SeqS primers are removed and subsequent PCR is performed with primers containing UPS sites as well as the sequences required for attachment to the sequencing instrument

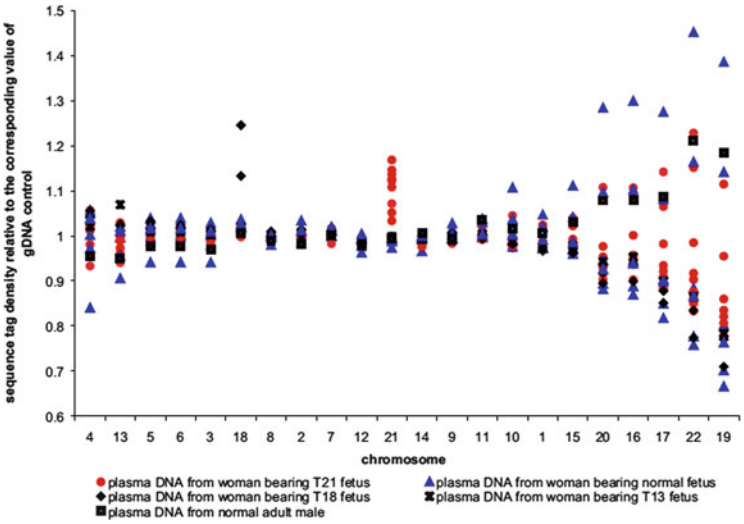

Fig. 14 Fetal aneuploidy is detectable by the overrepresentation of the affected chromosome in maternal blood. Sequence tag density relative to the corresponding value of genomic DNA control; chromosomes are ordered by increasing GC content [6]

detected (Fig. 14). Using this method, fetuses with trisomy 21 (Down syndrome), trisomy 18 (Edward syndrome) and trisomy 13 (Patau syndrome) were detected from maternal blood at gestational ages as early as the 14th week. Following this publication, there was an increasing attention from both academia and the health care industry to the emerging market of non-invasive prenatal testing. Large-scale clinical trials have been performed [18], and the first commercial non-invasive Trisomy 21 tests were launched in 2012, representing the first clinical application of next generation sequencing in cfDNA analysis. In 2012, both Fan et al. and, independently, Kitzman et al. [19] demonstrated that the whole-genome sequence of a human fetus could be reconstructed by sequencing the maternal plasma DNA. This breakthrough hints that comprehensive, non-invasive prenatal screening for Mendelian disorders may be clinically feasible in the near future (see also chapters "Fetal CNAPS – DNA/RNA" and "Other Body Fluids as Non-invasive Sources of Cell-Free DNA/RNA").

Since 2012, a number of papers have reported the analysis of tumor DNA in plasma by various sequencing methods. Using deep Amplicon sequencing targeted

Fig. 13 (continued) ("GP," *black*) to prepare the templates for massively parallel sequencing. When mutations preexist in template DNA before sample preparation, all of the sequenced daughter molecules sharing the same UID will contain the same mutation (a "supermutant"). In contrast, artifactual mutations caused by sample preparation or sequencing are unlikely to be observed in most other daughter molecules sharing the same UID ("Artifact"). Note that only one of two DNA strands is depicted for clarity [17]

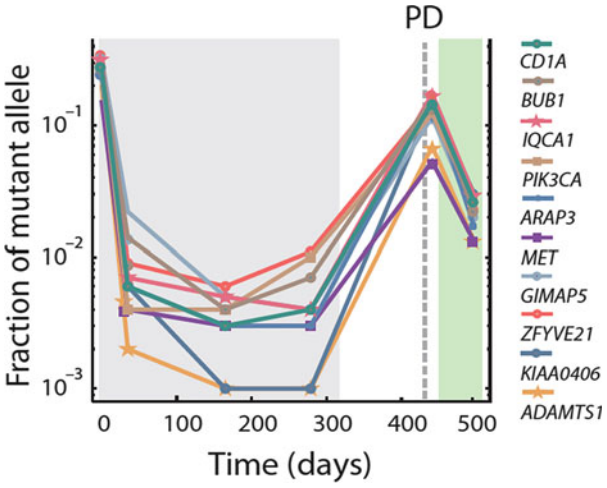

Fig. 15 Longitudinally monitoring tumor mutations in plasma by target sequencing. Dynamics of 10 tumor-specific mutations in the plasma of a breast cancer patient, PD, progressive disease [13]

to four cancer-related genes (*EGFR*, *BRAF*, *KRAS* and *PIK3CA*), Rosenfeld's group screened cancer point mutations present in cfDNA, non-invasively identified the origin of metastatic relapse in a patient with multiple primary tumors and longitudinally monitored tumor dynamics by tracking the concomitant mutations in plasma [13] (Fig. 15). Dawson et al. [14] extensively investigated the sensitivity of using tumor cfDNAs as biomarkers to monitor cancer treatment and compared them with other potential biomarkers including cancer antigen 15-3 and circulating tumor cells. In their proof-of-concept analysis, tumor cfDNA levels showed a greater dynamic range and greater correlation with changes in tumor burden than did either cancer antigen 15-3 or cell-free tumor cells. The follow-up work of the same group moved one step further when they established proof-of-principle that exome-wide sequencing analysis of tumor cfDNA could track acquired resistance to cancer therapy [12]. Recently, Leary et al. [8] detected chromosomal aberrations – copy number changes and rearrangements – in the circulation of cancer patients using massively parallel whole-genome sequencing (Fig. 16), the methodology of which is similar to that for the non-invasive detection of fetal aneuploidy.

Shotgun sequencing of cfDNA has also been used for the detection of organ transplant rejection [10] (Fig. 17). The fraction of donor-specific bases at particular SNP locations where the donor bears different bases from the recipient could be calculated by counting the number of reads mapped to that location and this fraction could indicate the rejection level of organ transplantation. Their results demonstrated that this method provides a quantitative measure of organ health that can be either complementary to or possibly replace other approaches for post-transplant monitoring.

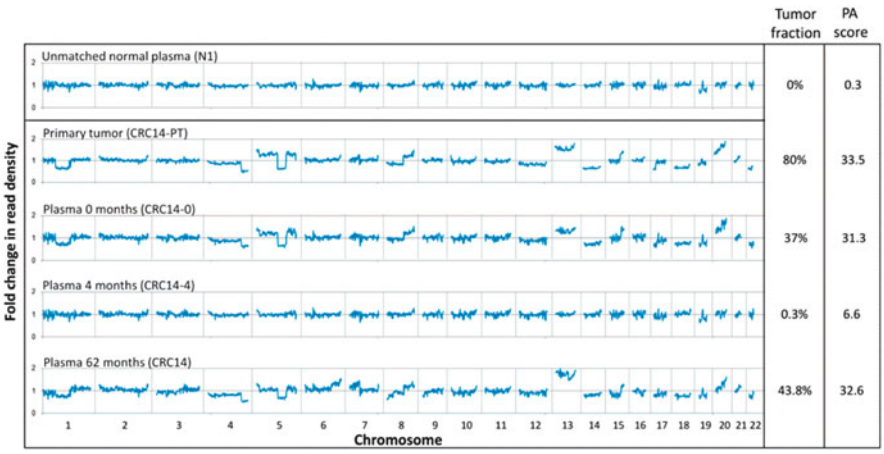

Fig. 16 Copy number analyses of tumor and serial plasma samples from patient with colorectal cancer. Primary tumor and plasma samples taken at various time points over 62 months of multimodality treatment were analyzed and compared with unmatched normal plasma. The plasma samples were obtained at the time of initial evaluation (0 months), after extensive chemotherapy and surgical intervention (4 months) and at the time of cancer recurrence (62 months) [8]

8 Profiling of cfRNA

8.1 Profiling of cfmRNA

The high throughput methods of microarray and RNA-seq provide a global view of the cfRNA level across different gene transcripts. In 2007, Maron et al. [20] used gene expression microarrays to profile the cffmRNA in maternal plasma and compared its transcriptome expression pattern with that of whole blood. They used a mixture of oligo-dT and random hexamers to synthesize first strand cDNA and amplified cDNA using SPIA (Single Primer Isothermal Amplification) technology. Their results showed that the genes that are highly expressed in the placenta have a higher expression in the antepartum plasma compared with the corresponding antepartum whole blood. Gene expression microarray has also been used to screen cfmRNA biomarkers for cancer patients. O'Driscoll et al. [21] have investigated the feasibility of identifying panels of cfmRNA biomarkers of breast cancer by profiling the gene expression in serum using whole genome microarray.

Compared with microarrays, the newer technology of RNA-seq provides higher sensitivity and wider dynamic range. We used RNA-seq to profile the cfRNA in maternal plasma in parallel with microarray. According to this study, the mRNA transcripts of 10,115 genes (51 % of known human genes) could be detected by RNA-seq with FPKM > 0 at the saturated sequencing depth (Fig. 18). Most of the

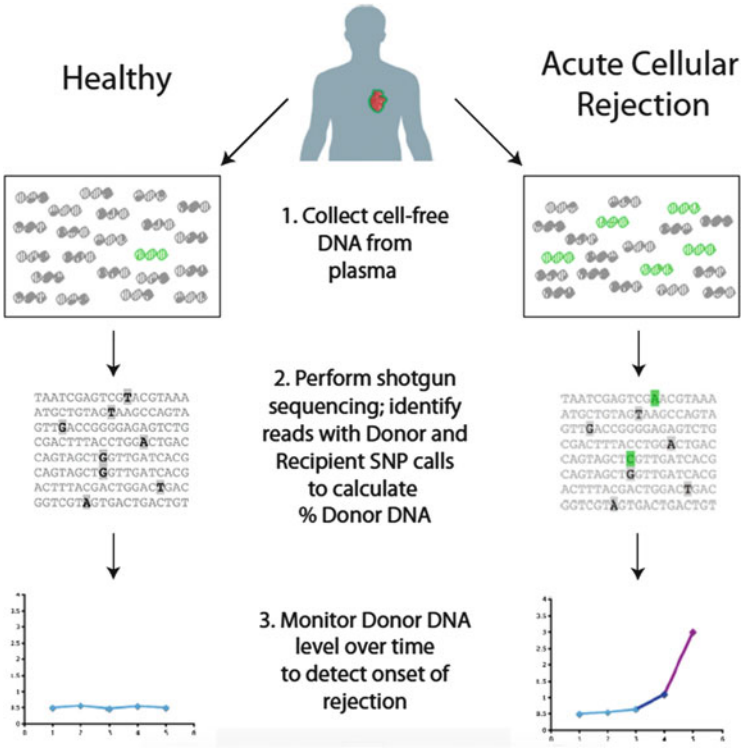

Fig. 17 General scheme for the universal detection of solid organ transplant rejection. cfDNA collected in plasma contains a majority of molecules from the recipient (*in gray*) but may also include some from the transplanted organ (*green*). Due to increased cell death in the organ during a rejection episode, more donor molecules are expected to be present in the blood at these times. Shotgun sequencing of the purified DNA allows for counting recipient versus donor molecules by looking at SNPs that vary between donor and recipient. Very high levels of donor DNA, particularly changes from past measurements, will indicate the onset of rejection [10]

genes detectable by RNA-seq are overlapped with the genes that are "on" in microarray. The gene expression measurements by RNA-seq are generally concordant with the measurement of microarray with an average correlation coefficient of 0.74 (Fig. 19). In the same study, 17 genes with their abundance changes over the time course of pregnancy were identified by both microarray and RNA-seq. Most of these genes are originated from the placenta and are involved in the pregnancy related pathways. This exploratory study implied the potential of using cfmRNA and cffRNA as biomarkers to monitor pregnancy complications and fetal development (Koh W, Pan W, Gawad C, Fan HC, Blumenfeld YJ, EI-Sayed YY, Quake SR unpublished data).

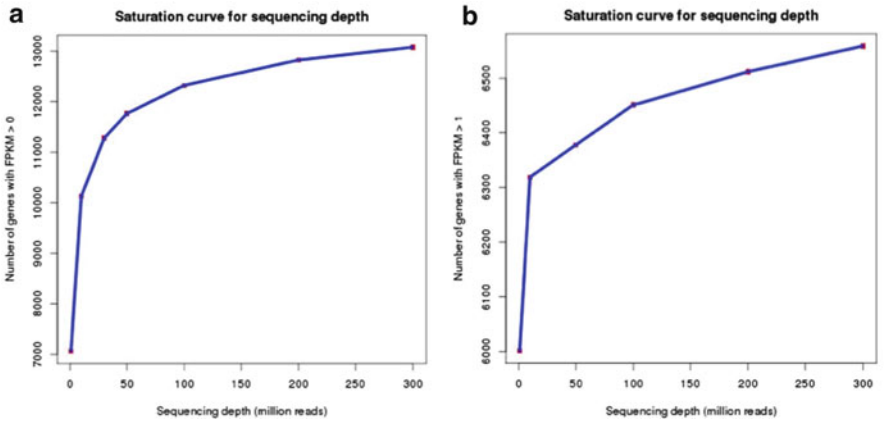

Fig. 18 The saturation curve for sequencing depth. (**a**) The number of genes detected with FPKM > 0 at different sequencing depth. (**b**) The number of genes detected with FPKM > 1 at different sequencing depth

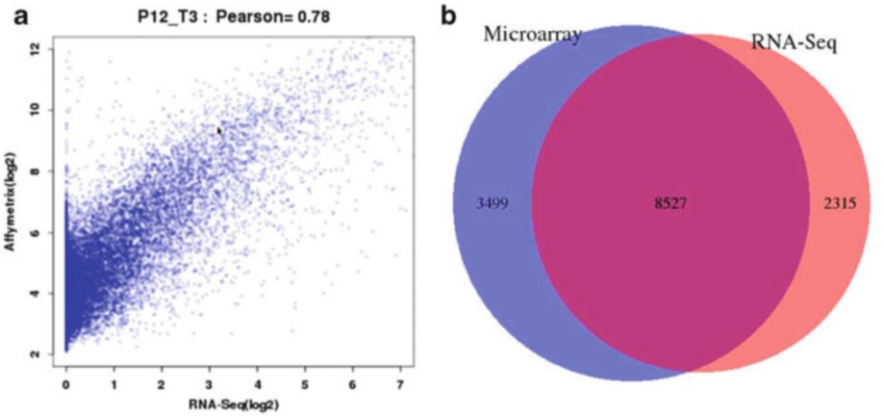

Fig. 19 Characterization of Maternal Plasma Transcriptome by RNA-Seq and microarray assays. (**a**) The scatter plot of the correlation between RNA-Seq and Affymetrix array assay (sample P12_T3). The Pearson correlation coefficient is 0.78. (**b**) Venn diagram displaying the genes detected by RNA-Seq and Microarray. The cutoff of RNA-Seq is FPKM > 0. The cutoff of Microarray is Intensity > 4. Sample P12_T3 is shown here as an example

8.2 Profiling of Cell-Free Small RNA

Besides cfmRNA, other RNA species existing in circulation also demonstrated potential application in non-invasive cancer and prenatal diagnosis. Recent work has characterized human plasma-derived exosomal RNA by deep sequencing [22]. These researchers have compared three small library preparation protocols for cell-free exosomal RNA. All three protocols require adaptor-ligation to small

RNA molecules before reverse transcription and cDNA amplification. They also classified the composition of cell-free exosomal RNA and the most abundant species were microRNAs. Another recent paper [23] profiled cfmiRNA from blood samples using deep sequencing and revealed that the vast majority of cfmiRNA originate from blood components and endothelial cells. More interestingly, tissue-specific miRNA, for example, from liver and gut, were present as well. Taking advantage of high throughput sequencing, Wang et al. [24] made a comprehensive survey of the source of the RNA molecules in plasma and observed that a significant fraction of these RNA molecules originated from exogenous species including bacteria and fungi.

9 Perspective

The use of high throughput sequencing for the detection of cfNAs in blood opens up enormous opportunities, but also challenges, for non-invasive clinical diagnosis. For cancer diagnosis, one major challenge is to be able to detect tumor mutation at an extremely low fraction of tumor DNA. The current technology can detect a mutant allele with a frequency as low as 2 %. However, the fraction of tumor DNA could be much lower than this level at an early tumor stage. One way to increase the sensitivity of detecting rare alleles is to decrease the sequencing error, which would be achievable with the advancement of sequencing techniques.

Compared with cfDNA, there are more unexplored areas for cfRNA. Recent work has started to globally profile RNA in plasma. Tissue-specific mRNA and miRNA has been identified in plasma, which might have the potential for use in monitoring the health status of different organs. However, more thorough investigation needs to be done before proceeding to the clinic. For example, cfRNA biomarkers with high sensitivity and specificity to certain disease phenotypes need to be discovered; the relationship between the level of tissue-specific RNA in plasma and the health status of its corresponding organ should be characterized in both patients and healthy controls (see also section “Choice of Appropriate Controls”).

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Part III
Applications

CNAPS and General Medicine

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Abstract The identification of blood-based markers that help clinicians to diagnose, predict and monitor diseases is a great challenge. In general, the earlier a precise diagnosis and therapy can be applied, the higher the probability of a successful treatment of the patients. Cell-free nucleic acids have promising clinical potential because they can critically be dysregulated during pathogenic processes. They are usually released during cellular stress or tissue injury and related to inflammatory responses caused by a coordinated expression of numerous genes that initiate, sustain and propagate immune responses and tissue remodeling. Although there is a potential for the application of cell-free nucleic acids as clinical assays, their use as potential biomarkers in pathologic conditions is still at the experimental stage, partly due to different qualities of the analyses employed. With the exception of minimally invasive prenatal diagnostic tests, the approaches on circulating, cell-free DNA, mRNA and microRNAs applicable for clinical practice currently remain somewhat elusive.

Keywords Circulating DNA/RNA • mRNA • microRNA • Transplantation • Aging • Burns • Sepsis • Cardio-vascular • Multiple sclerosis • Liver • Kidney • Stroke • Trauma • Hemodialysis • Pancreatitis • Predictive diagnosis

1 Introduction

This following discussion draws attention to the latest developments of the use of cfNAs (circulating, cell-free nucleic acids) with particular regard to general medicine, and discusses the utility of cfNA assays in predictive and personalized medicine. The succeeding survey of general diseases is not meant to be

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comprehensive, but is based on studies that offer substantial clinical insight in the scope of the application of cfNAs.

2 Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune-mediated, inflammatory, demyelinating and neurodegenerative disease of the central nervous system (CNS) and may have a fluctuating, wavelike course. The vast majority of MS patients suffer from relapsing-remitting MS, and have relapsing and remitting attacks of demyelination affecting different parts of the CNS at various times during the course of disease. In most patients, this initial inflammatory relapsing-remitting phase of MS is followed by a more chronic (secondary) progressive phase years later. Less frequently, patients have the rarer, primary progressive form of MS without bouts or exacerbations. The main problem of all MS types is to define standard procedures for monitoring the course of disease [1].

The most common clinical syndrome of MS, the relapsing-remitting MS, may be accompanied by a unique disease – and state-specific fingerprint of cfDNA (circulating, cell-free DNA) that provides significant clinical sensitivity and specificity. Using mass sequencing and assembly technologies, serum cfDNA motifs from 28 patients with definite relapsing-remitting MS and 50 healthy individuals were sequenced. Protein-coding genes that were differentially expressed in MS serum encoded cytoskeletal proteins, brain-specific regulators of growth and receptors involved in signal transduction of the nervous system. The cfDNA motifs characteristic for relapsing-remitting MS along with their disease activity could, thus, be promising as a clinical tool in monitoring patient responses to treatment modalities. Several repeat sequences, such as the L1M family of LINE (Long Interspersed elements), were also consistently differently expressed in all MS patients and even yielded the best separation from the healthy population ($p < 0.0001$) [2]. However, the role that this gene product may play in MS is unknown. It is presumed that these repeats have a function in the dynamic equilibrium of auto-reactive T lymphocytes that play a pivotal role in the prevention of autoimmune diseases, such as MS [3].

More recently, disease-associated changes in DNA methylation have particularly gained interest for biomarker development because the technique used is more disease-specific than measurements of the altered cfDNA levels. DNA methylation is an epigenetic process of chemical DNA modification leading to inactivation of gene expression [4] and such changes may also be involved in MS. In a previous assay, differences in methylation patterns were observed in exacerbation and remission of relapsing-remitting MS patients so identifying two different states of a single disease. Three patient cohorts were examined: 59 patients in either remission ($n = 30$) or exacerbation ($n = 29$) and 30 healthy individuals as controls. The DNA methylation patterns of 56 gene promoters were determined by a microarray-based assay (MethDet-56). These patterns permitted recognition of (a) relapsing-remitting MS patients in remission versus healthy controls (sensitivity of 79.2 %;

specificity of 92.9 %), (b) patients in exacerbation versus healthy controls (sensitivity of 75.9 %; specificity of 91.5 %) and (c) patients in exacerbation versus those in remission (sensitivity of 70.8 %; specificity of 71.2 %). These findings demonstrate that DNA methylation patterns of numerous genes involved in multiple signal pathways allowed identification of relapsing-remitting MS better than a single molecular marker and that the use of a composite DNA methylation pattern resulted in a higher accuracy. As DNA methylation reflects the molecular features of pathological processes, these cfDNA methylation pattern differences may be related to MS disease progression and its associated immune activation, inflammation and cell death [5].

Assays on numerous dysregulated cfmiRNAs (circulating, cell-free microRNAs) in the blood circulation of MS patients have also been performed [6]. Using qRT PCR and Exiqon Human Panel assays, 19 miRNAs were detected in serum derived from independent cohorts of 50 relapsing-remitting MS patients, 51 secondary progressive MS patients and 32 healthy controls. In particular, miR-92a-1*, involved in cell cycle regulation and cell signaling, and miR-454, the functions of which are largely unknown for immune cells, play a role in MS. MiR-92a-1* ($p = 0.001$) and miR-454 ($p = 0.005$) were differently expressed in relapsing-remitting MS and secondary progressive MS. MiR-92a-1* ($p = 0.002$) and miR-454 ($p = 0.005$) also showed association with the MS-specific Expanded Disability Status Scale (EDSS) [7].

As demonstrated by the above-described findings, MS patients may display disease- and state-specific changes in the levels of cfDNA and cfmiRNAs as well as in the profiles of cfDNA methylation that can be detected in the peripheral blood of these patients.

3 Cardiovascular Disease

The most common causes of cardiovascular (or heart) diseases are atherosclerosis and/or hypertension. Additionally, during aging, a number of physiological and morphological changes lead to increased risk of this disorder. Myocardial infarction is caused by the partial interruption of blood supply to the heart muscle based on the blockage of a coronary artery following the rupture of a vulnerable atherosclerotic plaque. The resulting ischemia and ensuing oxygen shortage lead to damaged or dying heart cells [8]. The gold standards in detection of acute myocardial infarction are increase in troponin, a marker of myocardial necrosis, and ST-segment elevation as measured with an electrocardiogram, when the trace in the ST segment is abnormally high above the isoelectric line. In particular, in geriatric patients who have frequently atypical symptoms and acute non-ST-segment elevation with non-diagnostic electrocardiograms, the detection of a modest elevation of cardiac troponins is often challenging for physicians. Unfortunately, non-coronary diseases, such as acute heart failure, may also cause elevated troponin values [8]. If combined with troponin measurements, the increased levels of cfDNA detected in

cardiovascular disease could reduce such drawbacks and improve patient screening, since soon, after the onset of chest pain, cfDNA concentrations rise in the blood circulation. This is due to acute cellular injury in cardiovascular disease, provoking apoptosis of both cardiac myocytes and non-myocytes, so leading to this disease-associated elevation of cfNAs. When additional complications occur, the levels may further rise and have a potential clinical value in monitoring disease progression of patients with acute myocardial infarction [9].

The housekeeping, gene ß-globin, was quantitatively measured by qPCR in blood samples taken from 58 patients with chest pain of apparent cardiac cause and 21 healthy individuals. Median plasma cfDNA concentrations were higher in patients who later either developed heart failure [1,060 versus 500 kilogenome equivalents L^{-1} (kGE L^{-1}), $p < 0.01$] or reinfarcted (1,000 versus 530 kGE L^{-1}, $p < 0.03$) or who had a cardiac arrest in their admission (1,350 versus 525 kGE L^{-1}, $p = 0.04$) or were readmitted within 6 months of discharge (725 versus 475 kGE L^{-1}, $p = 0.04$) than in their counterparts. Hence, the increased levels of cfDNA could predict post-acute coronary syndrome complications, such as cardiac failure, cardiac reinfarction and cardiac arrest [10]. During hospitalization serial determinations of cfDNA levels were performed by RT qPCR on the plasma of 47 patients with acute myocardial infarction once daily (235 samples) and once with the plasma of 100 healthy subjects. cfDNA concentrations were significantly higher in patients throughout hospitalization compared to the levels of healthy subjects ($p < 0.001$). The median maximum plasma cfDNA concentration was 3.5-fold higher in 20 patients with complicated post-acute myocardial infarction course (range 117–4,996,212 kGE L^{-1}) than in 27 patients without complications (range 56–4,715 kGE L^{-1}, $p = <0.004$). cfDNA levels rose significantly on the day the complication occurred and remained elevated on the day after the complication [11]. Using a branched DNA-based Alu assay, cfDNA concentrations were also determined in the plasma of 137 patients with acute coronary syndrome, 13 patients with stable angina and 60 healthy individuals. Patients with acute coronary syndrome (range 916–4,857 ng ml^{-1}), especially patients with ST-segment elevation (range 4,013–8,643 ng ml^{-1}) displayed a significant increase in plasma levels of cfDNA compared with patients with stable angina (range 112–256 ng ml^{-1}) and healthy controls (range 81–221 ng ml^{-1}). Thus, in acute coronary syndrome, cfDNA may be a promising marker for diagnosing and predicting the severity of coronary artery lesions and risk stratification [12].

Since cfmiRNAs offer many attractive features as biomarkers, specific profiles of cfmiRNAs are emerging as blood-based biomarkers for cardiovascular diseases, such as coronary artery disease, myocardial infarction, hypertension or heart failure [13]. In patients with advanced renal failure the diagnosis of acute myocardial injury could be improved by quantifying cfmiRNAs. The plasma levels of 6 miRNAs (miR-1, miR-21, miR-133a, miR-208a, miR-423-5p and miR-499-5p) in 92 patients with acute non-ST-segment elevation myocardial infarction (complicated by acute heart failure in 74 % of cases), 81 patients with acute heart failure without acute myocardial infarction and 99 healthy controls were analyzed. MiR-499-5p was >80 times higher in patients with acute non-ST-segment

elevation myocardial infarction than in controls. Interestingly, the ability of this cfmiRNA to discriminate myocardial infarction from acute heart failure was comparable to that of cardiac troponins. At initial contact with patients with modest elevation of troponins, miR-499-5p enhanced the diagnostic accuracy as sensitive biomarker. Its diagnostic accuracy with AUC of 0.86 was higher than that of cardiac troponin with AUC of 0.70 in differentiating non-ST-segment elevation myocardial infarction and acute heart failure patients with modest cardiac troponin at presentation. MiR-499 regulates the actin-based cytoskeletal myosin isoforms and modulates cardiac kinase and phosphatase pathways [14]. In a previous study, miR-499 and miR-208b were analyzed in the plasma of 397 patients with ST-segment elevation and 113 patients with non-ST-segment elevation myocardial infarction. Patients with ST-segment elevation had significantly higher cfmiRNA concentrations than patients with non-ST-segment elevation ($p < 0.001$). Both miRNAs correlated with peak concentrations of creatine kinase and cardiac troponin. cfmiRNAs and cardiac troponin were already detectable in plasma 1 h after the onset of chest pain, and 3 h after the onset of pain miR-499 and troponin were still found in 93 % and 88 % of patients, respectively. In this study, miR-499 and cardiac troponin provided a comparable diagnostic value with an AUC of 0.97 [15]. A combination of multiple cfmiRNAs in a diagnostic test could still further advance the diagnostic accuracy, because different causes of heart diseases can result in dysregulated levels of cfmiRNAs. In this regard, the combination of circulating miR-1, miR-133 (a and b) and miR-208 (a and b) in 444 patients with coronary syndrome was assessed and associated with the development of cardiac hypertrophy. Although these cfmiRNAs identified patients with myocardial infarction after the onset of complaints, they showed a large overlap between patients with unstable angina and myocardial infarction. In univariate and age- and gender-adjusted analyses increased miR-133a ($p > 0.03$) and miR-208b ($p < 0.05$) levels were significantly associated with the risk of death [16]. In contrast, it was observed that circulating miR-1, miR-133 and miR-499 were down-regulated in the hearts of diabetic rats [17]. miR-1, miR-133 and miR-208 are associated with the development of cardiac hypertrophy and influence myocardial contractile function. In the heart, miR-1 supports cardiomyocyte Ca2-cycling and contractility by targeting the transmembrane protein junction and is involved also in cell proliferation [18, 19]. A prominent role was ascribed for miR-133 and miR-208, but not for miR-1, in the cardiac adaption to and/or remodeling of the ischemic heart [20].

In summary, these findings show that cardiac injury is accompanied by changes in the levels of cfDNA and cfmiRNAs that may represent potential therapeutic targets for modulation of the cardiac function and remodeling during heart disease progression.

4 Stroke

Due to the transient or permanent reduction in cerebral blood flow, ischemic stroke is caused by either an embolus or local thrombosis. The interaction of complex pathophysiological processes, such as excitotoxicity, inflammation and apoptosis, leads to ischemia tissue damage and release of cfNAs into the bloodstream [21]. Soon after the onset of acute ischemic stroke an increase in nuclear and mitochondrial cfDNA followed by a gradual decrease were observed in blood. In 50 acute ischemic stroke patients and 50 control subjects at risk, plasma nuclear and mitochondrial cfDNA levels were serially measured by qPCR using β-globin (specific for nuclear DNA) and *MT-ND2* gene (specific for mitochondrial DNA), respectively. Elevated circulating nuclear cfDNA in plasma persisted until 1 month after the acute stroke. Levels of plasma nuclear cfDNA were 5,575 kGE L^{-1} in the poor and 5,120 kGE L^{-1} in the good outcome group, whereas levels of plasma mitochondrial cfDNA were 3,121 kGE L^{-1} in the poor and 2,333 kGE L^{-1} in the good outcome group. This study indicates that in acute stage patients the levels of cfDNA reflects the clinical severity of ischemic stroke and may be useful for risk stratification [22]. A further study, showing the correlation between plasma cfDNA concentrations and the volume of cerebral hematoma, quantified the levels of cell-free β-globin in 70 patients with ischemic stroke, 11 patients with intracerebral hemorrhage and seven patients with transient ischemic attacks. Median plasma cfDNA concentrations taken within 3 h of symptom onset were higher in patients who died than those who survived at discharge (6,205 versus 1,334 kGE L^{-1}). Plasma cfDNA concentrations of >1,400 kGE L^{-1} had a sensitivity of 100 % and a specificity of 74.4 % for predicting hospital mortality after stroke with an AUC of 0.89. Accordingly, the cfDNA concentrations were related to the extent of brain damage and predicted short- and long-term neurobehavioral morbidity as well as post-stroke mortality [23]. Also, in patients with clinical stroke who had no obvious acute cerebral lesions by either computerized tomography or magnetic resonance imaging, the cfDNA levels were an independent predictor for stroke outcome. In this study, 17 of 44 patients were classified as patients with post-stroke modified Rankin score (mRS) grades 3–6. Determining the quantity of β-globin gene by qPCR, the median plasma cfDNA concentration of this group of patients was significantly higher than that of patients with post-stroke mRS grades 0–2. The median concentrations of the relatively specific neurobiochemical S100 protein did not show significant differences between the two groups. Plasma cfDNA concentrations of >800 kGE L^{-1} had a sensitivity of 42 % and a specificity of 100 % for predicting 6-month post-stroke mRS (grades 0–2) with an AUC of 0.74, whereas serum S100 protein concentrations of >0.09 μg L^{-1} had a sensitivity of 48 % and specificity of 75 % with an AUC of 0.54. Thus, plasma cfDNA concentrations predict post-stroke morbidity and mortality in patients with negative neuro-imaging, and may be more effective than S100 protein measurements [24]. In contrast to this study, it was reported that S100 protein is a better biomarker for early stroke diagnosis than the non-specific increase in cfDNA levels because

serum S100 was increased in 126 ($p < 0.001$) but plasma β-globin cfDNA was increased in only 36 stroke patients. Serum S100 protein was also shown to be a better risk stratification and prognostic marker of long-term mortality than plasma cfDNA, but plasma β-globin cfDNA alone was better at discriminating hemorrhagic (1,725 kGE L^{-1}) from non-hemorrhagic stroke (100 kGE L^{-1}). However, the use of combined selected cut-off levels of plasma β-globin cfDNA and S100 protein reached the highest ratio of 16.55 than cfDNA alone (4.24) [25]. These findings emphasize, again, that the assessment of combined markers provide the best results in diagnostics.

In the pathophysiology of stroke, the detection of specific cfmiRNAs in the peripheral blood is still in their infancy with respect to addressing their potential use as diagnostic and prognostic markers [26].

5 Sepsis

Sepsis is a vascular infection disorder accompanied by a systemic activation of inflammatory and coagulation pathways in response to microbial infection of normally sterile areas of the body. Severe sepsis leads to acute organ dysfunction with a mortality rate of 30–50 % [27]. In the pathogenesis of multiple organ dysfunction, excessive amounts of inflammatory cytokines are released and may cause apoptosis of cells resulting in elevated plasma levels of nucleosomes [28]. In patients with severe sepsis the quantification of cfDNA measured by UV absorbance at 260 nm provided high prognostic accuracy and enhanced risk-stratification of the patients. The serial measurements of cfDNA in the plasma of these 80 patients demonstrated that the ICU (intensive care unit) mortality may be predicted within 24 h, i.e. the AUC for cfDNA was 0.97 and a cfDNA cut-off value of 2.35 ng μL^{-1} (sensitivity of 88 %; specificity of 94 %). By combining the quantification of cfDNA with markers essential for sepsis pathophysiology, such as the levels of protein C (an anticoagulant factor) or MODS, the predictive power of cfDNA could be increased [29]. Although the plasma cfDNA concentrations may be an independent predictor for ICU mortality, its clinical value does not seem to be qualified for hospital mortality in severe sepsis and septic shock. When plasma β-globin cfDNA of 255 patients was measured by qPCR, cfDNA concentrations were found to be higher in ICU non-survivors (15,904 kGE L^{-1}) than in survivors (7,522 kGE L^{-1}; $p < 0.001$) and were an independent predictor for ICU mortality ($p = 0.005$), but not for hospital mortality [30].

The diagnoses of mild sepsis, severe sepsis and septic shock are based on clinical judgments. In respect to the clinical aspect, miR-223 and miR-499-5p might be of marked interest being associated with organ failure. In 166 patients with sepsis and 24 healthy controls, the expression levels of several miRNAs were determined by RT qPCR. Serum levels of miR-223 ($p < 0.04$) and miR-499-5p ($p < 0.001$) were significantly different between patients with mild sepsis and those with severe sepsis and septic shock [31]. Serum miR-499-5p, also a biomarker of acute

myocardial infarction [14], had the highest AUC of 0.69 followed by serum miR-223 with an AUC of 0.61 [31]. The levels of miR-223 could also differentiate sepsis patients from patients with systemic inflammatory response syndrome (SIRS). In this study, the levels of miR-223 and miR-146a were quantified for 50 sepsis patients, 30 SIRS patients and 20 healthy controls. miR-223 (AUC = 0.804) and miR-146a (AUC = 0.858) levels were significantly lower in the cohort of sepsis patients than in the cohorts of SIRS patients and healthy controls. However, miR-223 expression is also dysregulated in other diseases such as influenza or hepatitis B infection, Crohn's disease, type 2 diabetes, leukaemia and lymphoma. miR-223 modulates inflammation, infection and cancer development [32] and is able to suppress pro-inflammatory activation of macrophages [33]. MiR-146a also controls pro-inflammatory signals in endothelial cells, repressing the pro-inflammatory NF-κB pathway as well as the MAP kinase pathway and so may, consequently, affect vascular inflammatory diseases. Its expression is induced upon exposure of endothelial cells to pro-inflammatory cytokines. Over-expression of miR-146a moderates endothelial activation, while loss of miR-146a has a stimulatory effect [34].

These studies highlight the association of increased cfDNA and dysregulated cfmiRNA levels with the severity of sepsis and their prognostic value to predict, with high specificity and sensitivity, the mortality of the patients.

6 Hemodialysis

During hemodialysis sessions, elevated cfDNA concentrations have been reported in numerous studies. It is assumed that these increased cfDNA levels in blood circulation are caused by apoptotic cells on the dialysis membranes. Patients with end-stage renal disease undergoing hemodialysis are characterized by a chronic inflammatory disorder that includes aberrant and chronic production of inflammatory cytokines, such as IL-6 (interleukin-6) [35]. IL-6 released by monocytes may induce elevated levels of cfDNA indicating that this process may also contribute to the increase in cfDNA levels and to the pro-inflammatory environment observed in hemodialysis patients [36].

In addition to changes in cfDNA concentrations, significant and specific changes in DNA methylation are involved in hemodialysis patients. cfDNA from randomly selected patients before and after hemodialysis sessions were assayed twice daily with a 4-h interval. The extent of promoter methylation of 24 genes involved in the immune response was examined using the EpiTect Methyl quantitative PCR array. In this pilot study, changes in DNA methylation patterns of the immune response gene promoters (IL-7, IL-13, IL-17C and tyrosine kinase TYK2) were detected, indicating that DNA methylation profiling on cfDNA may provide additional information about the actual state of immune response in hemodialysis patients [37].

In patients with acute kidney injury dysregulated levels of cfmiRNAs were observed during dialysis therapy. Although miRNAs are small in size, miR-21 and miR-210 were not removed by various dialysis membranes [38]. In contrast, in patients with advanced renal failure, hemodialysis affected the levels of cfmiRNAs. In this assay plasma miR-499 and serum troponins were quantified in 41 patients and 41 healthy controls. Both parameters were elevated in the blood circulation of hemodialysis patients compared to controls ($p < 0.001$). In contrast to the levels of troponins that were unaffected by hemodialysis, there was a 6.5-fold decrease ($p = 0.002$) in miR-499 levels, reducing the potential of miR-499 as a biomarker for patients with end-stage renal disease [39]. These observations in patients with kidney diseases show that it remains unclear if and how the dialysis procedure affected the levels of cfmiRNAs.

7 Liver and Kidney Diseases

Liver and kidney diseases are frequently caused by viral infections, alcohol abuse and toxic chemical exposure. They can be examined by biopsies, but these examinations are invasive and not routinely performed. Thus, a marker with an increased specificity would be helpful for evaluating the presence of these diseases. In contrast to cfDNA analyses, particular attention was drawn to the investigations of cfmiRNAs in liver and kidney diseases. The concentration of miR-34a was reported to be significantly altered by chronic ethanol feeding of rats resulting in a poor functional state of the liver and a serious inhibition of its regenerative ability [40].

Disease-specific changes in the levels of miR-34a and miR-122 were observed in 53 patients with chronic hepatitis C infection who had no alcoholic or fatty liver. The serum levels of miR-34a associated with cell-cycle checkpoint failure and increasing cell proliferation and of miR-122 involved in the replication of hepatitis C virus correlated positively with the histological disease severity of simple steatosis to steatohepatitis. These changes in the levels of both cfmiRNAs also correlated with liver enzymes levels, fibrosis stage and inflammation activity [41]. Increases in miR-122 concentrations were also observed in 82 of 83 patients with histopathologic change and they were reflected in the severity of liver disease. The plasma levels of miR-122 significantly correlated with alanine aminotransferase activity, a biomarker commonly used for the diagnosis and assessment of liver disease ($p < 0.001$) [42]. Moreover, the quantification of serum miRNAs in 48 patients with chronic type B hepatitis and 101 patients with hepatocellular carcinoma showed that the levels of miR-122 ($p < 0.0001$) and miR-21 ($p = 0.0004$) in patients with chronic hepatitis were higher than in patients with hepatocellular carcinoma [43]. miR-21 has biological functions in inflammatory conditions and correlates with the pathogenesis of numerous other disorders including autoimmune diseases such as type 1 diabetes, systemic lupus erythematosus, systemic sclerosis, psoriasis and multiple sclerosis [19]. miR-21 also plays a role in

allergic diseases, including atopic dermatitis, a hyperproliferative cutaneous disorder associated with a defective skin barrier, and allergic rhinitis [44]. Functional analyses showed that miR-21 also is involved in the regulation of the polarization of adaptive immune responses and activation of T cells [44]. The potential use of circulating miR-21, as well as of miR-122 and miR-192 as a novel, predictive, and reliable blood-based marker panel for alcohol-, viral- and chemical-induced liver injury was also reported [42, 43, 45]. Serum miR-122 ($p < 0.0001$) and miR-192 ($p < 0.0004$) were substantially higher in 53 patients with acetaminophen-induced acute liver injury than in 11 patients with chronic kidney disease [45]. In kidney, miR-192 was described to be involved in TGF-β1-mediated fibrogenesis [46].

With respect to pathophysiological changes in acute kidney injury, miR-210 was measured in the plasma of 77 patients. The altered levels of miR-210 predicted the mortality of the patients ($p = 0.03$) and could, therefore, serve as an independent and powerful predictor of 28-day survival [47]. Findings providing novel insights into the angiogenesis mechanism of this injury revealed that after renal ischemia/reperfusion injury miR-210 targeted the VEGF signaling pathway to regulate angiogenesis [48].

Accordingly, these studies provide evidence for the specific role of miRNAs in inflammatory processes and their potential use as biomarkers of alcohol-, viral- and chemical-induced injuries.

8 Pancreatitis

Acute pancreatitis is usually a short lasting mild disease, but in 20 % of cases the disease takes a severe course with high mortality rates despite treatment. The disease may be cellular triggered by acinar injury and its subsequent progression to a systemic illness involves a complex interplay between the pancreatic parenchymal microvasculature, circulating soluble cytokine mediators, cellular mediators of inflammation and regional endothelial beds, especially those in lung, liver and kidneys [49].

Severe acute pancreatitis is characterized by inflammation and accelerated apoptotic and necrotic pathways. A previous study measured cfDNA using qPCR with a TaqMan RNase P kit on 43 patients with severe acute pancreatitis, 12 patients with pancreatic cancer and 28 non-cancer controls undergoing laparoscopic cholecystectomy. Plasma cfDNA levels in patients with acute pancreatitis (median of 0.40 ng μL^{-1}, range of 0.05–0.79 ng μL^{-1}) were significantly lower than in controls (median of 1.60 ng μL^{-1}, range of 0.45–9.10 ng μL^{-1}, $p < 0.001$). During the disease course, cfDNA levels in patients with acute pancreatitis significantly fell to a median value of 0.08 ng μL^{-1} (range of 0–0.53 ng μL^{-1}, $p < 0.001$) [50]. To investigate whether or not cfDNA correlates with the extent of pancreatic necrosis, cfDNA was measured in the serum of 30 patients with acute pancreatitis. On the first day following admission, patients who developed severe pancreatitis had significantly higher serum cfDNA levels (median of 0.271 ng μL^{-1}) than those

with mild disease (0.059 ng μL^{-1}, $p < 0.001$) with an AUC of 0.97. There was a significant correlation between Balthazar score (CT severity index) and serum cfDNA concentrations ($p = 0.012$) [51]. To confirm the value of cfDNA and test its practical usage in pancreatitis, cfDNA in both serum and plasma samples of 33 with severe and 71 with mild acute pancreatitis was quantified. The plasma levels of cfDNA differentiated between patients with mild and severe acute pancreatitis with a sensitivity of 91 % and a specificity of 80 % using a cut-off value of >0.304 ng μL^{-1}. Although the parameters were somewhat lower for serum cfDNA levels (a sensitivity of 88 % and a specificity of 68 % using a cut-off >0.363 ng μL^{-1}), both plasma and serum cfDNA showed a better sensitivity and specificity than either C-reactive protein or two scoring systems (Ranson and APACHE II scores) that are, in practice, the mostly used clinical predictors for acute pancreatitis [52].

These data demonstrate that quantification of cfDNA can be a possible early marker of the severity of acute pancreatitis.

9 Transplantation

Transplantation medicine is another field of medicine that could benefit from the analyses of cfNAs. In spite of the advances in immunosuppressive treatment, graft rejection is still a severe problem in human transplantation. The levels of donor-derived cfDNA and cfRNA in the recipient's blood circulation could serve as a marker for transplant rejection. To date, donor-derived cfDNA could be isolated from the blood of liver, kidney, pancreas and bone-marrow transplant recipients [53]. In this regard, a sex-mismatched bone marrow transplantation model was used to obtain information about the source of cfDNA in transplant patients and whether cfDNA is derived from hematopoietic and/or non-hematopoietic cells. In this assay, plasma of 22 sex-mismatched bone marrow transplant patients was examined and showed that the median percentage of Y-chromosome cfDNA in female patients receiving bone marrow from male donors (59.5 %) significantly differed from that in male patients receiving bone marrow from female donor ($p < 0.001$). These findings show that plasma cfDNA in the bone marrow of transplant recipients was of predominant hematopoietic origin [54].

Kidney transplantation is the most desirable and cost-effective modality of renal-replacement therapy for patients with irreversible chronic kidney failure. In the early postoperative phase kidney transplant patients have the highest risk of complications and the first 3 months are critical for acceptance or rejection of the graft [55]. Procalcitonin, a precursor of the hormone calcitonin, and produced by cells after surgery is used as a general marker for infection. In the early postoperative period, immunological monitoring at the molecular level along with serial quantification of plasma procalcitonin and cfDNA has been demonstrated to detect complications of renal transplantation, such as acute rejection or sepsis. Total cfDNA and donor-derived cfDNA were quantified in both plasma and urine of

100 renal transplant recipients by qPCR for the *HBB* (hemoglobin ß) and the *TSPY1* (testis specific protein Y-linked 1) genes, respectively. Concentrations of plasma cfDNA markedly increased during acute rejection and returned to reference values after anti-rejection treatment. A cut-off level of 12,000 kGE L^{-1} classified acute rejection and non-acute rejection in 86 % of post-transplantation complications (sensitivity of 89 %; specificity of 85 %). The use of the combination of plasma cfDNA and procalcitonin improved the diagnostic specificity to 98 %. Although these biochemical markers will not replace renal biopsy as a definitive method for diagnosing medical complications after renal transplantation, they could support the diagnosis of patients displaying contraindications to biopsy and also reduce the number of biopsies [56].

Accordingly, the further development of such non-invasive assays on cfDNA and cfmiRNAs could improve the identification of patients with acute rejection of the graft and predict long-term transplant function in the future.

10 Trauma

Trauma is a physiological wound or injury caused by external situations. Brain trauma, also known as intracranial injury is caused by e.g. falls, vehicle accidents, acceleration alone and violence. Following this injury, a variety of events can occur in terms of minutes and days and include alterations in cerebral blood flow and pressure within the skull. These processes substantially contribute to the damage of the initial injury. Severe traumatic brain injury is still related to a high rate of unfavorable outcome and associated with a mortality rate of 35–70 % [57]. DNA is released early from the injured tissue into the blood circulation of trauma patients. The cfDNA levels increase within an hour and rapidly continue decreasing at 24 h after trauma. The amount of cfDNA is related to the severity of tissue damage and predicts post-traumatic complications including mortality, but cfDNA concentrations may also considerably vary in blood [58].

A qPCR study was made of plasma cfDNA from 84 patients who had sustained an acute blunt traumatic injury and 27 control subjects using the housekeeping, gene β-globin. The median plasma cfDNA concentrations in the control, 47 minor/moderate trauma (Injury Severity Score <16) and 37 major trauma (Injury Severity Score ≥ 16) groups were 3,154 kGE L^{-1}, 13,818 kGE L^{-1} and 181,303 kGE L^{-1}, respectively. Plasma cfDNA concentrations in patients with adverse outcomes, including acute lung injury, acute respiratory distress syndrome and death, had a 12-fold higher plasma cfDNA concentration than those who did not develop these complications. At a cut-off level of 232,719 kGE L^{-1}, the sensitivities of plasma cfDNA analysis for the prediction of acute lung injury, acute respiratory distress syndrome and death were 100 %, 100 % and 78 %, respectively. The respective specificities were 81 %, 80 % and 82 % [59]. To analyze the degree of cfDNA decline after severe traumatic brain injury, plasma cfDNA concentrations of 65 patients during 96 h in the Intensive Care Unit were also quantified by qPCR

using the β-globin gene. After the following 4 days, a 51 % and a 71 % decrease in cfDNA levels during the first 24 h and after 48 h were observed, respectively. The decrease was more pronounced in survivors than in non-survivors. A cut-off ratio of 1.95 was established for the detection of patients who will not survive after the injury with a sensitivity of 70 % and a specificity of 66 % [60]. To examine whether or not the cfDNA concentration could also predict the later development of posttraumatic organ failure and multiple organ dysfunction syndrome, plasma cfDNA of 83 patients was measured as a consequence of major trauma by qPCR using the β-globin gene. The sensitivity of plasma cfDNA for predicting posttraumatic organ failure and multiple organ dysfunction syndrome ranged from 50 to 100 %, the specificity ranged from 74 to 95 % and the likelihood ratio ranged from 3.89 to 10.50. Using a classification and regression tree, plasma cfDNA and aspartate transaminase predicted organ failure and multiple organ dysfunction syndrome with an overall correct classification of 93 and 87 %, respectively. Along with other typical predictors for trauma, such as the maximal abbreviated injury score and shock index, the cfDNA quantification may further aid an early diagnosis of organ failure and mortality and so guide clinicians' decisions considering admission to intensive care or modifications in therapy [61].

The deadly China Wenchuan earthquake also caused changes in cfDNA concentrations in the plasma of the Chinese people involved. In a cohort of 1,187 healthy adults and 283 trauma patients the cfDNA levels were measured by a duplex qPCR assay using the β-actin gene. The median plasma cfDNA concentration of females (16.9 ng ml^{-1}) was significantly lower than that of males (22.6 ng ml^{-1}, $p < 0.0001$). During the early stage of injury, the median plasma cfDNA levels of patients increased above 100 ng ml^{-1}, i.e. five times that of the healthy controls. There were statistically significant correlations of plasma cfDNA concentrations with the Injury Severity Score ($p < 0.0001$) and the presence of organ injury ($p = 0.001$) [62].

Acute trauma also includes skin burns that involve an extensive vascular damage and an intense inflammatory response. Stages of the repairing process of skin wounds include processes of cell proliferation, tissue remodeling and angiogenesis. During the wound healing process bone marrow-derived circulating endothelial progenitor cells (EPCs) migrate to sites of neovascularization and support angiogenesis. In burned patients there is an increase in number of EPCs that is associated with the burnt body surface area. Likewise, a rise in the levels of both cfDNA and cfmRNA was observed in the blood circulation of burned patients. In this pilot study, cfDNA and the cfmRNA were measured in the plasma of 19 burned patients at days 1–3 and week 10 following acute thermal injury and in 19 healthy controls by qPCR using two endothelial specific genes *EndoPDI* and *ECSM2*. The increased levels of cfNAs detected in burned patients were related to the severity of burn, in terms of the percentage of the burnt body surface area, and consequently, to the levels of EPCs and also correlated with the levels of cytokines in blood ($p < 0.05$) [63].

To avoid time-consuming quantification of cfDNA by qPCR, neutrophil-derived extra-cellular traps (NETs) containing cfDNA (NET/cfDNA) were quantified by a

fluorescence-based assay in a prospective study [64]. Neutrophils actively release NET/cfDNA together with cytoplasmic effector molecules in order to trap and kill pathogens extracellularly ([65]; chapter "The Biology of CNAPS"). Blood samples from 32 patients with severe burn injuries were sequentially obtained on days 1, 3, 5 and 7 after admission. Seven patients died within a month following burn injury. On day 1, differences of NET/cfDNA values were already significant between survivors (220 ± 20 ng ml^{-1}) and non-survivors (645 ± 163 ng ml^{-1}). By day 3 the values decreased in survivors (175 ± 9 ng ml^{-1}) and non-survivors (322 ± 88 ng ml^{-1}), but remained elevated over 7 days. These increased NET/cfDNA values may reflect neutrophil hyperactivity rather than only tissue damage alone. To verify the prognostic potential of NETs/cfDNA for patient mortality after burn injury, ROC curves were established and showed an AUC of 0.815 on day 1 and of 0.883 on day 3 at a cut-off of 255 ng ml^{-1}; NET/cfDNA had a sensitivity of 0.80 and a specificity of 0.74. These findings show that NET/cfDNA may be a rapid and valuable marker for prediction of the mortality of burned patients [64].

Changes in the levels of numerous cfmiRNAs were detected in the blood circulation of patients with traumatic brain injury. To determine whether cfmiRNAs can identify patients with traumatic brain injury, plasma from 21 patients within the first 24 h post-injury and 10 healthy controls was used. This study showed that miR-16 (AUC of 0.89), miR-92a (AUC of 0.82) and miR-765 (AUC of 0.86) might be promising markers of severe traumatic brain injury. In combination with established clinical practices, such as imaging, neurocognitive and motor examinations, these cfmiRNAs markedly increased their diagnostic accuracy. With a specificity of 100 % and a sensitivity of 100 %, they have the potential to improve classification and management of patients with traumatic brain injury. In patients with mild traumatic brain injury, the plasma levels miR-765 were unchanged, while the levels of miR-92a (AUC of 0.78) and miR-16 (AUC of 0.82) were significantly increased within the first 24 h of injury compared to healthy volunteers [66].

These findings indicate that cfNAs in traumatic patients may provide a useful tool for new therapeutic interventions, monitoring and prediction of patient outcome.

11 Implications of cfNAs in Aging

Aging is accompanied by increased cellular senescence and cell death. With advanced age, exposure to a variety of damaging substances, such as free radicals, leads to cellular damage along with a chronic low-grade inflammation. Moreover, the fragility of lymphocytes also increases [67]. As a result of inflammation and cell death, the accumulation of cfDNA and its somatic mutations have been demonstrated to increase in the blood circulation of elderly people [68]. To examine whether or not aging is reflected in the appearance of cfDNA in blood, plasma of 12 women aged 90+ years and 11 healthy control women (aged 22–37 years) was

used. cfDNA was quantified directly in plasma by the Quant-iTTM DNA high-sensitivity assay kit, the amplifiable cfDNA was assessed using qPCR and the quality of cfDNA was analyzed by a DNA Chip assay. The concentration of cfDNA was significantly higher in nonagenarians than in controls ($p < 0.05$). The quality of the cfDNA also displayed a marked difference between nonagenarians and controls. A fragmented pattern of low-molecular weight cfDNA was observed in the majority of the nonagenarians whereas in controls, cfDNA was intact and had an appearance of high-molecular weight DNA. These findings show that not only the quantity, but also the quality of cfDNA displays a striking difference between nonagenarians and young women [69]. Moreover, the plasma levels of gene-coding cfDNA, Alu repeat cfDNA, cfmitDNA (circulating, cell-free mitochondrial DNA) copy number and the amounts of unmethylated and total cfDNAs were measured in 144 nonagenarian participants (aged 90+) and 30 young controls. In the nonagenarians, higher levels of total DNA ($p = 0.002$), unmethylated cfDNA ($p = 0.001$) and cfmitDNA copy number ($p < 0.03$) were associated with increased frailty. Higher levels of total and unmethylated cfDNAs were also associated with immunoinflammatory activation in the nonagenarians but plasma cfmitDNA appeared to be inert in terms of inflammatory activation [70]. The levels of nucleosomes in the blood also increase with age ($p = 0.0001$). The concentrations of nucleosomes were quantified in the serum of 140 healthy subjects (age 15–70 years) using a Cell Death Detection ELISA kit [71].

These data demonstrate that apart from pathologies, inflammatory factors also play an essential role in age-related processes resulting in increased levels of cfDNA in the blood circulation of elderly individuals.

12 Clinical Relevance of cfNAs in General Medicine

The above-described studies are intended to give a short overview on the research dealing with the changes in the levels of cfNAs and their association with general medicine. They summarize the potential use of cfNAs in general medicine and show the limitations of these studies using different analytical variables.

The concentrations of cfDNA are usually early elevated in the blood circulation after onset of different diseases and may be highest in patients with disease-specific complications and a high risk of death. Accordingly, the elevation in cfDNA levels is not specific for a definite disease and varies among the patients within a patient cohort, but may correlate with the severity of disease. These observations indicate that cfDNA levels, if increased in the blood of an individual, may reflect pathological processes in her/his body. However, aging and altered physiological states that may also display increases in cfNA levels should additionally be considered. The diagnostic accuracy of the established markers and possibly, patient classification and management could be improved by a combination of these disease-specific markers with the quantification of cfDNA concentrations. Since cfDNA levels may change during the course of disease and parallel with the severity of disorder, their

measurement could be a potentially useful marker for the assessment of adverse outcome and might allow clinicians to make a rapid risk stratification for more rational therapeutic decisions.

Several studies have revealed the presence of methylated cfDNA in patient serum or plasma. The detection of gene-specific changes in the methylation pattern of cfDNA represents one of the most promising approaches for detection and risk assessment of various diseases and is more specific than the measurement of cfDNA concentrations. Although epigenetic alterations are not unique for a single disease, there are particular genes that are frequently and specifically methylated and that expression is down-regulated in a certain disorder. Nevertheless, to improve the assay conditions and the clinical relevance of methylated cfDNA, it is essential to select the appropriate disease-related genes from a long list of candidate genes known to be methylated. In sick individuals, this disease-related methylation of cfDNA can be analyzed by gene-specific methylation sensitive PCR.

Apart from cfDNA, much attention and effort have been put into the study on cfmiRNAs over the last few years. In the future, minimally invasive blood analyses of cfmiRNAs may have great potential to complement the existing biomarkers. In particular, some identified miRNAs appear repeatedly significant for different diseases and show promising prognostic associations with disease outcomes, suggesting that miRNA-coordinated regulatory pathways are common for several diseases. Thus, functional studies on these miRNAs will be necessary and may provide a better understanding of the mechanisms underlying diverse disorders. However, an important question that has to be addressed for a miRNA-based therapy is that a single miRNA can target numerous mRNAs and consequently, prevent the translation of many different proteins that are involved in several (disease-relevant) signal transduction pathways. Although the impact of miRNAs on these signal pathways offers additionally potential therapeutic targets in the treatment of diseases, it also leads to multiple changes in the signal transduction of a cell and possibly adverse effects. Therefore, each miRNA and its influence on the different signaling pathways need to be identified and considered before applying miRNAs as targeted therapy for patients. Moreover, for the development of a useful and clinically relevant panel of cfmiRNAs as biomarkers, the establishment of an endogenous reference miRNA with constant values is especially important to normalize the values of circulating candidate cfmiRNAs.

Currently, there are too few substantial progresses to establish a blood-based assay of cfDNA or cfmiRNAs that can stand up clinical trials. Much more emphasis needs to be placed on improving the quality of the methodology to reach consistency of data between laboratories before such an approach can be introduced into the clinic. Pre-analytical and analytical aspects of cfNA analyses have to be standardized. Pre-analytical parameters implicate blood collection, processing of plasma or serum and storage. Analytical parameters include extraction, quantification and assessments of cfNAs (Chapter III; Chapter VI). Another issue that has also to be addressed is that the analyses of cfNAs vary in assay sensitivity and specificity. To date, no approach has been developed that is consistent, robust, reproducible and validated on a large-scale or prospective multicentre patient population.

Usually, in the studies the number of patients analyzed is small and the follow-up period is, likewise, short.

For the most acute clinical syndromes, it is likely that multiple markers rather than a single marker will give the best diagnosis and prognosis. At optimal cut-off levels, higher odds ratios are achieved using combined selected cut-off points. If these technical problems and common deficiencies can be solved and the reliability of such tests and universal standardization of data comparison can be demonstrated, a blood-based assay on either cfDNA or cfmiRNAs could be introduced into clinical routine testing of patients with different diseases in the future.

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Fetal CNAPS – DNA/RNA

Stephanie Cheuk Yin Yu

Abstract The discovery of fetal circulating nucleic acids in maternal plasma and serum has revolutionized prenatal genetic testing by providing a non-invasive source of fetal genetic material. Since fetal DNA coexists with a high background of maternal DNA in the maternal plasma, early studies in the field have been focused on the detection of paternally inherited sequences that are absent from the maternal genome. This approach has been applied to fetal sex and blood type determination, as well as the detection of paternally inherited mutations causing single-gene disorders. The emergence of single molecule counting technologies, such as digital PCR and massively parallel sequencing, have allowed the detection of subtle allelic imbalances and the precise quantification of sequences in the maternal plasma. This precise quantification has enabled the deduction of maternally inherited fetal monogenic diseases, as well as the accurate detection of fetal chromosomal aneuploidies. While some of the applications of fetal circulating nucleic acid have been rapidly incorporated into clinical practice, a number of ethical, legal and social issues have been raised regarding the current and potential use of this technology. Overall, research on fetal circulating nucleic acids in maternal plasma and serum is a rapidly developing and exciting area. It is envisioned that the use of fetal circulating nucleic acids in maternal plasma and serum will play an increasingly important role in prenatal care.

Keywords Fetal circulating DNA/RNA • Fetal sex • Fetal rhesus D • Fetal aneuploidy • Single gene disorders • Fetal genome • Mosaicism • Predictive diagnosis • Preventive medicine

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1 Introduction

Chromosomal and genetic abnormalities are important causes of perinatal morbidity and mortality. Worldwide, 1 in 200 newborns has a major chromosomal abnormality and 1 in 100 has a single-gene abnormality [1]. In view of the high incidence and clinical significance of these abnormalities, prenatal genetic testing, which comprises both non-invasive screening and invasive diagnostic testing, has become an essential part of modern obstetric care.

Currently, prenatal screening tests for a number of conditions are clinically available: for instance, prenatal screening of aneuploidy by a combination of maternal serum biochemical markers and ultrasonographic markers [2] and prenatal screening of cystic fibrosis via carrier testing [3]. These screening tests are either offered to all pregnant women or based on either ethnic background or family or personal history. These screening tests provide pregnant women with a risk assessment for certain fetal chromosomal or genetic abnormalities.

Nevertheless, definitive prenatal diagnosis still requires the sampling of fetal genetic material by invasive procedures, such as chorionic villus sampling (CVS) or amniocentesis, which are associated with a small but significant risk of fetal loss (~1 %) [4, 5].

Over several decades, researchers have been searching for non-invasive methods to sample fetal genetic material. One approach is to isolate fetal nucleated cells from maternal blood [6]. However, such cells are extremely rare – about one cell in each milliliter of maternal blood. In addition, selected fetal cell populations may persist in the maternal circulation after delivery [7] and the isolation of fetal cells is technically demanding. Such difficulties have motivated researchers to look for alternative approaches for non-invasive prenatal testing (NIPT).

An important advance in NIPT came in 1997 when it was reported that cffDNA is present in the plasma and serum of pregnant women [8]. Since then, the field of NIPT has seen rapid developments.

This chapter will provide a comprehensive review of the applications of fetal CNAPS starting with a brief history and summary of the discovery and the biological characteristics of fetal CNAPS. An outline of the general approaches will be given for various clinical applications of fetal CNAPS, including NIPT of paternally inherited traits, aneuploidy and monogenic diseases, with a particular emphasis on the strengths and pitfalls of different approaches. Several important technical and analytical aspects which may be important for clinical implementation will be highlighted. The chapter will conclude with a survey of several unexplored areas and future directions in the field of fetal CNAPS.

2 Characteristics of Fetal Circulating Nucleic Acids

2.1 Circulating cffDNA

In 1997, Lo and colleagues demonstrated the presence of circulating cffDNA by showing the presence of Y chromosome sequences in the plasma and serum of women carrying male fetuses [8]. Subsequent qPCR experiments showed that circulating cffDNA is present in the maternal plasma and serum at a surprisingly high absolute concentration, accounting for a mean of 3–6 % of the total plasma DNA [9]. Recent studies with the use of more precise quantification methods, such as dPCR and MPS, show that the fractional cffDNA concentration may be some two- to three-fold higher [10–12].

In general, cffDNA molecules are detectable in maternal plasma from about the seventh week of gestation onwards [13], increasing in concentration as gestation progresses [11, 13]. Following delivery, circulating cffDNA is cleared rapidly from the maternal plasma [14, 15]. It has been suggested that clearance of circulating cffDNA may occur in two phases: in an initial rapid phase, with a mean half-life of 1 h and in a subsequent slow phase, with a mean half-life of 13 h [15]. cffDNA does not persist in the maternal circulation – its final disappearance is about 1–2 days after delivery, a phenomenon that has been confirmed in a number of studies [15–17].

The relatively high concentration and lack of persistence following delivery are two important advantages of cffDNA as a source of genetic material for non-invasive prenatal testing.

Several pieces of evidence support the placenta as the predominant source of cff DNA: first, in cases of confined placental mosaicism (in which the fetus and placenta have different chromosomal constitutions), placenta-specific genetic signatures can be detected in the maternal plasma [18]; second, cffDNA molecules with placenta-specific epigenetic signatures can be detected in the maternal plasma [19, 20]; and third, in cases of anembryonic pregnancy (in which the placenta is present but no fetus is developing), cffDNA levels are comparable to those of normal pregnancies [21].

Maternal blood cells are believed to be the predominant source of maternally-derived DNA in maternal plasma [22]. Using a sex-mismatched bone marrow transplantation model, researchers have showed that most of the plasma cfDNA molecules of the recipient show the sex genotype of the transplant donor [22]. This conclusion has been extrapolated to the scenario of maternal plasma cffDNA in pregnancy and appears to be accepted by many workers in the field [23–25].

Plasma DNA molecules are fragmented in nature. In maternal plasma, cffDNA is generally shorter than maternal cfDNA [26]. By using paired-end massively parallel sequencing, researchers have constructed the size profile of total (predominantly maternal) plasma DNA and fetal-specific plasma DNA [27]. The size distributions of both total and cffDNA show a series of peaks [27]. They both have a peak at 166 bp, another peak at 143 bp and a distinctive 10-bp periodicity

below 143 bp [27]. The most marked difference between fetal and total plasma DNA is that plasma cffDNA has a reduced 166 bp-peak and a more prominent 143 bp-peak [27].

2.2 *Circulating cffRNA*

Three years after the discovery of circulating cffDNA, Poon et al. [28] demonstrated the presence of circulating cffmRNA in maternal plasma by the detection of Y-chromosome-specific mRNA in the plasma of women carrying male fetuses. This finding was surprising because mRNA was believed to be labile in plasma due to the existence of plasma ribonucleases. Later, plasma mRNA was found to be associated with subcellular particles that render them remarkably stable, probably by protecting them against degradation by plasma ribonucleases [29]. Structurally, mRNA molecules in maternal plasma are similar to plasma DNA molecules in that they are also fragmented, showing various degrees of degradation at their 3′ ends [30].

In a recent study by Quake's group, transcripts from fetal brain and fetal liver have also been detected in the maternal plasma. cffmRNA of placental origin, such as *human placental lactogen* (*hPL*), the β-subunit of *human chorionic gonadotropin* (β-*hCG*) and *corticotropin-releasing hormone* (*CRH*), is readily detectable in maternal plasma [31]. Ng et al. [31] quantified *hPL* and β-*hCG* levels in maternal plasma using RT-qPCR and found that the plasma cffmRNA levels of *hPL* and β-*hCG* correlate with their corresponding protein levels at various gestational ages. Both *hPL* and β-*hCG* mRNA can be detected in the maternal plasma as early as the fourth week of gestation and their concentrations increase as gestation progresses. Furthermore, like plasma cffDNA, cffmRNA is rapidly cleared from maternal plasma following delivery [14, 32]. Circulating *hPL* cffmRNA has been shown to be cleared from the maternal plasma with a mean half-life of 14 min [32].

3 Fetal Epigenetic Markers

Epigenetic modifications are heritable molecular changes that regulate gene expression, but are not associated with changes in DNA sequence. DNA methylation, one of the best characterized epigenetic mechanisms, is a process by which a methyl group is added to the 5′ carbon of a cytosine residue to give 5-methylcytosine, often occurring on the cytosine residues of CpG dinucleotides.

In 2002, Poon et al. [33] used an imprinted locus to demonstrate that it is possible to detect fetal-specific epigenetic signatures in maternal plasma. The group studied an imprinted region between the *insulin-like growth factor 2* (*IGF2*) and *H19* genes, which is methylated when inherited from the father and unmethylated when inherited from the mother (Fig. 1) [33]. They were able to detect an allele on this

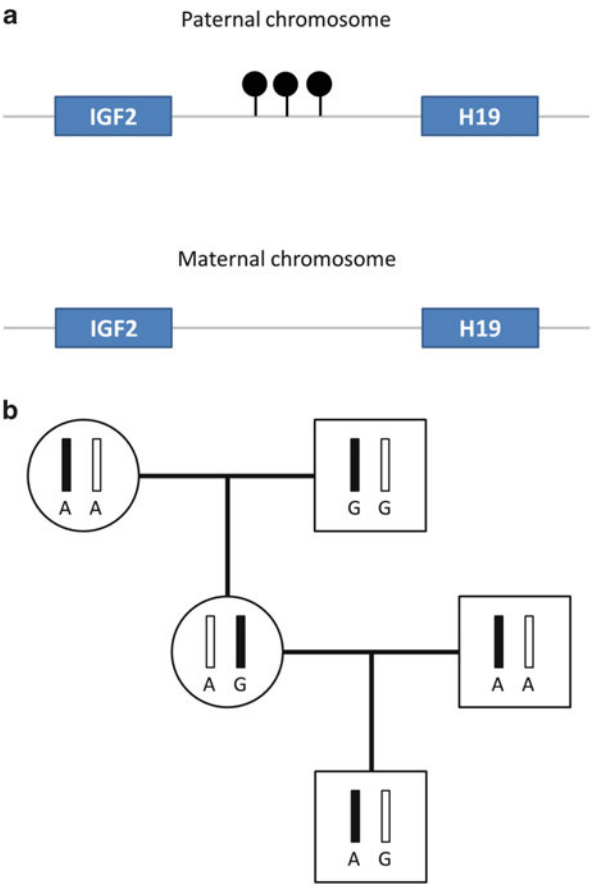

Fig. 1 First fetal-specific epigenetic signature detected in maternal plasma. (**a**) The imprinted region between the *insulin-like growth factor 2* (*IGF2*) and *H19* genes is methylated when inherited from the father and unmethylated when inherited from the mother. *Filled circles* indicate DNA methylation. (**b**) The G allele that the mother has inherited from the grandfather is methylated. When the G allele is passed from the mother to the fetus, it will become unmethylated. *Filled bars* represent methylated sequences whereas *open bars* represent unmethylated sequences [33]

imprinted locus that the fetus had inherited from the mother in the maternal plasma (Fig. 1), demonstrating for the first time that maternally-inherited cffDNA sequences can also be detected in maternal plasma [33].

DNA methylation is one of the mechanisms that have contributed towards tissue-specific gene expression [34]. Since plasma fetal and maternal DNA originates predominantly from different tissues, namely the placenta and maternal blood cells, respectively, the DNA methylation pattern between them may be postulated to be different. Therefore, fetal-specific epigenetic markers may be found by distinguishing the DNA methylation patterns between the placenta and maternal blood cells.

The *maspin* (*SERPINB5*) gene promoter is the first sequence that has allowed the demonstration that a placenta-specific epigenetic signature can be detected in maternal plasma [20]. The *SERPINB5* promoter is hypomethylated in the placenta and hypermethylated in maternal blood cells [20]. Hypomethylated *SERPINB5* promoter sequences can be detected in maternal plasma samples from all three trimesters of pregnancy [20].

On the other hand, several fetal epigenetic markers have opposite methylation patterns in the placenta and maternal blood cells; for instance, the *Ras association domain family 1A* (*RASSF1A*) gene promotor is hypermethylated in the placenta [35] and hypomethylated in maternal blood cells [19]. Hypermethylated *RASSF1A* sequences are also readily detectable in maternal plasma during pregnancy [19].

Characterization of fetal DNA using this epigenetic approach yields consistent results with methods using genetic markers. Positive correlation between the levels of fetal epigenetic markers and fetal genetic markers in the maternal plasma has been demonstrated [19, 20]. Sequences with fetal-specific epigenetic signatures are cleared rapidly (within 24 h) from the maternal plasma after delivery [19, 20].

4 Detection of Paternally Inherited Traits

Early studies in the field of fetal CNAPS focused on the qualitative detection in the maternal plasma or serum of fetal-specific DNA sequences inherited from the father and absent from the maternal genome. Two representative applications are prenatal fetal sexing and rhesus D genotyping, both of which are now clinically available in a number of countries.

4.1 Fetal Sex Determination

Prenatal fetal sex determination is useful in pregnancies at risk for X-linked recessive diseases such as Duchenne muscular dystrophy and hemophilia [36]. Since males have only a single copy of chromosome X, the male fetus of a mother who is a carrier of an X-linked recessive disorder has a 50 % chance of inheriting the defective gene and hence developing the disorder. Therefore, if a carrier woman is pregnant with a male fetus, invasive prenatal diagnosis would be recommended. On the contrary, if a carrier mother is bearing a female fetus, invasive testing can be avoided.

Prenatal fetal sexing is also useful in pregnancies at risk for congenital adrenal hyperplasia (CAH) [37]. CAH is an autosomal recessive disorder resulting from defects in certain enzymes involved in the biosynthesis of cortisol from cholesterol. As a consequence of the enzyme deficiencies, accumulated cortisol precursors e.g. pregnolone are converted into androgens. While excessive synthesis of androgens causes virilization of female fetuses, male fetuses are not affected. Therefore, antenatal dexamethasone treatment, which can ameliorate the effect of virilization, is given to female fetuses.

Currently, ultrasound examination is the standard method of assessing fetal sex non-invasively however, it is unreliable before 11 weeks of gestation. With the discovery of circulating cffDNA in maternal blood, fetal sex can now be determined non-invasively by the detection of Y chromosome sequences in either the

maternal plasma or serum [8, 36, 37]. Positive amplification of Y chromosome sequences indicates a male fetus; however, negative amplification should be interpreted with caution since it may be due to low levels of cffDNA in maternal blood or even complete absence of plasma or serum cffDNA. Therefore, it is of prime importance to verify the presence of cffDNA for the validation of negative amplification. To ascertain detectable levels of cffDNA in maternal blood samples, biallelic insertion/deletion polymorphisms [38] or fetal epigenetic markers can be used [19].

There are two systematic reviews and meta-analyses on the use of cffDNA for prenatal fetal sex determination [39, 40]. Both reviews have shown that test performance is generally high (Sensitivity: 95.4 and 96.6 %; Specificity: 98.6 and 98.9 %) [39, 40].

Thus far, there are more than a 100 studies on prenatal sex determination using circulating DNA. Most of the studies analyze plasma samples and some use serum, but both of them show similar performance [40].

Reported studies mainly used qPCR and conventional PCR [39]. The uses of other methods such as matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) [41] and pyrophosphorolysis-activated polymerization [42] have also been reported. Among these methods, PCR appears to give the best performance.

Different Y chromosome sequences have been used, including *SRY*, *DYS14*, *DYS1*/*DAZ*, *DYS3* and *AMELY*. Among them, *SRY* and *DYS14* (a multi-copy sequence) are being used more frequently and both of them appear to perform equally well [40]. Using this method, fetal sex can be reliably determined as early as 7 weeks of gestation. Although test performance increases with gestational age, test performances in the late first and early second trimesters are comparable.

In a few studies, inconclusive results or failed tests have been reported, which would require retesting with another blood sample obtained at a later stage of pregnancy. According to Wright and colleagues, the reported reasons for either inconclusive or false-positive or false-negative results include: blood samples not being processed within 48 h of collection, poor plasma and serum quality, variable or low concentration of cffDNA in the maternal blood samples and a suboptimal diagnostic threshold, the cutoff value of the threshold cycle used for assigning positive or negative detection of Y chromosome in a sample, used for fetal sex determination [40].

Non-invasive determination of fetal sex using circulating cffDNA in maternal blood has already been incorporated into routine clinical practice in a number of countries including the United Kingdom, the Netherlands, France and Spain. Several companies have also begun to offer this test through the internet as a direct-to-consumer test, so raising concerns of potential abuse for non-medical purposes [23].

4.2 Fetal Rhesus D Genotyping

Like antigens in the ABO blood group system, Rhesus (Rh) factor is a red blood cell surface antigen. There are five main Rh antigens, namely D, C, c, E and e. Among them, the D antigen is the most immunogenic. A person with RhD-positive phenotype has at least one functional copy of the *RHD* gene, whereas a person with RhD-negative phenotype lacks functional *RHD*.

In the context of pregnancy, RhD incompatibility occurs when a RhD-negative woman is pregnant with a RhD-positive fetus. Maternal alloimmunization against the D antigen is the major cause of hemolytic disease of the fetus and the newborn (HDFN). The pathogenesis of HDFN usually involves a primary and a secondary exposure. Potential sensitizing events can be a previous pregnancy with a D-positive fetus or previous blood transfusion with D-positive blood. If a woman who has been previously sensitized becomes pregnant with a D-positive fetus, then secondary exposure to the D-positive blood would cause the sensitized woman to produce a large amount of anti-D antibodies that can cross the placenta and attack the D-positive red cells of the fetus.

Therefore, prenatal determination of fetal RhD genotype is clinically useful in the management of pregnancies in RhD-negative women. If the fetus is RhD positive, administration of anti-D immunoglobulin would reduce the chance of sensitization in a non-sensitized woman. Even though anti-D prophylaxis does not work for sensitized women, knowing the fetal RhD status gives clinicians sufficient time to plan for further tests and treatments. Conversely, if the fetus is RhD negative, no further testing and treatment would be needed.

Traditionally, fetal genetic material for fetal RhD typing was obtained through amniocentesis or CVS. However, in addition to the inherent risk of miscarriage, these invasive procedures could induce fetomaternal hemorrhage, which would increase the risk of maternal sensitization.

With the demonstration of cffDNA in the maternal plasma, non-invasive prenatal RhD typing became feasible [43, 44]. In the genome of a RhD-negative woman, the *RHD* gene is typically absent. Thus, any *RHD* sequences detected in the maternal plasma can be presumed to be from the fetus and inherited from the father. In cases with negative detection of *RHD*, similar to the negative detection of Y chromosome sequences in fetal sexing, it is critical to ascertain the presence of cffDNA to minimize false negatives.

Initial studies of fetal Rh typing amplified a single region on *RHD*, with either conventional or qPCR. Advances in the molecular characterization of rhesus genes revealed extensive polymorphisms in the Rh blood group system [45]. There are more than 150 alleles for the *RHD* gene and the frequencies of these *RHD* variants vary widely among different populations [45, 46]. In the Caucasian population, the majority of D-negative phenotypes are caused by a complete deletion of the *RHD* gene whereas, the majority of D-negative Africans have an intact but nonfunctional *RHD* pseudogene, the *RHDψ* or the *RHD-CE-D* hybrid gene. Therefore, to avoid false-positive results due to these common gene variants, more recent studies have

amplified at least two distinct regions on the *RHD* gene (commonly exon 5 and 7) [47–50].

Furthermore, maternal-fetal incompatibility of other blood cell antigens such as the c antigen of the Rh blood group system and the K antigen of the Kell blood group system can also cause HDFN. In the light of this, a few studies have analyzed circulating cffDNA in maternal plasma for non-invasive prenatal *RHCE* and *KEL* genotyping and have demonstrated accurate test results [50, 51].

Currently, in the United Kingdom [52], the Netherlands [50], Denmark [47], France [53] and Sweden [54], non-invasive prenatal testing of fetal rhesus status is already in clinical use. Before its clinical introduction, antenatal anti-D treatment was usually given to all RhD-negative women regardless of the fetal RhD status at about 28 weeks of gestation. Since 40 % of RhD-negative women actually carry a RhD-negative fetus, these women would receive the treatment unnecessarily [48, 53]. Anti-RhD immunoglobulin is a pooled human blood product from RhD-negative donors who have been exposed to RhD-positive red cells to stimulate the production of RhD antibodies [48]. Therefore, targeted anti-D prophylaxis would prevent pregnant women from unnecessary exposure to human blood product and would reduce the risk of infection [55]. In addition, this approach would minimize the usage of anti-D immunoglobin, which is only available in limited amounts.

5 Detection of Fetal Aneuploidy

Fetal chromosomal aneuploidy is the main reason why pregnant women choose to undergo prenatal diagnosis. Aneuploidy refers to an abnormal number of chromosomes; more specifically, having one extra or one less copy is termed trisomy or monosomy, respectively. While the majority of chromosomal aneuploidies are non-viable, leading to spontaneous abortion, a subset may survive to term and beyond. Fetuses with trisomy appear to be more viable than those with monosomy. The three most common autosomal trisomies detected in newborns are: (a) trisomy 21 (Down syndrome), with an incidence of 1 in 800 live births; (b) trisomy 18 (Edwards syndrome), with an incidence of 1 in 8,000 live births; and (c) trisomy 13 (Patau syndrome), with an incidence of 1 in 20,000 live births.

The detection of fetal chromosomal aneuploidy is much more challenging than the determination of fetal sex and RhD status since the former requires the quantitative analysis of fetal chromosome dosage, while the latter simply involves the detection of specific cffDNA sequences in the maternal plasma. Since cffDNA represents only a small proportion of the DNA in maternal plasma, the overwhelming maternal DNA background makes the assessment of fetal chromosome dosage difficult.

In fact, increased levels of cffDNA in the maternal plasma and serum have been reported in trisomy 21 and trisomy 13, but not in trisomy 18 [56–58]. However, the maternal plasma and serum cffDNA concentrations in euploid and aneuploid

pregnancies significantly overlapped. Therefore, it seemed difficult to unambiguously distinguish euploid from trisomic fetuses just by measuring cffDNA concentration in maternal plasma.

5.1 Enrichment of Fetal Nucleic Acid

After the realization that cffDNA only represents a minor fraction of the DNA in maternal plasma, a number of researchers have attempted to increase the fractional fetal DNA concentration with either physical enrichment or molecular enrichment.

Physical Enrichment of cffNAs

Physical enrichment of cffDNA in maternal plasma entails either the selective enrichment of the cffDNA by using size or the reduction of the background maternal cfDNA by using formaldehyde.

Based on the fact that cffDNA in maternal plasma is generally shorter than the maternal cfDNA [26], Li et al. [59] targeted the shorter DNA molecules in plasma to enrich for cffDNA. This size-based enrichment has been applied in the detection of paternally inherited fetal mutations in maternal plasma [60]. On the other hand, whether the degree of enrichment attained by using this approach is enough to detect fetal aneuploidy in the maternal plasma is still unknown. A drawback to this method used by Li et al. [59] is that it is relatively labour-intensive and prone to contamination, involving agarose gel electrophoresis for size separation and manual excision of targeted gel slices for size fractionation. However, using an automated size fractionation system could potentially solve these problems.

An alternative approach to increasing the relative proportion of fetal DNA in the maternal plasma is to suppress the maternal background DNA. Dhallan et al. [61] treated blood samples with formaldehyde, which they claimed could inhibit cell lysis and reduce the release of DNA from maternal blood cells into the maternal blood since formaldehyde stabilizes cell membranes. In addition, formaldehyde inhibits nucleases in the maternal blood, which may increase the recovery of fetal DNA by reducing the degradation of plasma DNA [61]. Dhallan et al. [61] showed significantly increased fetal proportions in the formaldehyde-treated samples as compared with the untreated samples. However, other groups were unable to reproduce this result [62, 63]. Zhang et al. [64] suggested that this discrepancy might be due to the difference in sample processing time after blood collection.

Molecular Enrichment of cffNAs

Another approach to overcoming the high maternal DNA background is to target the nucleic acids that are specifically derived from the fetus. Since the predominant

source of cffDNA and maternal cfDNA in the maternal plasma is the placenta and the maternal blood cells, respectively, one can identify fetal-specific nucleic acid markers from the different methylation and expression patterns between the placenta and the maternal blood cells. One strategy whereby these placenta-specific epigenetic signatures and placenta-derived RNA can be used for detecting aneuploidies is when their sequences are located on a disease-causing chromosome. Since most common aneuploidies are on chromosomes 21, 18, and 13, researchers have looked to identify epigenetic and RNA fetal markers on these chromosomes.

Fetal Epigenetic Markers

Epigenetic markers are based on a difference in methylation levels between the placenta and the maternal blood cells in the same sequence. The *SERPINB5* gene is hypomethylated in cffDNA and hypermethylated in maternal cfDNA and was the first fetal epigenetic marker used in the detection of fetal aneuploidy since it is located on chromosome 18 [20, 65]. Subsequent efforts were made to systematically search for fetal epigenetic markers on chromosome 21 [66, 67]. Candidates such as *HLCS*, *AIRE*, *SIM2* and *ER,* have been identified and so may potentially be used for NIPT of trisomy 21 [66, 67].

To detect fetal epigenetic markers in maternal plasma DNA, two methods are commonly used: bisulfite modification and restriction enzyme digestion. When DNA is treated with sodium bisulfite, cytosine, but not 5-methylcytosine, is converted into uracil. Therefore, plasma DNA molecules with identical DNA sequences but different methylation status are now distinguishable. Such sequences are then detected either via a methylation-specific PCR (MSP), in which PCR primers are specifically designed to amplify either the altered or unaltered sequence, or via sequencing, in which cytosine and 5-methylcytosine in the original DNA sequences will be shown as thymine and cytosine, respectively, in the sequence trace. A major drawback to this approach is the massive DNA degradation (over 90 %) that occurs during the process of bisulfite conversion. This is problematic in NIPT as cffDNA is already present in very low quantities in the maternal plasma.

On the other hand, restriction enzyme digestion uses methylation-sensitive restriction enzymes (MSREs). Similar to other restriction enzymes, MSREs recognize and cleave at specific sequences but are different in that they are also sensitive to cytosine methylation and cannot cleave methylated DNA. When using a hypermethylated fetal marker, for instance *RASSF1A* [19], treatment with MSRE cleaves the hypomethylated maternal DNA sequences, while the hypermethylated cffDNA sequences remain intact and can be subsequently amplified by various PCR methods. Conversely, when using a hypomethylated fetal marker, for instance *SERPINB5* [20], MRSE cleaves the cffDNA sequences, which could then be amplified by a stem-loop primer [68]. Altogether, these methods of distinguishing epigenetic differences in DNA combined with the fetal epigenetic markers in potentially aneuploid chromosomes can be used for the analysis of fetal aneuploidy.

cffRNA Markers

Other than epigenetic markers, another fetal-derived source of nucleic acids is placenta-derived RNA. Scientists have systematically searched for placental mRNA markers using high throughput microarray-based expression profiling of the placenta and maternal blood cells [69]. Differential expression of the candidate markers was then confirmed by RT-qPCR.

The best cffRNA transcript markers for the detection of fetal aneuploidy are not only transcribed from the potentially aneuploid chromosomes, but also have a high absolute expression level in placenta since this combination of characteristics makes them more specifically and readily detectable in maternal plasma [70]. Transcripts with a large difference in the relative expression levels between the placenta and maternal blood cells are also preferred. In this regard, scientists have used *PLAC4* mRNA, which has both a high absolute expression in the placenta and a large difference in the relative expression between the placenta and maternal blood cells, for the detection of trisomy 21 [70]. With cffRNA markers, researchers can specifically detect cffNAs, without interference from the maternal nucleic acid background in their analysis.

To tackle the inherent problems of low cffDNA fraction and high maternal cfDNA background in analyzing maternal plasma cfDNA, researchers have used either physical enrichment or molecular enrichment. Physical enrichment actually changes the absolute number of DNA molecules in the plasma by either isolating cffDNA by size or suppressing maternal DNA release. On the other hand, molecular enrichment retains the original composition of the plasma and instead specifically targets cffNAs for detection and analysis.

5.2 Determining the Fetal Chromosome Dosage of Enriched cffNAs

Because molecular enrichment identifies the fetal subset of nucleic acids without the interference of maternally derived nucleic acids, one can directly analyze and determine the fetal chromosome dosage. Two approaches, namely the allelic ratio analysis and the relative chromosome dosage analysis, have been used in combination with the molecular enrichment strategy to determine the fetal chromosome dosage.

Allelic Ratio Analysis

The core concept behind allelic ratio analysis is to determine the ratio of alleles at a heterozygous locus on a potentially aneuploid chromosome. Theoretically, the heterozygous locus would have an allelic ratio of 1:1 for a euploid fetus or an allelic ratio of either 2:1 or 1:2 for a trisomic fetus (Fig. 2). Allelic ratio analysis can

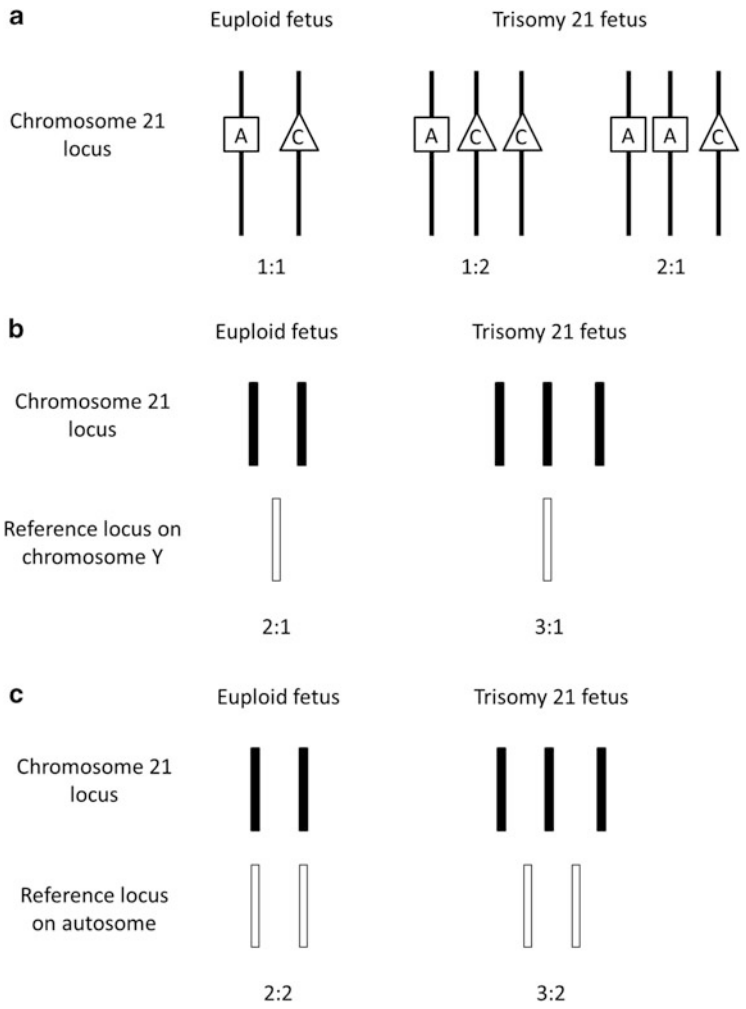

Fig. 2 Approaches for the determination of fetal chromosome dosage. (**a**) Allelic ratio approach. (**b**) Relative chromosome dosage approach using a reference locus on the Y chromosome. (**c**) Relative chromosome dosage approach using a reference locus on an autosome

be applied to either fetal epigenetic or RNA markers. For epigenetic markers, the process of analysis has been referred to as the Epigenetic Allelic Ratio (EAR) approach [65]; for RNA markers, the process is named the RNA-SNP allelic ratio approach [70].

Having identified a placenta-specific DNA methylation marker, the hypomethylated *SERPINB5* sequence, in maternal plasma, Tong et al. [65] determined the allelic ratio of a SNP located on these hypomethylated *SERPINB5* sequences. Briefly, their process is as follows: first, the method involves bisulfite conversion and methylation-specific PCR to amplify fetal-specific hypomethylated

SERPINB5 sequences; then, primer extension and mass spectrometric analysis are used to determine the allelic ratio. To distinguish between a euploid and a trisomic fetus, a reference interval of allelic ratios would need to be established with plasma samples from women with euploid fetuses. A drawback of the EAR approach is that it requires at least 4,000 template molecules, which is around 16 mL of plasma, in the beginning before bisulfite conversion to distinguish between trisomy and euploid pregnancies.

Similarly, the RNA-SNP allelic ratio approach applies allelic ratio analysis on placental expressed mRNA. Lo et al. [70] used the *PLAC4* mRNA in maternal plasma, which is transcribed from chromosome 21 and expressed specifically from the placenta, to detect fetal trisomy 21. Basically, Lo et al. [70] performed a RT-qPCR on the maternal plasma RNA sample and determined if there was an allelic imbalance of a SNP on the *PLAC4* mRNA using primer extension and mass spectrometry. One important assumption of this approach is that the allelic ratio in the plasma reflects the allelic ratio in the placenta. The sensitivity and specificity of this RNA-SNP allelic ratio approach is 90 % and 96.5 %, respectively, and the accurate detection of fetal trisomy 21 requires an estimated minimum of 1,000 template molecules of *PLAC4* mRNA.

Instead of using mass spectrometry, dPCR can also be used in RNA-SNP analysis [70]. With dPCR, individual alleles can be counted, resulting in a more precise quantification of alleles when compared with a mass spectrometry-based method. Lo et al. [70] used a statistical algorithm called the sequential probability ratio test (SPRT) [71] to determine if there is an allelic imbalance. The presence of an allelic imbalance of a fetal heterozygous SNP would suggest trisomy.

In fact, Dhallan et al. [72] have also used the allelic ratio method in conjunction with their cffDNA enrichment strategy by formaldehyde for the determination of fetal chromosome dosage.

The major limitation of these allelic ratio approaches is that the fetus must be heterozygous for the analyzed SNP. Multiple SNP markers are needed to achieve a broad population coverage using the EAR and RNA-SNP approaches. However, gathering multiple SNP markers is difficult because the SNP must also be located relatively close to the fetal-specific marker (either epigenetic or RNA). This requirement is to ensure that the heterozygous SNP and the fetal-specific marker would coexist on the same plasma DNA molecule and be quantified. Therefore, achieving broad population coverage with either the EAR or the RNA-SNP approach would be difficult.

Epigentic-Genetic Chromosome Dosage Method

Another approach to determining the fetal chromosome dosage for non-invasive fetal diagnosis is to analyze the relative chromosome dosage. The core concept behind relative chromosome dosage analysis is to assess the ratio between the loci of a potentially aneuploid chromosome and the loci of a reference non-aneuploid chromosome (Fig. 2).

Applying this relative chromosome dosage approach to analyze fetal-specific epigenetic locus/loci is called the epigenetic-genetic (EGG) chromosome-dosage approach. Tong et al. [73] used the relative dosage of a fetal-specific epigenetic marker on chromosome 21, the hypermethylated holocarboxylase synthetase (*HLCS*) sequence, and a fetal-specific genetic marker on a reference chromosome, *ZFY* on chromosome Y. Since there is only one Y chromosome, the expected chromosome ratio would be 2:1 for a euploid male pregnancy and 3:1 for a trisomic male pregnancy (Fig. 2). The reference fetal-specific marker is not an epigenetic marker; instead, any paternally inherited fetal-specific genetic marker could potentially be used [74]. However, when the reference locus is located on an autosome, the expected ratio for a euploid and a trisomic fetus would be 2:2 and 3:2, respectively (Fig. 2). Therefore, the EGG chromosome-dosage approach can be used to detect fetal trisomy 21 from maternal plasma DNA [73, 74].

One advantage of the EGG approach is that the epigenetic and the genetic markers do not need to be in the same genomic region. Therefore, it is easier to achieve broader population coverage than those approaches that rely upon allelic ratio analysis, such as the EAR approach.

Single-Molecule Counting Approach

The advent of single molecule counting technologies, such as dPCR and MPS, has changed the field of maternal plasma nucleic acid-based prenatal detection of fetal aneuploidy. With these technologies, the enrichment of the fetal subset of nucleic acids is no longer necessary prior to analysis. Instead, it is now possible to directly infer the fetal chromosome dosage in maternal plasma by counting single molecules. The theory behind the single molecule counting approach to diagnosing fetal aneuploidy requires that a trisomic fetus will release an increased amount of DNA into the maternal plasma due to its extra chromosome. The amount of increase depends on the proportion of cffDNA in the maternal plasma sample, which is around 10 % in early pregnancy. With a cffDNA fraction of around 10 %, a trisomic fetus would contribute around 5 % more fetal DNA from the aneuploid chromosome than a euploid fetus. This small 5 % increase in the chromosome dosage of a particular chromosome would be challenging to be measured precisely without either dPCR or MPS. The accurate quantification provided by either dPCR or MPS has revolutionized the field of fetal aneuploidy detection.

dPCR allows individual DNA molecules to be counted [75]. This is in contrast to conventional qPCR, which only has a discrimination power of 2-fold, a power inadequate for detecting the small increase in chromosome dosage contributed by the fetal aneuploid chromosome. The theory and process behind using dPCR to quantify maternal plasma DNA is as follows: (a) the sample is diluted and partitioned into hundreds to thousands of wells such that each well contains either a single or no target molecule; (b) each well performs an individual PCR and each reaction will be either positive or negative for a target amplicon; (c) the number of

target molecules in the sample is then measured by counting the number of positive PCRs [75, 76].

With the precise quantification provided by dPCR, the theory of relative chromosome dosage can be applied to a maternal plasma sample without prior molecular enrichment of cffDNA. This method directly assesses whether or not a chromosome 21 locus is overrepresented when compared with a locus on a reference chromosome [77]. For a euploid pregnancy, the number of positive wells for the two target loci would be approximately equal whereas for a trisomy 21 pregnancy, the number of positive wells for the chromosome 21 locus would be greater than those for the reference chromosome.

Since the degree of overrepresentation depends on the cffDNA fraction in the maternal plasma sample, scientists have estimated the number of molecules required for reliable detection of fetal trisomy at different fetal fractions using DNA mixtures and computer simulations [77]. From their simulations, they determined that a maternal plasma sample with a fetal fraction of 25 % would require the analysis of approximately 7,680 DNA molecules to detect fetal trisomy (correct classification in 97 % of cases) [77].

Initially, labor-intensive, manual partitioning of DNA samples into hundreds of wells was required. However, the development of automated dPCR platforms, such as the microfluidic dPCR chips, emulsion PCR and droplet dPCR, has allowed dPCR to be performed in a high-throughput fashion, hence greatly increasingly its feasibility for routine clinical applications.

The major limitation of using dPCR for fetal aneuploidy detection is that it is a locus-specific method, meaning that only those plasma DNA fragments with a specific locus targeted by the PCR primers would be analyzed (Fig. 3) [78]. These fragments must also encompass the full amplicon, as only those that allow the binding of both PCR primers will be amplified. This requirement implies that most of the DNA fragments in a particular maternal plasma sample that are derived from a potentially aneuploid chromosome would be 'wasted'. As a consequence and for illustration purposes, to capture the 7,680 target molecules necessary for analyzing trisomy in a fetal fraction of 25 %, around 15 mL of maternal plasma would be needed. Therefore, fetal aneuploidy detection using dPCR to quantify one locus on a potentially aneuploid chromosome relative to another locus on a non-aneuploid chromosome would typically require a large volume of plasma sample.

MPS In contrast to dPCR, MPS does not require a DNA fragment to contain a particular pair of primer-binding sites (Fig. 3), meaning that all plasma DNA fragments could be counted. Therefore, it makes a more efficient use of the limited amount of DNA molecules in the maternal plasma. In addition, MPS allows very precise quantification as millions of plasma DNA molecules are analyzed in a single run.

There are three major MPS-based methods developed for prenatal detection of fetal chromosomal aneuploidy in maternal plasma, namely the whole genome random sequencing approach, the chromosome-selective targeted sequencing approach (targeted sequencing of nonpolymorphic loci on the chromosome of

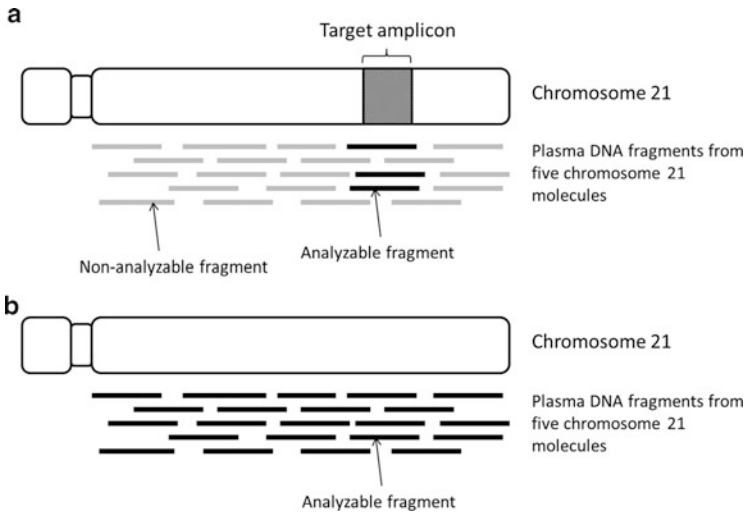

Fig. 3 Comparison between locus-specific and locus-independent methods for DNA quantification. (**a**) When a locus-specific method is used, only those fragments that contain the complete locus and allow the binding of both PCR primers will be amplified. (**b**) Alternatively, when a locus-independent method is used, virtually all fragments originated from that chromosome could be used. Therefore, with the same amount of plasma DNA input a locus-independent DNA quantification method would be more precise as more molecules are being counted (Figure adapted, with permission, from Chiu et al. [78]. Copyright, 2009; Elsevier)

interest) and SNP-based targeted sequencing approach (targeted sequencing of polymorphic loci on the chromosome of interest).

Whole Genome Approach

The whole genome sequencing approach entails the random sequencing of a representative portion of the DNA molecules in maternal plasma. Each sequence tag is aligned to the reference human genome to determine its chromosomal origin; the number of sequence tags aligned to each chromosome is then counted (Fig. 4). The fetal chromosome dosage can then be assessed in two different ways: (1) the proportional representation of the at-risk chromosome can be determined and compared with that of a group of euploid pregnancies [79]; (2) the counts of the at-risk chromosome can be normalized with the counts of other chromosomes expected to be disomic [80]. The robustness of these approaches for the noninvasive detection of fetal T21 was first demonstrated in two initial proof-of-concept studies [79, 81]. In a case report, Lun et al. [82] demonstrated that the whole genome sequencing approach could similarly be applied for the detection of Down syndrome due to unbalanced Robertsonian translocation.

Scientists have used different statistical approaches to analyze data and report the results. Most reported studies used a z-score which is defined as the number of

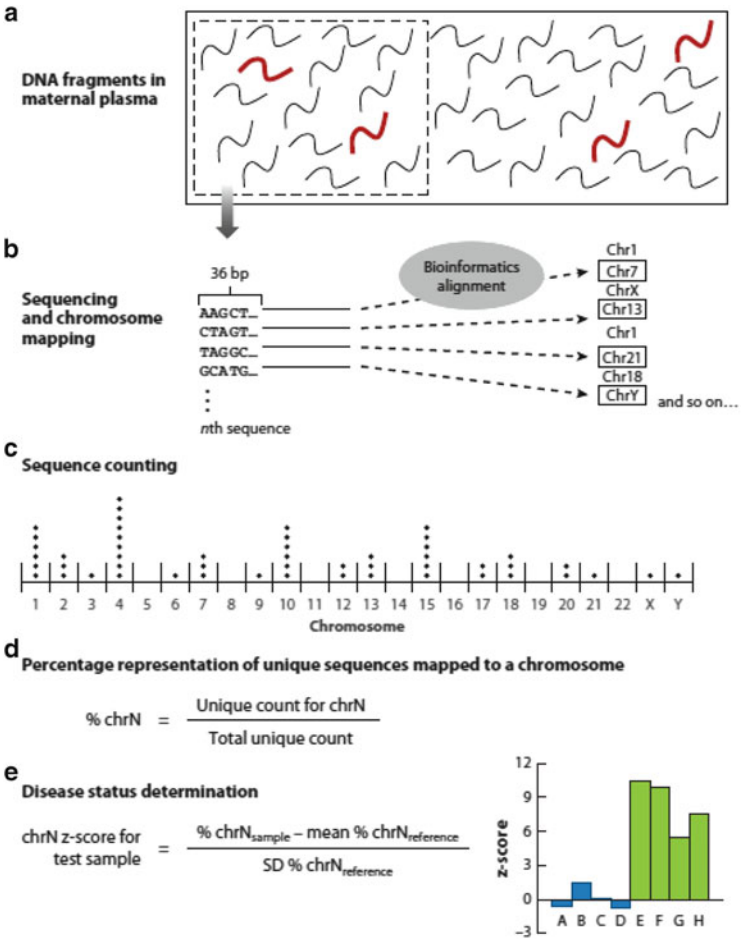

Fig. 4 Procedural framework for the whole genome random sequencing approach for the non-invasive detection of fetal chromosomal aneuploidy. (**a**) cffDNA (*thick red fragments*) circulates in maternal plasma as a minor population in a high background of maternal DNA (*black fragments*). A sample containing a representative profile of DNA molecules in maternal plasma is obtained. (**b**) As an illustration, one end of each plasma DNA molecule was sequenced for 36 bp using massively parallel sequencing. The chromosomal origin of each 36-bp sequence was identified through mapping to the human reference genome by bioinformatics analysis. (**c**) The number of unique sequences mapped to each chromosome was counted and then (**d**) expressed as a percentage of all unique sequences generated for the sample, termed % chrN for chromosome N. (**e**) Z-scores for each chromosome and each test sample were calculated using the formula shown. The z-score of a potentially aneuploid chromosome is expected to be higher for pregnancies with an aneuploid fetus (cases E–H, *green bars*) than those without an aneuploid fetus (cases A–D, *blue bars*) (Figure reproduced, with permission, from Chiu et al. [79]. Copyright, 2008; National Academy of Sciences, U.S.A.)

standard deviations away from the mean of a reference dataset [12, 79, 80]. In studies using a z-score many have reported the results as either "positive" or "negative" for trisomy [12, 79], with a number of other studies reporting an additional category of "no call" [80], based on predefined cutoff values of the z-score. On the other hand, one group of researchers used a binary hypothesis t-test where one hypothetical model corresponds to a euploid fetus, while the other corresponds to an aneuploid fetus. They then calculated a relative logarithmic likelihood odds ratio between the binary hypotheses (named as the L-score) for classification [83].

To increase throughput, plasma DNA molecules from different samples can be labeled with unique tags such that multiple samples can be pooled together and sequenced [10, 84, 85]. While most studies have used the Genome Analyzer or the HiSeq sequencing platform from Illumina, researchers have also applied the whole genome tag counting approach to other MPS platforms, such as the ABI Sequencing by Oligonucleotide Ligation and Detection (SOLiD) platform [86] and the Ion Torrent Personal Genome Machine (PGM) sequencer [87] (see also chapter "Genomic Approaches to the Analysis of Cell Free Nucleic Acids").

The accuracy and precision of the whole genome sequencing approach to diagnosing aneuploidy are affected by several factors. For cases with an aneuploid fetus, the degree of quantitative perturbation would positively correlate with the fetal fraction. A minimum fetal fraction (i.e. 4 %) is usually required to ensure that there is a sufficient amount of cffDNA in the maternal plasma for precise quantification. Furthermore, the absolute number of DNA molecules analyzed would influence the precision of the measurement. The latest studies performed sequencing 4–6 samples per lane on the Illumina HiSeq 2000 sequencer, corresponding to 20–25 million reads per case [84, 88].

Detecting Trisomy 18 and Trisomy 13 with MPS

Soon after the accurate detection of fetal trisomy 21 with MPS-based NIPT was initially reported, the test was expanded to include the other common autosomal aneuploidies on chromosomes 18 and 13 [84, 88, 89].

Indeed, the non-invasive prenatal detection of fetal trisomy 18 and trisomy 13 appears to be more challenging [89]. MPS technologies contain a GC bias, meaning that the read coverage of different genomic regions can vary depending on the genomic GC content [90]. The GC content affects the efficiency of the PCR steps during sample preparation or analysis by MPS. The chromosomal GC content may also partly account for the variation in precision of the MPS platforms in measuring the proportional representation of different chromosomes [81, 89]. While chromosome 21 has a GC content that is average relative to that of all human chromosomes, both chromosomes 18 and 13 have relatively low GC content when compared with chromosome 21 [81, 89].

To reduce GC-associated quantitative biases, bioinformatics algorithms have been developed to adjust the sequencing read counts to the local genomic GC

content [88, 89, 91]. Alternatively, one could also normalize the number of counts of the potentially aneuploid chromosome with the counts of a reference chromosome with a similar GC content [80]. In fact, different sequencing platforms appear to show different patterns of GC bias [81, 86]. For the Genome Analyzer from Illumina, chromosomes with low GC contents are underrepresented while chromosomes with high GC contents are overrepresented [81]. For the SOLiD system from ABI, the opposite pattern was observed [86]. This may be due to the different sequencing chemistries of the two platforms: the Genome Analyzer uses sequencing-by-synthesis, whereas SOLiD uses sequencing-by-ligation. Even correcting for the effects of the GC content and the sequencing platform with bioinformatics, measurements of the genomic representations of chromosomes 18 and 13 still appeared to be less precise than those of chromosome 21 on both platforms [86].

Since PCR is one of the processes known to introduce GC-associated quantitative bias in the sequencing data, the use of single-molecule sequencing platforms, which require no PCR amplification step during sample preparation and sequencing [92], could potentially reduce the bias. Using a single-molecule sequencing platform, the sequencing data show no GC bias and a more distinct separation between trisomic and euploid samples occurs [93]. So far, they have accurately detected trisomy 21 and trisomy 18, but not trisomy 13, with the use of the Helicos single-molecule sequencing platform [93, 94]. It is speculated that factors other than just GC content might influence the measurement precision of chromosome 13 [94].

Sex Chromosomal Aneuploidy

While early studies mainly focused on detecting fetal autosomal aneuploidies, detection of sex chromosomal aneuploidies is also of clinical importance. The incidence of sex chromosomal aneuploidies is estimated to be 1 in 500 live births. Although patients with sex chromosomal aneuploidies generally have less severe phenotypes compared with patients with autosomal aneuploidies, a proportion of such subjects still suffer from severe morbidities. Common sex chromosomal aneuploidies include the Klinefelter syndrome (47, XXY), 47, XYY syndrome (47, XYY), triple X syndrome (47, XXX), and Turner syndrome (45, X).

In this regard, Bianchi et al. [84] developed a complex classification algorithm for the detection of sex chromosomal aneuploidies based on the z-scores for both chromosomes X and Y. However, testing for detecting sex chromosomal aneuploidy appears to be not as sensitive as testing for autosomal aneuploidy. For instance, with non-mosaic Turner syndrome (45, X), the detection rate was 75 %, and the false-positive rate was 0.2 % (Table 1) [84].

On the other hand, Mazloom et al. [95] used a training cohort to establish various cutoffs for a classification system to determine the copy number of the two sex chromosomes (Fig. 5). In pregnancies with euploid male fetuses, the proportion of X chromosome reads in plasma (containing both maternal and fetal X chromosome) depends on the fetal fraction. As the fetal fraction in maternal plasma increases, the proportional representation of chromosome Y increases, while the proportional

Table 1 Performance of massively parallel sequencing of maternal plasma DNA for non-invasive prenatal testing of fetal sex chromosomal aneuploidies

	45, X		47, XXX		47, XXY		47, XYY	
	DR % (n)	FPR % (n)	DR % (n)	FPR % (n)	DR % (n)	FPR % (n)	DR % (n)	FPR % (n)
Bianchi et al. [84]	75 % (15/20)	0.2 % (1/417)	75 % (3/4)	–	67 % (2/3)	–	100 % (3/3)	–
Mazloom et al. [95]	81 % (17/21)	0.3 % (1/390)	100 % (1/1)	0 % (0/410)	100 % (5/5)	1.0 % (4/406)	67 % (2/3)	0 % (0/408)
Liang et al. [96]	100 % (5/5)	0.3 % (1/407)	100 % (1/1)	0 % (0/411)	100 % (1/1)	0 % (0/411)	100 % (1/1)	0 % (0/411)

DR detection rate, *FPR* false-positive rate

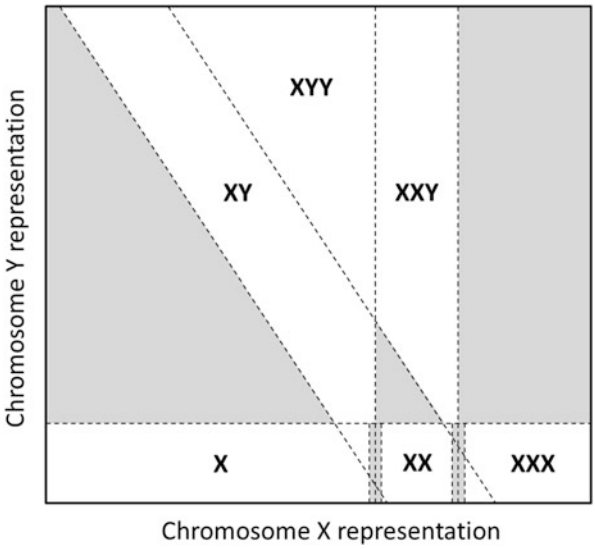

Fig. 5 The sex chromosomal aneuploidy classification system. The x- and y-axes show the X and Y chromosome representations, respectively. The *grey areas* mark the regions with non-reportable results for sex chromosomal aneuploidy (Figure adapted, with permission, from Mazloom et al. [95]. Copyright, 2013; John Wiley and Sons, Ltd.)

representation of chromosome X decreases (Fig. 5). Therefore, for cases with a putative male fetus, one needs to examine the proportional representations of both chromosomes X and Y, and determine if they have fallen outside the confidence interval for euploid male fetuses established with the training dataset. On the other hand, for cases with a putative female fetus, one could determine the chromosome X dosage by comparing against the pregnancies with euploid female fetuses (Fig. 5). By using this algorithm, the detection rate for Turner syndrome, triple X syndrome, Klinefelter syndrome and 47, XYY syndrome were 83 % (25/30), 83 % (5/6), 85 % (11/13) and 75 % (3/4), respectively [95]. Liang et al. [96] used a similar approach to detect sex chromosomal aneuploidy, but had fewer affected cases in the study (Table 1).

Overall, these studies indicate that it is feasible to detect sex chromosomal aneuploidy albeit with a lower accuracy than with the detection of autosomal aneuploidy (Table 1). Nevertheless, to date, there are only a few studies on the prenatal testing of fetal sex chromosomal aneuploidies (Table 1).

Several factors may contribute to the suboptimal performance of NIPT of sex chromosomal aneuploidies. Firstly, the sequence similarity between parts of chromosomes X and Y causes difficulty in mapping. Using current bioinformatics algorithms, a small number of plasma DNA molecules from pregnancies with female fetuses would align to the Y chromosome [79]. Secondly, copy number analysis can typically only be performed on a small number of unique Y-chromosome loci, only 2.2 % of the Y chromosome, leading to a large variation in the representations of chromosome Y [95]. Lastly, mosaicism for sex chromosomal aneuploidies is common, so complicating the quantitative analysis of sex chromosomes.

Chromosome-Selective Approach

When one is only interested in quantifying a particular chromosome, such as chromosome 21, for disease diagnosis, only a small fraction of the sequencing data generated using the whole genome sequencing protocol is derived from that chromosome. For example, chromosome 21 represents only 1.3 % of the entire genome. A more efficient strategy is to selectively sequence genomic regions on the chromosome of interest so that sequencing power is focused on genomic regions of diagnostic interest. This would help to reduce the cost and increase the throughput.

In this regard, Sparks et al. [97] developed an assay called Digital ANalysis of Selected Regions (DANSR) to specifically amplify non-polymorphic loci on chromosomes 21 and 18. For each targeted locus, they used a set of three locus-specific oligonucleotides and a pair of universal PCR primers for the amplification [97]. Initially, they targeted 384 non-polymorphic loci on chromosome 21 and on chromosome 18; subsequently, they expanded the DANSR assay to include 576 loci on each chromosome.

In the same assay, a user of this approach would also simultaneously target SNP loci on chromosomes not usually involved in an aneuploidy (chromosomes 1–12) for the determination of the fetal fraction [98]. They have used an algorithm that takes into account the fetal fraction when determining the risk of fetal trisomy in each pregnancy [98].

This chromosome-selective sequencing approach has been stated to require approximately one million mappable reads per sample, which appears to be less than that required by the whole genome sequencing approach [97]. One disadvantage of this approach is that other off-target chromosomal aneuploidies will not be detected.

SNP-Based Approach

Liao et al. [99] reported an MPS-based approach to detect fetal aneuploidy by using targeted enrichment and sequencing of SNP loci. They used an in-solution hybridization-based capture strategy to enrich 2,906 SNP loci on chromosomes 7, 13, 18 and 21, after which they sequenced the DNA by MPS [100]. They applied this approach for the detection of trisomy 21 in this proof-of-concept study as follows: first, they analyzed the informative SNPs in which the mother was homozygous and the fetus was heterozygous and calculated the ratio between the fetal-specific alleles and the shared alleles, the F-S ratio (F-SR) (Fig. 6). Then, by subsequently comparing the F-SR between the target and the reference chromosomes, they determined the fetal ploidy status and the parental origin of the extra chromosome (Fig. 6). This approach is founded upon the observation that the original allelic ratio of the targeted SNPs was maintained even after target enrichment [100]. However, as 85 % of trisomy 21 cases are maternally derived, it should be noted that the detection of maternally derived trisomy 21 is less robust than that of paternally

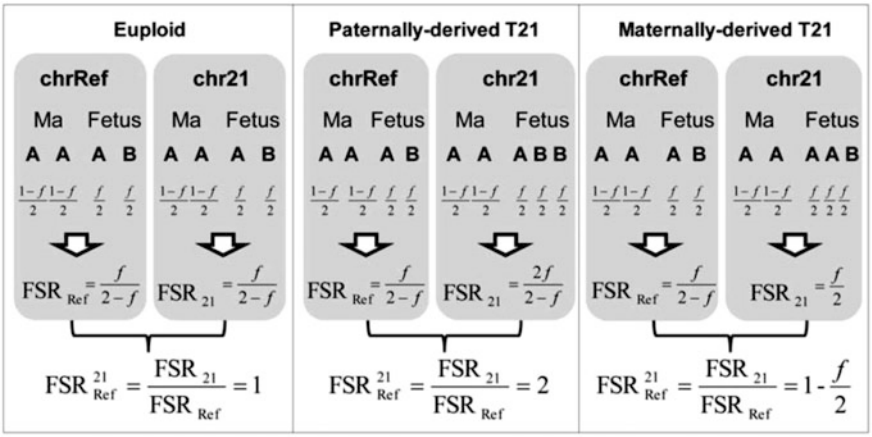

Fig. 6 Principle of trisomy 21 detection by F-S ratio calculation. Assuming the fractional cffDNA concentration in chrRef is f, the F-S ratio would be f/(2-f) on chrRef irrespective of the aneuploidy status of the fetus. On the other hand, the F-S ratio on chr21 would be f/(2-f) if the mother was carrying a euploid fetus, 2f/(2-f) if the mother was carrying a paternally-derived trisomy 21 fetus, and f/2 if the mother is carrying a maternally-derived trisomy 21 fetus. Therefore, the FSR21Ref would be 1 if the mother is carrying a euploid fetus, would become 2 if the mother is carrying a paternally-derived trisomy 21 fetus, and would become (1-f/2) if the mother is carrying a maternally-derived trisomy 21 fetus (Figure reproduced from Liao et al. [99])

derived trisomy 21 using this method. The difference in robustness is because the perturbation in the allelic ratio in maternally derived trisomy 21 cases depends on the fetal fraction in the maternal plasma (Fig. 6). Compared with the non-polymorphic tag counting approach, this SNP-based approach requires parental genotype information and more sequencing reads if the fetal fraction in the maternal plasma is low.

Zimmermann et al. [101] have evaluated a SNP-based MPS approach to detect fetal aneuploidy. They used a PCR-based enrichment strategy, followed by MPS to detect fetal aneuploidy in chromosomes 13, 18, 21, X and Y [101]. Initially, they performed multiplex amplification of 11,000 SNP loci on chromosomes 13, 18, 21, X and Y in a single reaction [101]. Later, they increased the targeted SNP number to 19,488, corresponding to over 3,000 SNPs per chromosome evaluated [102]. For sequencing data analysis, they developed an algorithm that uses maternal genotypes and recombination frequencies to generate billions of possible hypotheses about the fetal genotypes. Based on the observed allelic distributions, their algorithm determines the relative likelihood of each hypothesis and selects the hypothesis with the maximum likelihood to infer the copy number for each of the five analyzed chromosomes. In addition, a sample-specific calculated accuracy was reported for each of the analyzed chromosomes.

The combination of this SNP-based method and the algorithm to detect aneuploidy in chromosomes 13, 18, 21, X and Y was validated in a blinded study led by an independent group [102].

It is also possible to detect triploidy (the condition of having an additional haploid set of maternal or paternal chromosomes) with this SNP-based method [103]. In addition, it is also possible to detect uniparental disomy by using this method.

6 Clinical Implementation

Existing prenatal screening tests for fetal aneuploidy use a combination of maternal age, fetal nuchal translucency thickness and maternal serum β-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein-A (PAPP-A) concentrations to assess the risk of fetal aneuploidy [2]. Currently, prenatal screening is routinely offered to all pregnant women regardless of maternal age. These screening tests can achieve a detection rate of 90–95 % for fetal trisomies 21, 18 and 13, with a false-positive rate of 2.5–5 % [2]. Cases with a positive screening test result require confirmation via invasive diagnostic testing.

Clinically, MPS-based non-invasive prenatal tests can be performed after 10 weeks of gestation with a turnaround time of 8–10 days [104]. Although a number studies have demonstrated that it is feasible to simultaneously detect all fetal whole chromosome aneuploidies in a single assay [96, 105], most clinical validation studies have been focused on evaluating the performance of NIPT for trisomies 21, 18 and 13 [10, 12, 83, 85, 88, 98, 106–109]. There are also a few studies evaluating the performance for detecting sex chromosomal aneuploidies [84, 102].

Among the three MPS-based approaches (see sections "Whole Genome Approach", "Detecting Trisomy 18 and Trisomy 13 with MPS", "Sex Chromosomal Aneuploidy", "Chromosome-Selective Approach", "SNP-Based Approach" above) whole genome random sequencing and chromosome-selective sequencing have been more extensively validated (Table 2). The overall detection rates for trisomy 21, trisomy 18 and trisomy 13 are 99.4 %, 97.4 % and 86.2 %, respectively. While the detection of trisomy 21 and trisomy 18 were consistently more accurate across different studies, the detection of trisomy 13 was comparatively less accurate in both the whole-genome sequencing and chromosome-selective sequencing approach (Table 2). Furthermore, the number of trisomy 13 cases studied is also comparatively less.

Overall, the false-positive rates for trisomy 21, trisomy 18 and trisomy 13 are 0.1 %, 0.1 % and 0.4 %, respectively (Table 2). Thus, the introduction of maternal plasma nucleic acid NIPT could possibly reduce the number of invasive tests that would be performed. However, the false-positive rate associated with NIPT is still at a clinically significant level; therefore, it could not yet replace invasive diagnostic test.

In response to the results of these clinical validation studies, several professional bodies and expert societies have published guidelines on the use of NIPT for fetal aneuploidy. Currently, NIPT for aneuploidy is being recommended as an advanced

Table 2 Performance of massively parallel sequencing of maternal plasma DNA for non-invasive prenatal testing of fetal autosomal aneuploidies

			T21		T18		T13	
	Study design	Approach	DR %	FPR %	DR %	FPR %	DR %	FPR %
Ashoor et al. [106] Ashoor et al. [107]	Case-control	Chromosome-selective	100 % (50/50)	0 % (0/297)	98 % (49/50)	0 % (0/297)	80 % (8/10)	0.05 % (1/1939)
Bianchi et al. [84]	Case-control	Whole genome	98.9 % (89/90)	0 % (0/410)	92.1 % (35/38)	0 % (0/463)	68.6 % (11/16)	0.62 % (3/485)
Chiu et al. [10]	Prospective and case-control	Whole genome	100 % (86/86)	2.10 % (3/146)	–	–	–	–
Ehrich et al. [85]	Prospective	Whole genome	100 % (39/39)	0.24 % (1/410)	–	–	–	–
Guex et al. [105]	Retrospective	Whole genome	100 % (39/39)	0 % (0/237)	95.8 % (23/24)	0 % (0/252)	100 % (15/15)	0 % (0/261)
Liang et al. [96]	Prospective	Whole genome	100 % (39/39)	0 % (0/372)	100 % (13/13)	0 % (0/398)	(4/4)	0.25 % (1/408)
Nicolaides et al. [102]	Prospective	SNP-based	100 % (25/25)	–	(3/3)	–	(1/1)	–
Norton et al. [109]	Prospective	Chromosome-selective	100 % (81/81)	0.03 % (1/2888)	97.4 % (37/38)	0.07 % (2/2888)	–	–
Palomaki et al. [12] Palomaki et al. [88]	Case-control	Whole genome	98.6 % (209/212)	0.20 % (3/1471)	100 % (59/59)	0.28 % (5/1688)	97.1 % (11/12)	0.90 % (16/1688)
Sparks et al. [98]	Case-control	Chromosome-selective	100 % (36/36)	0.81 % (1/123)	100 % (8/8)	0.81 % (1/123)	–	–
Total			99.4 % (693/697)	0.1 % (9/6354)	97.4 % (227/233)	0.1 % (8/6109)	86.2 % (53/58)	0.4 % (21/4781)

second-tier screening test for pregnant women identified as high-risk by conventional methods such as ultrasound scanning and maternal serum biochemistry. Positive NIPT results still need to be confirmed by invasive testing as NIPT has a small yet clinically significant false-positive rate.

Recently, results of a few studies on unselective general population screening have become available [108, 110]. In a study using the chromosome-selective approach to routinely screen a first trimester population [108], a false-positive rate of 0.1 % in detecting trisomies 21 and 18 was reported, highlighting the possibility of using this test for the general population. Despite this, there is still a relative paucity on studies reporting evidence about the performance of the test in the general population. Furthermore, the cost of MPS-based NIPT of Down syndrome is still too expensive for routine clinical implementation as a primary screening test [111]. Therefore, whether NIPT via cffNAs in maternal plasma would be implemented as a primary screening test would require more studies with low-risk and average-risk women and also a substantial reduction in the cost of sequencing.

7 Mosaicism

Mosaicism refers to the presence in an individual of two or more genetically different cell lineages arising from the same zygote. Overall, the incidence of fetal mosaicism is 0.15 % [112]. The presence of fetal mosaicism presents considerable challenges to the detection of fetal aneuploidy as it reduces the effective fetal fraction [113].

At present, only a few studies have reported the NIPT of mosaic cases [84, 113]. The sensitivity of these maternal plasma-based MPS approaches to detect fetal aneuploidy in cases with different levels of fetal mosaicism remains to be determined. Furthermore, phenotypes of individuals with mosaic karyotypes can be highly variable. The clinical significance of mosaicism depends on the proportion and the tissue distribution of the abnormal cells. Thus, mosaicism poses a challenge for genetic testing and counseling.

8 Aneuploidy Detection for Twin Pregnancies

Despite the robustness of MPS-based approaches for the non-invasive detection of fetal aneuploidy from maternal plasma, most studies only assess the use of MPS-based NIPT of fetal aneuploidy for singleton pregnancies. There are two types of twins, namely identical and fraternal twins. Identical twins are monozygotic (derived from the same fertilized egg) and therefore genetically identical; fraternal twins are dizygotic (derived from two fertilized eggs) and therefore not genetically identical. A number of groups have applied the same analytic algorithm

used to detect fetal trisomies in singleton pregnancies to pregnancies with multiple gestations [80, 114, 115]. In fact, a number of companies are now offering sequencing-based NIPT for twin pregnancies.

As discussed previously, it is important to assess the fetal fraction in maternal plasma to avoid possible false-negative detection due to insufficient cffDNA content in a particular maternal plasma sample. In this respect, Canick et al. [114] determined a combined fetal fraction and assumed that each twin fetus had contributed sufficient cffDNA molecules for aneuploidy detection if the combined fetal fraction exceeded the minimum requirement. However, this assumption might not be valid in some cases. To tackle this problem, Qu et al. [116] proposed an MPS-based method to non-invasively determine the zygosity of a twin pregnancy and the fetal fraction for each fetus of a twin pregnancy. Genetic differences between dizygotic twins would result in fluctuations in the apparent fetal fractions across multiple genomic regions. This method has been applied to two discordant dizygotic twin pregnancies (with one aneuploid fetus from each pair of dizygotic twins) to assess the twin zygosity and to determine the fetal fraction contributed by each twin fetus [117].

9 Abnormal NIPT Results

With more widespread use of plasma DNA-based NIPT, a number of reports have described discordant results between NIPT and fetal metaphase karyotyping. At least part of the discordant results might be attributed to the fact that circulating DNA molecules in the maternal plasma originate from both the mother and the placenta. As a consequence, NIPT results may not genuinely reflect the fetal karyotype.

For instance, confined placental mosaicism (CPM), a condition where a second cell lineage exists only in the placenta but not in the fetus, is one of the situations that may lead to a discordant result [118, 119]. Overall, the incidence of CPM is approximately 1 % [112]. While one-fifth of pregnancies with CPM show prenatal or perinatal complications such as intrauterine growth retardation (IUGR) and spontaneous fetal loss, most of them show a normal pregnancy outcome [120]. Impact of CPM on NIPT has been discussed in detail elsewhere [121].

On the other hand, NIPT by MPS of maternal plasma may sometimes reveal the presence of maternal chromosomal abnormalities. In this regard, a case of an abnormal NIPT result due to undiagnosed maternal mosaicism has been reported [122]. To distinguish maternal mosaicism from true fetal mosaicism and CPM, one may sequence the maternal buffy coat to confirm or exclude maternal mosaicism. When a copy number aberration originates from the fetus, the degree of under- or over-representation would be correlated with the fetal fraction. On the contrary, an abnormally large degree of under- or over-representation may indicate maternal chromosomal abnormalities instead.

Abnormal NIPT results may also be attributed to a maternal malignancy. Analogous to cffDNA in the maternal plasma, circulating tumour cfDNA is also present in the plasma of cancer patients. In fact, cancer in pregnancy is not uncommon; it complicates approximately 0.1 % of all pregnancies [123]. A case of discordant NIPT results in a patient who was subsequently diagnosed with a metastatic disease has been reported [124]. Another unusual case of discordant NIPT results, where a double aneuploidy for chromosomes 13 and 18 were detected, was consistent with a maternal malignancy diagnosed after delivery [125].

Therefore, one should always bear in mind when interpreting MPS-based NIPT results that circulating DNA in maternal plasma is of both maternal and placental origin.

10 Detection of Subchromosomal Aberrations

In light of the successful detection of fetal copy number changes involving a whole chromosome, a number of groups have further expanded the diagnostic spectrum of non-invasive plasma-based testing to subchromosomal copy number changes [126–129]. Although such aberrations occur less frequently than aneuploidies, they are associated with serious fetal conditions, such as developmental disabilities or congenital anomalies.

Techniques commonly used for detecting subchromosomal aberrations include metaphase cytogenetic analysis, chromosomal microarray analysis (CMA), fluorescence *in situ* hybridization (FISH) and quantitative fluorescent PCR (QF-PCR). Both metaphase karyotyping and CMA provide a genome-wide snapshot of all the chromosomes of an individual, whereas FISH and QF-PCR allow for targeted analyses of only one or a few regions of the entire genome. CMA offers higher resolution (can resolve up to 100 kb) than metaphase karyotyping which typically detects copy number variations (CNVs) of 5 Mb or greater. It has been demonstrated that CMA can detect 2 % more clinically relevant CNVs when used with pregnancies with standard indications for prenatal diagnosis (such as advanced maternal age and positive prenatal screening result) and 6 % more when used with an anomaly on ultrasound screening, respectively [130]. Several expert groups have also recommended the use of CMA as a first-tier diagnostic test for individuals with developmental disabilities or congenital anomalies [131, 132]. The major disadvantage of metaphase karyotyping and CMA is that the fetal genetic material for the tests is obtained through either amniocentesis or CVS that may pose a risk of miscarriage.

In light of this, two studies have investigated the possibility of using MPS-based analysis of maternal plasma to detect fetal subchromosomal copy number aberrations [126, 127]. Peters et al. [127] were able to detect a 4.2 Mb paternally inherited deletion on the fetal chromosome 12 in a maternal plasma sample. On the other hand, Jensen et al. [126] were able to detect a 3 Mb deletion on the fetal chromosome 22 in two maternal plasma samples. Even though both studies performed

whole fetal genome sequencing on the maternal plasma cffDNA, their statistical analyses were solely focused on one or a small number of genomic regions.

Conversely, Srinivasan et al. [128] and Yu et al. [129] have attempted to detect fetal subchromosomal copy number changes across the whole genome through MPS of maternal plasma cffDNA using the random sequencing approach. The two studies used different bioinformatics approaches to detect and classify CNVs, but their underlying principles were similar. In fact, both of their analyses for detecting subchromosomal CNVs were similar to that for detecting whole chromosome aneuploidy. To detect subchromosomal aberrations, the genome was divided into non-overlapping bins of a fixed-size. By counting the number of plasma cffDNA fragments aligned to each bin and comparing them with a reference (either a different bin with a presumably normal copy number within the same sample or a set of samples with a normal copy number for the same bin), it was possible to deduce whether or not the copy number has changed from the normal.

While Srinivasan et al. [128] used a higher threshold to identify amplified or deleted regions in the genome, Yu et al. [129] used the additional criterion of requiring three consecutive bins outside the reference interval, all showing changes in the same direction, to call a positive result.

Both studies demonstrated the detection of fetal *de novo* copy number changes across the whole genome. In particular, Srinivasan et al. [128] detected a microdeletion as small as 300 kb, which is similar to the genomic resolution using CMA. The detection of subchromosomal aberrations requires a higher depth of sequencing than the detection of whole-chromosome aneuploidies (which typically requires 2.5–5 million reads). By using computer simulations, Yu et al. [129] predicted that to achieve a diagnostic resolution of 2 Mb and 1 Mb with a 99 % sensitivity, 240 million and 480 million plasma cfDNA molecules would need to be analyzed. To achieve a diagnostic resolution of 100 kb, one would need to analyze approximately one billion plasma cfDNA molecules [128].

Occasionally, metaphase karyotyping would reveal the presence of additional material of unknown origin. Srinivasan et al. [128] have demonstrated that it is possible to identify the chromosomal origin of the additional material by using MPS. Similar to CMA, fetal *de novo* balanced rearrangements, such as balanced translocation and inversion, cannot be detected with the whole-genome MPS-based approach because there is no gain or loss of genetic material [128, 130].

At present, only a few proof-of-principle studies, involving a small number of cases, have been reported [126–129]. These methods should be validated in large-scale prospective studies with first or early second trimester samples. Nonetheless, prenatal detection of fetal subchromosomal deletions and duplications through MPS-based maternal plasma analysis is feasible. Its resolution appears to be able to match that offered by CMA, and it has an additional advantage of being non-invasive. However, an additional cost would be added to the current aneuploidy test if subchromosomal CNVs are to be included as a higher sequencing depth is required. Alternatively, this test can be offered only when an abnormal ultrasound is indicated. In addition, the detection of CNVs of unknown clinical significance poses a challenge for counselling and can cause unnecessary anxiety. Conversely, it

may also be possible to target those clinically relevant pathogenic copy number variants by targeted sequencing; however, this raises the question as to which conditions should be tested.

11 Detection of Single-Gene Disorders

The global prevalence of monogenic diseases is approximately 1 in 100 newborns. Examples of common monogenic disorders include thalassemia, sickle cell anemia, cystic fibrosis and hemophilia. Pregnancies at high risk for monogenic diseases are usually identified when there is a positive family history or when parents are confirmed to be carriers through carrier screening programs. Sometimes, an abnormal fetal ultrasound finding may also be an indication for genetic testing. A number of fetal anomalies can be picked up by an ultrasound scan. However, a number of prenatal features may be associated with multiple conditions, making definitive diagnoses in these pregnancies challenging.

Initial efforts to achieve non-invasive prenatal diagnosis of monogenic diseases focused on the qualitative detection of a paternally inherited mutation or a *de novo* mutation that is absent from the maternal genome. The principle is the same as that for detecting paternally inherited traits (i.e. the detection of chromosome Y sequences in prenatal sex determination and the detection of *RHD* sequences in RhD-negative women). For instance, if the father is affected by an autosomal dominant disorder, one could determine whether or not the fetus has inherited the paternal mutation either by directly detecting the mutation [133] or indirectly detecting the polymorphic markers linked to the mutation. Different methods have been used for the detection of paternally inherited base substitutions, insertions and deletions that cause single-gene disorders [134]. In particular, the PCR-based approach is commonly used and is relatively straightforward.

If the father and the mother carry different mutations for an autosomal recessive condition, the detection of the paternal unique mutation in the maternal plasma would indicate that the fetus has a 50 % chance of being affected. On the other hand, the absence of the paternal mutation would exclude the fetus from being affected by the recessive condition and manifesting the severe disease. Therefore, invasive diagnosis can be avoided in these pregnancies. This exclusion strategy has been applied to prenatal diagnosis of recessive conditions such as β-thalassemia [135–137], congenital adrenal hyperplasia [136] and cystic fibrosis [138]. To exclude the fetal inheritance of a recessive condition with confidence, it is crucial to ascertain the presence of detectable cffDNA in the maternal plasma sample, thereby avoiding false-negative results. Commonly used fetal markers include Y-chromosome sequences (e.g. *SRY* and *DYS14*) for male pregnancies, a panel of polymorphic short tandem repeats (STRs), SNPs or insertion/deletion markers, and epigenetic markers (e.g. hypermethylated RASSF1A) [19]. The major limitation of this exclusion approach for the prenatal diagnosis of recessive conditions is that it cannot be applied if the father and mother are carrying the same mutation.

In addition, whether or not the fetus will have inherited the maternal mutation is not known.

To prenatally diagnose recessive conditions when the parents have the same mutation, Lun et al. [139] introduced a strategy called Relative Mutation Dosage (RMD). They took advantage of the analytic precision of dPCR that allows for the discrete counting and hence precise quantification of plasma cfDNA molecules [75, 77]. Briefly, they detected and counted the number of mutant and normal alleles and determined the relative dosage of the two alleles in the maternal plasma. If the mother is a heterozygous carrier, she would contribute the mutant and normal alleles in a 1:1 ratio. There would be three possible scenarios: (1) the fetus has inherited the mutant alleles from both parents and contributes additional mutant alleles to the maternal plasma, resulting in an overrepresentation of the mutant allele relative to the normal allele; (2) the fetus has inherited the normal alleles from both parents, resulting in an overrepresentation of the normal allele in the maternal plasma; and (3) the fetus has inherited one mutant and one normal allele from its parents and contributes an equal amount of the mutant and normal alleles to the maternal plasma, resulting in an allelic ratio of 1:1. This degree of allelic imbalance positively correlates with the fetal fraction in the maternal plasma [139]. These authors [139] used a statistical approach called the sequential probability ratio test (SPRT) to determine whether or not a statistically significant allelic imbalance exists. SPRT is a method that allows for the testing of a hypothesis while data accumulate [71]. Each SPRT curve is case-specific, and its construction is based on the fetal fraction and the experimentally derived average template concentration. The RMD approach has been successfully applied to recessive conditions such as thalassemia [139] and sickle-cell anemia [140].

Similarly, this approach could also be used to determine whether or not the fetus has inherited the maternal mutation in a situation where the mother is heterozygous for an autosomal dominant condition when the expected allelic ratio would be 1:1. However, if the fetus has inherited the normal allele, the latter would be over-represented in the maternal plasma.

Furthermore, this strategy could also be applied to X-linked diseases such as hemophilia [141]. When a pregnant woman is a carrier of an X-linked mutation, her male fetus would be at risk for the X-linked condition. Since a male fetus has only one copy of chromosome X that must be inherited from the mother, it would have a 50 % chance of inheriting the mutation. As a consequence, if the fetus has inherited the mutant allele from the mother, the mutant allele would be over-represented in the maternal plasma. On the other hand, if the fetus has inherited the normal allele from the mother, the normal allele would be over-represented.

Undoubtedly, the use of dPCR allows for the precise quantitative analysis of allelic imbalance in maternal plasma. Together with the RMD approach, and despite the interference of a large background of maternal DNA, it is possible to detect a disease-causing mutation that is also present in the maternal genome that the fetus has inherited from the mother in the maternal plasma. Nonetheless, there are several limitations to the use of dPCR for the diagnosis of monogenic diseases. Technically, a separate set of probes specific for the mutant and wild-type alleles is

required for each mutation. Clinically, prior knowledge about the specific mutation involved in each family is required.

12 Decoding the Fetal Genome

After the development of targeted assays for monogenic diseases, the next question is whether or not it is possible to screen, non-invasively, the entire fetal genome for genetic diseases. To achieve this, the entire fetal genome sequence would need to be decoded from the circulating cfDNA in the maternal plasma. So far, there are four studies demonstrating the recovery of the entire fetal genome through deep sequencing of maternal plasma cfDNA (Table 3) [27, 142–144]. Despite differences in some key technical details (Table 3), the general principle for constructing a fetal genetic map from maternal plasma sequencing data is similar across different studies:

Firstly, parental genotypes at each locus are determined. This step is crucial as it provides information about the possible fetal genotypes at each locus. Parental genotypes are determined by either array-based SNP-genotyping or whole genome sequencing of parental DNA. While array-based SNP-genotyping restricts the analysis to the more common SNPs on the array, whole genome sequencing could potentially reveal the genotypes in most of the positions in the parental genomes.

Subsequently, each genomic position is assigned to one of the following five categories, according to the parental genotypes at the corresponding site (Table 4). Sites with different combinations of parental genotypes require different strategies for inferring the inheritance of the fetus (Table 4). For instance, sites where both parents are homozygous either for the same or a different allele, the fetal inheritance can be inferred directly. For the remaining sites where one or both parents are heterozygous, the fetal inheritance would need to be deduced from the maternal plasma cfDNA sequencing data. The five categories of polymorphic sites together with the strategy needed for inferring fetal inheritance for each category are as follows:

(i) For sites where both parents are homozygous for the same allele, the fetus will be homozygous for the parental allele, a category accounting for >99.9 % of sites in the genome [145].
(ii) For sites where the parents are homozygous for different alleles, the fetus would be an obligate heterozygote. This category accounts for <0.03 % of sites in the genome [145].
(iii) The paternal inheritance of the fetus at sites where the father is heterozygous and mother is homozygous can be deduced by detecting the paternal-specific allele at each of these sites. The underlying principle is the same as that for

Table 3 Comparison of different fetal genome recovery approaches used in the four studies

	Lo et al. [27]	Kitzman et al. [144]	Fan et al. [143]	Chen et al. [142]
Samples	Mother: plasma, whole blood	Mother: plasma, whole blood	Mother: plasma, whole blood	Mother: plasma, whole blood
	Father: plasma, whole blood	Father: saliva		Father: plasma, whole blood
	CVS			Four grandparents: saliva
Sequencing depth of maternal plasma DNA	65×	78×	52.7×	44×
Genotyping	Microarray-based SNP genotyping of maternal and paternal DNA	Sequencing of maternal (32×) and paternal DNA (39×)	Microarray-based SNP genotyping of maternal DNA	Sequencing of maternal (20×) and paternal DNA (20×); microarray-based SNP genotyping of grandparents' DNA
Haplotyping	Maternal haplotype deduced from CVS (obtained by invasive procedure)	Whole-genome experimental phasing of maternal chromosomes: use of clone pool dilution sequencing	Whole-genome experimental phasing of maternal chromosomes: use of an automatable microfluidics device to separate chromosomes from a single cell in metaphase, followed by SNP genotyping	Computational phasing of both maternal and paternal chromosomes: use of trios and population haplotype frequency to deduce

fetal sex determination through detecting Y chromosome sequences in the maternal plasma. If the paternal-specific allele is not detected, it would imply that the opposite allele has been transmitted. More specifically, if the paternal-specific allele is transmitted, sequence reads containing the paternal-specific allele would contribute to half of the fetal-derived reads at this locus, i.e., half of the fetal cffDNA fraction. Therefore, the accuracy of this approach for determining paternal inheritance at these sites is dependent on the fetal fraction in maternal plasma, as well as the sequencing depth.

(iv) In theory, the maternal inheritance of the fetus at sites where the mother is heterozygous and father is homozygous could be deduced by determining if there is an allelic imbalance in the maternal plasma on a site-by-site basis, which is conceptually similar to the RMD approach described in the previous

section. However, this approach would require several thousand fold coverage to accurately determine the maternal inheritance at each maternal heterozygous site, and genome-wide sequencing to such depth is impractical at present. To solve this problem, Lo et al. [27] introduced an analytical strategy called Relative Haplotype DOsage (RHDO). Instead of deducing the maternal inheritance of the fetus at an individual SNP site, the RHDO approach determines the fetal inheritance of the maternal haplotype, which is a series of neighbouring alleles on a single chromosome of a homologous pair that are inherited together (Fig. 7). To achieve this, heterozygous sites in the maternal genome need to be phased into a series of haplotype blocks (to identify which alleles of a series of heterozygous sites are present on each of the two maternal haplotypes). These SNPs are then classified into two types, namely α and β, which are analyzed separately. When classifying the fetal inheritance of the maternal haplotype, the paternal genotype information on the maternal heterozygous sites is also needed. This method of combining the counts from dozens to hundreds of maternal heterozygous sites into a haplotype block allows the maternal inheritance of the fetus to be deduced with a lower depth of coverage.

(v) The deduction of the paternal and maternal inheritance of a fetus at sites where both parents are heterozygous is more complex because there are three possible fetal genotypes at these sites (Table 4). To resolve the inheritance at these sites, haplotype information from both the mother and the father would be required. The inherited paternal haplotype could be deduced using a tagging SNP (a SNP that is homozygous in the mother and heterozygous in the father on the haplotype blocks). Inheritance of the tagging SNP at a specific allele would represent the inheritance of the corresponding haplotype, and the paternal inheritance at other sites on the same haplotype could then be inferred. After knowing the paternal inheritance at these sites, maternal inheritance could be deduced by performing RHDO analysis.

In addition to the inherited component, a complete fetal genetic map should also include fetal *de novo* mutations which are newly arisen mutations in the maternal or paternal germ line that do not exist in the somatic cells of the parents. It is estimated that the number of *de novo* mutations in a fetus is approximately 50–100 [146]. In this regard, Kitzman et al. [144] attempted to detect fetal *de novo* mutations noninvasively. Ideally, fetal *de novo* mutations would be variants detected in the maternal plasma that are absent from the parental genomes. However, current sequencing technologies are far from perfect with errors being introduced during the sequencing process, and these sequencing errors are inherently indistinguishable from true *de novo* mutations. Despite their efforts to filter the noise (sequencing errors) at the expense of the sensitivity, they came up with thousands of candidate *de novo* mutations <1 % of which were indeed true *de novo* mutations. Therefore, the non-invasive detection of fetal *de novo* mutations remains a challenge at present, requiring further development.

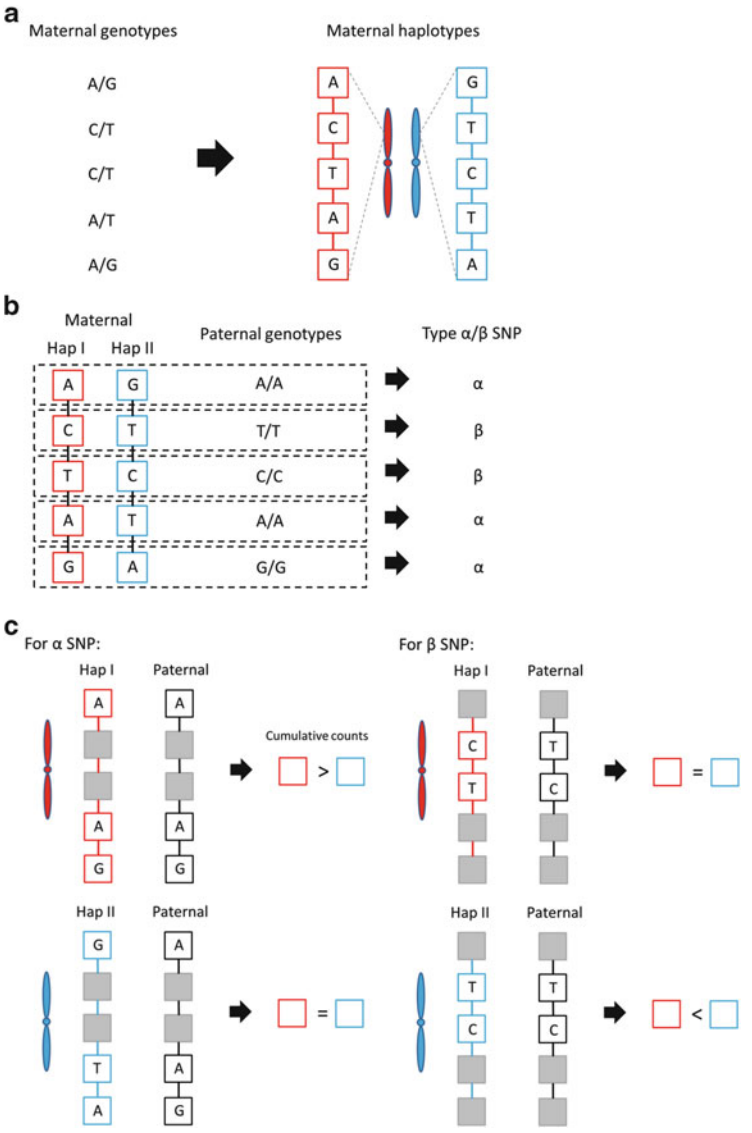

Fig. 7 Procedural steps for the determination of maternal inheritance (**a**) Heterozygous sites in the maternal genome are phased into a series of haplotype blocks. (**b**) These SNPs are then classified as either type α or β SNPs based on the paternal genotypes at the corresponding sites. Type α SNPs are those for which paternal alleles are identical to the alleles on the maternal Hap I whereas type β SNPs are those for which paternal alleles are identical to the alleles on the maternal Hap II. (**c**) These two types of SNPs are analyzed separately. For type α SNPs, alleles on Hap I would be overrepresented if the fetus has inherited Hap I. On the other hand, alleles on Hap I and Hap II would be equally represented if the fetus has inherited Hap II. On the contrary, for type β SNPs, alleles on Hap II would be overrepresented if the fetus has inherited Hap II whereas alleles on Hap I and Hap II would be equally represented if the fetus has inherited Hap I

Table 4 The five possible combinations of maternal and paternal genotypes

SNP category	Maternal genotype	Paternal genotype	Possible fetal genotypes
1	AA	AA	AA
2	AA	CC	AC
3	AA	AC	AA, AC
4	AC	AA	AA, AC
5	AC	AC	AA, AC, AC

The accuracy and completeness of the fetal genetic map could be affected by a number of factors. Firstly, fetal fraction and maternal plasma sequencing depth may affect the accuracy of fetal genotype deduction. For instance, as the fetal fraction decreases, a higher depth of maternal plasma cfDNA sequencing would be needed to infer the fetal genotypes at a predefined level of accuracy. Secondly, the length of the phased haplotype block may affect the precision of RHDO analysis. A longer haplotype block would contain more heterozygous sites for haplotype inference. Finally, the completeness of haplotype phasing (fraction of heterozygous sites phased) would affect the resolution of the fetal genetic map. In this regard, a number of experimental and computational haplotype phasing methods are currently available [147]. However, all of the currently available phasing methods are not perfect. In this respect, Browning and Browning [147] have provided a comprehensive review of the haplotype phasing methods that are available, discussing the merits and demerits of each approach and the practical aspects for their application.

Maternal plasma DNA sequencing for fetal genome scanning was used to determine if a fetus had inherited β-thalassemia mutations from its parents, carriers for different β-thalassemia mutations [27]. Nonetheless, this fetal genome-wide scanning approach for detecting fetal genetic disorders is relatively expensive due to the high cost associated with the deep sequencing of maternal plasma DNA. In addition, large amounts of data are generated, which require complex data analysis and interpretation.

Recently, the use of targeted MPS of maternal plasma cfDNA for the prenatal diagnosis of β-thalassemia has been reported [148]. It has been demonstrated that one could perform RHDO analysis with target-enriched MPS data. Specifically, Lam et al. [148] determined if the fetus had inherited the haplotype blocks containing the mutant allele from its parents. By using targeted sequencing, sequencing and analysis are targeted to genomic regions of interest. It is also possible to target multiple, clinically relevant, genomic regions in the same assay. As a consequence, it would be more cost-effective to use targeted MPS for the prenatal diagnosis of monogenic diseases.

13 Conclusion

13.1 The Present

The discovery of circulating cffNAs in maternal plasma and serum has revolutionized prenatal genetic diagnosis and testing. Prior to the availability of NIPT, the potential loss of a normal fetus due to the inherent risk associated with invasive procedures has deterred some pregnant women from receiving prenatal testing. Now, with NIPT made only by a simple blood draw, more pregnant women may choose to undergo prenatal testing [149]. The main advantages of NIPT are that (1) it can reduce the number of invasive tests performed [149], thus reducing the number of unnecessary miscarriages caused by invasive procedures, and (2) it can be performed earlier in pregnancy than can the current tests. Having the test earlier in pregnancy, possibly at around the tenth week of gestation, may give pregnant women more time to consider their options, such as whether to continue or terminate the pregnancy and whether to receive further testing.

In this chapter, the general approaches for some of the possible applications of cffNAs have been reviewed, including fetal sexing for sex-linked disorders and congenital adrenal hyperplasia, fetal rhesus blood group genotyping for guiding management in RhD-negative pregnant women, fetal chromosomal aneuploidy and subchromosomal CNV detection and fetal monogenic disease detection. Some of these applications – fetal sexing, fetal RhD genotyping and fetal aneuploidy detection – have been translated into clinical practice. In particular, the successful demonstration of the use of MPS for fetal aneuploidy detection was rapidly followed by clinical validation and commercialization. Lastly, it also worth mentioning that the rapid development in the field has been accompanied by growing concerns of the ethical, legal, and social implications of NIPT (see chapter "Societal Aspects: Ethics").

13.2 The Future

While most of the current applications of cffNAs have been based on circulating cffDNA, researches in the area of circulating cffRNA are less well-established. The advent of MPS for cffRNA (RNA-seq), given its high sensitivity and high throughput, may change this landscape by allowing for the genome-wide expression profiling of the placenta through sequencing of the cffRNA isolated from the maternal plasma. On the other hand, non-coding RNAs, such as miRNA and large intergenic non-coding RNA (lincRNA), have also aroused great interest in the scientific community in the recent years because of their regulatory role in gene expression (see Sect. 3.2).

In a recent proof-of-concept study, scientists have demonstrated the use of genome-wide bisulfite sequencing of the maternal plasma DNA for analyzing the

fetal and placental methylomes [24]. In the same study, they have also demonstrated the feasibility of this approach for the detection of fetal trisomy 21. This approach to assessing the fetal and placental DNA methylation levels could be used for other pregnancy-related disorders, notably for those known to have altered epigenetic profiles, and for biomarker discovery.

Besides prenatal genetic testing, another possible prenatal application of cffNA analysis is the prediction and monitoring of pregnancy-associated conditions such as preeclampsia and preterm delivery. For instance, it has been reported that women with preeclampsia have increased cffDNA concentrations compared with normal pregnancy controls [150–153]. Some evidence suggested that this increase might occur before the onset of preeclampsia [154] and that the degree of increase correlates with the severity of the disease [150, 152, 153]. However, due to a large inter-individual variability in cffDNA concentrations (which may be due to factors such as maternal weight and ethnicity), cffDNA levels show much overlap between women who subsequently developed preeclampsia and those who do not. Similarly, it has been reported that some cffmRNAs, such as *CRH*, *PLAC1* and *selectin-P*, showed increased concentrations in preeclamptic pregnancies compared with controls [155, 156]. Nonetheless, cffNAs may be used in combination with existing biochemical and sonographic markers to screen for at-risk pregnancies, and to monitor preeclampsia.

Besides preeclampsia, a number of pregnancy-related disorders are also associated with abnormal fetal DNA concentrations in the maternal plasma. These include preterm delivery [157–159], hyperemesis gravidarum [160], invasive placentation [161] and fetal growth restriction [162]. Future research in these areas might expand the use of cffNAs to disease prediction and monitoring.

To conclude, research on circulating cffNAs in maternal plasma and serum is a rapidly developing and exciting area. One could envision that the use of cffNAs could likely play an increasingly important role in prenatal care.

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Circulating Nucleic Acids and Diabetes Mellitus

Asif Butt and Ramasamyiyer Swaminathan

Abstract Diabetes, which is a major health problem throughout the world is associated with increased mortality and morbidity. Early detection of those at risk and early detection of complications will help to reduce the adverse consequences. Current methods used for early detection of DM and its complications either have limitations or are expensive. Circulating miRNA and mRNA offer an exciting new approach to the diagnosis of diabetes and its complications. Several recent studies have reported that this approach is feasible. For example, mRNA for retinal specific proteins was reported to give good discrimination between different grades of diabetic retinopathy. In this chapter the use of miRNA and mRNA in diabetes and its complications is discussed.

Keywords Diabetes mellitus • Circulating DNA/mRNA/microRNA • Diabetic retinopathy • Diabetic nephropathy • Diabetic neuropathy • Macrovascular complications • Predictive, preventive and personalized medicine

1 Introduction

Diabetes mellitus (DM) remains one of the major global health concerns today [1]. It has been estimated that nearly 400 million people suffer form DM and the incidence is increasing rapidly both in the developed as well as in the developing countries. It is increasing in children as well as in adults due to changes in lifestyle, reduced physical activity and obesity as well as due to an aging population. By 2030 the number of people with diabetes is estimated to increase to 552 million [1, 2]. Diabetes is associated with micro and macrovascular complications. Microvascular complications include retinopathy, nephropathy and neuropathy and macrovascular

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complications include cardiovascular disease, stroke and peripheral vascular disease. These complications are often present at the time of diagnosis.

There are two main types of diabetes mellitus: Type 1 (T1DM) and Type 2 (T2DM). T1DM accounts for 5–8 % of all cases of diabetes mellitus and generally develops during childhood or in young adults [3]. T1DM is an autoimmune disorder where there is destruction of the insulin-producing β-cells. During this process, leucocytes infiltrate the pancreatic islets and secrete pro-inflammatory cytokines that help to recruit cytotoxic T lymphocytes causing β-cell dysfunction and death [4]. The net result is a progressive destruction of β-cells leading to severe or complete insulin deficiency.

T2DM accounts for 92–95 % of all diabetic cases and is characterized by insulin resistance in peripheral tissues including skeletal muscle, liver and adipose tissue as well as failure of pancreatic β-cells to secrete adequate amounts of insulin to maintain blood glucose concentration. The development of T2DM is closely linked to genetic, environmental and/or lifestyle factors, such as lack of exercise and obesity. When insulin resistance develops in the early stages, pancreatic β-cells compensate by increasing insulin secretion. However, in susceptible individuals the β-cells are unable to maintain this increased demand for insulin, leading to chronic hyperglycemia and T2DM [5]. Diabetes and its complication cause increased mortality and morbidity and the economic cost of diabetes is high [6]. Mortality and morbidity due to DM could be reduced if treatment is implemented early in the course of the disease. In order to do this good biomarkers are necessary to detect those who are at risk of DM, diagnose DM early and detect its associated complications.

In this review, a brief account of the role of miRNA in the pathogenesis of the disease is followed by an account of the role of cfNAs in the diagnosis and management of DM.

1.1 Pathogenesis of Diabetes and miRNA

A well-defined set of miRNAs are expressed in pancreatic β-cells and insulin sensitive tissues. Most of these miRNAs are not cell-specific and are distributed widely in many tissues, the one exception being miR-375 that is expressed in the pancreas to a greater extent and regulates the section of insulin. It is also involved in the increase in β-cell mass in response to insulin resistance [7, 8]. In patients with T1DM and T2DM, the expression profile of miRNA in the β-cell and in insulin sensitive tissues is altered [9–11]. In an animal model of T1DM, the NOD mouse, several miRNAs, including miR-21, miR-34a, miR-29 and miR-146a, are increased in the pre-diabetic state [12, 13]. Similar alterations in miRNA expression have been described in the islets of animal models of T2DM, namely *ob*/*ob* and *db*/*db* mice [14, 15]. The expression of miR-29, miR-34a, miR-802 miR-143, miR-107 and miR-103 are also increased in insulin sensitive tissues in these mouse models indicating that they may contribute to insulin resistance [16, 17]. In the skeletal

muscle of patients with T2DM, expression of miR-143 is up regulated and that of miR-206 and miR-133a, two muscle specific miRNAs, are down regulated [18]. It is worth noting that in patients with impaired glucose tolerance some of these miRNAs are already down regulated [18]. In addition expression of miRNAs in tissues affected by DM namely, blood vessels, retina and kidney are also altered [11, 19, 20].

miRNA has also been implicated in diabetic complications. In an experimental model of diabetic nephropathy miR-192, miR-200b/c, miR-216a and miR217 are up-regulated [21–24]. In a recent review Kato et al. [24] concluded that miR-192 is a key regulator of events leading to diabetic nephropathy. In diabetic retinopathy up-regulation of miR-155, miR-132 and miR-21 have been reported [25]. Similarly alterations in several miRNAs have been reported in diabetic cardiovascular complications. A summary of the possible role of miRNAs in diabetic complications is shown in Figs. 1 and 2.

In addition to the regulation of gene expression with in the cells, miRNAs are found in extracellular fluids being carried in exosomes or in HDL particles. They can be readily transferred into cells and it has been suggested that extracellular miRNA may act as signaling molecules between cells e.g. between endothelial cells and vascular smooth muscle cells ([27, 28]; see also chapter "The Biology of CNAPS").

1.2 Circulating Nucleic Acids as Biomarkers

cfNAs (cfDNA, cfmRNA and cfmiRNA) have been reported as potential biomarkers in the diagnosis of DM as well as in the detection of diabetic complications. Table 1 gives a summary of the potential of cfNAs that have been suggested as being useful in the diagnosis and management of diabetes and its complications.

2 Identification of the at Risk Population

2.1 Type 1 DM

Clinical T1DM develops when more than 80–90 % of the pancreatic β-cells are destroyed by the immune response [41]. This may take months or even years. The interval between the start of the immune reaction destroying the β-cell to clinical presentation is a useful period to identify and attempt to treat these subjects with immune modulating therapy. Such therapy helps to stop the progression and thereby preserve β-cell mass and delay the clinical onset of T1DM. Therefore, it is important to identify this group of patients as early as possible in order to initiate therapy. Auto-antibodies against islet cells, insulin, tyrosine phosphatase IA-2 and IA-2β, glutamate decarboxylase and zinc transporter are some of the useful

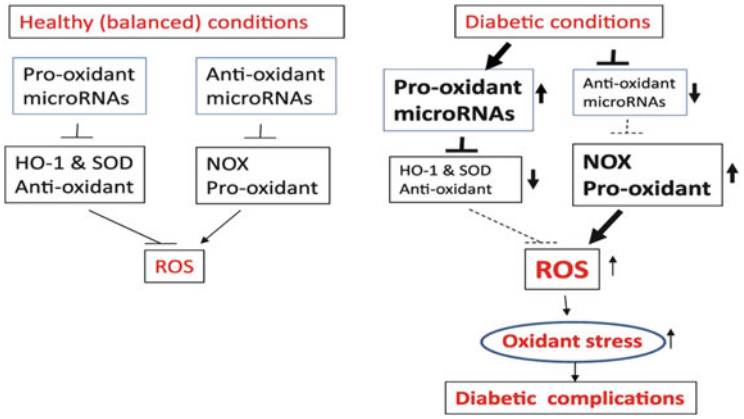

Fig. 1 Mechanism of diabetic complications (Adapted from Kato et al. [26])

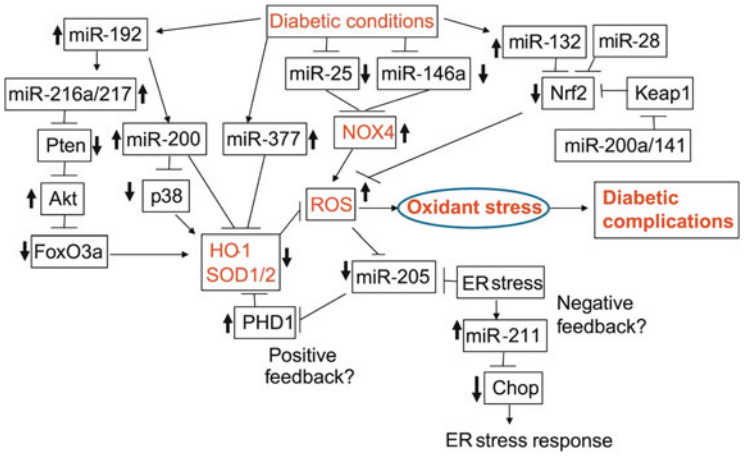

Fig. 2 A summary of possible miRNA in the pathogenesis of diabetic complications (Adapted from Kato et al. [26])

biomarkers to identify individuals at risk of developing T1DM [41, 42]. However, these are not useful to start treatment early. Furthermore, not all subjects with antibodies go on to develop clinical DM and they are not useful to monitor immune modifying therapy as auto-antibodies do not disappear quickly from the circulation [42]. These limitations have prompted attempts to find/develop new biomarkers for T1DM.

It has been suggested that miRNAs may be useful biomarkers for the prediction of β-cell destruction as well as to assess residual β-cell function. In one study, Nielsen et al. [35] compared newly diagnosed patients with T1DM with an age-matched control group. They identified several miRNAs (miR-24, miR-25, miR-26a, miR-27a, miR-27b, miR-29a, miR-30a-5p, miR-148a, miR-152,

Table 1 Summary of circulating miRNA changes associated with diabetes mellitus

Author	Groups	Up-regulated miRNA	Down-regulated miRNA ↓
Karolina et al. [29]	T2DM & control	144,150,192,29a, 320	146a,182,30d
Karolina et al. [30]	Metabolic syndrome	150,192,27a,320a, 375	
Kong et al. [31]	T2DM, IFG	9,29a,30d,34a,124, 146a, 375	
Zametaki et al. [32]	T2DM	28-3p	20b,21,24,15a, 126,191,197,223, 320,486
Heneghan et al. [33]	Obese & normal weight	122,143	17-5p,132,34a,99a, 145,195
Ortega et al. [34]	Morbid obesity & controls	140-5p,142-3p,222	532-5p,125b,130b, 221,15a,423-5p, 520-c-3p
Bajomo et al. (2013), unpublished data	Obese & control	17-5p	
Neilson et al. [35]	T1DM	24,25,26a,27a,27b,29a, 30a-5p,148a,152, 181a,200a,210	
Erener et al. [36]	T1DM mouse model	375	
Sebastiani [37]	T1DM	31,349,146a,155,181a,199a	
Salas-Perez et al. [38]	T1DM		21a,93
Sebastiani et al. [39]		326	
Complications			
Zampataki et al. [32]	T2DM	miR-126 associated with arterial disease	
Caporali et al. [40]		miR-16,21,210 &638 correlated with GFR	

miR-181a, miR-200a and miR-210) that were different in TIDM compared to the control group. Previous studies have shown that some of these miRNAs are involved in the regulation of apoptosis and β-cell function [35]. In addition, these workers found that residual β-cell function (assessed by the conventional test – levels of C-peptide) correlated with the concentration of miR-25 in circulation.

Another study, [65] compared the profile of circulating miRNAs in newly diagnosed patients with T1DM with a control group. They detected 206 miRNAs in the serum and of these, 64 were found to be differently expressed in the patients with T1DM including miR-31, miR-146a, miR-155, miR-181a and miR-199a, miR-9 and miR-34a. miR-375, which is expressed abundantly in the islets of Langerhans [36], has been suggested as a possible marker to predict the development of T1DM in animal models. In streptozocin induced diabetic mice, circulating levels of this miRNA increased in association with β-cell destruction. In NOD mice,

the plasma concentration of miR-375 was higher before the onset of T1DM [36] but decreased rapidly within a week.

Salas-Perez et al. [38] reported that expression of miR-21a and miR-93 in circulating mononuclear cells was lower in patients with T1DM than that in healthy controls. They also observed that if mononuclear cells were incubated with glucose (25 mmol L^{-1}) the expression of miR-21a, but not that of miR-93, was reduced showing that hyperglycemia may have been responsible for the reduction in miR-21a observed in T1DM patients. Another study, [39] found miR-326 expression in circulating lymphocytes to be higher in patients with T1DM and that this was related to the autoimmune reaction. These studies indicate that miRNA in serum or blood could be used to monitor the autoimmune response in T1DM.

In addition to miRNA, methylated DNA has been shown to be useful in the detection of β-cell destruction [43]. These workers developed a specific assay for DNA methylation and found that circulating levels of demethylated *Ins 1* DNA in an experimental model of T1DM was increased. In addition, they showed increased levels of demethylated DNA for *Ins1* in newly diagnosed T1DM when compared to age-matched controls. These authors suggest that methylation-specific PCR could be useful in detecting β-cell death in subjects who are at risk of developing T1DM.

In an *in vitro* study, Rani et al. [44] demonstrated that a number of β-cell gene transcripts (Pdx1, Egr1 and Chgb) could be demonstrated in the culture medium and that the amount of these reflects the β-cell mass. This approach could be used to assess the remaining β-cell function in T1DM prone subjects.

2.2 Type 2 DM

T2DM is a heterogeneous metabolic disorder as a result of interaction between genetic factors and environmental/life style factors. One of the important life style factor predisposing to T2DM is obesity, especially central or abdominal obesity. Therefore, methods identifying those at risk of developing DM from the large number of obese subjects is important. At present, such methods to identify these subjects are either not very sensitive or are expensive. Several studies have looked at cfNAs as potential markers. Heneghan et al. [33] demonstrated differential expression of miRNAs in the omental (abdominal) fat compared to that in the subcutaneous fat. In addition, they showed that these differences in miRNA expression could be demonstrated also in the circulation. Circulating levels of miR-122 and miR-143 were increased while miR-132 and miR-349 were lower in obese subjects with increased omental fat. Bajomo et al. have recently shown that circulating levels of miR17-5p was 21.3 folds higher in obese compared to normal weight subjects. In subjects with a history of cardiovascular disease, the expression of miR17-5 was 39.1 fold greater than in those who were free from cardiovascular disease. Circulating levels of miR17-5p also correlated significantly with age ($r = 0.3458$, $p < 0.05$) and waist circumference ($r = 0.3430$, $p < 0.05$) (Bajomo et al. 2013, unpublished observations).

In a recent study, Ortega et al. [34] analyzed plasma miRNA in morbidly obese individuals before and after weight loss and compared it with that in normal weight subjects. In morbidly obese subjects miR-140-5p, miR-142-3p and miR-222 were higher while that of miR-532-5p, miR-125b, miR-130b, miR-221, miR-15a, miR-423-5p, and miR-520c-3p were lower. Using discriminant function analysis they found that miR-15a, miR-520c-3p, and miR-423-5p were specific for morbid obesity and had a diagnostic accuracy of 93.5 %. After bariatric surgery, plasma miR-140-5p, miR122, miR-193-5p and miR-16-1 decreased and miR-221 and miR-199-3p increased. This was not seen after diet induced weight loss. These studies permit the possibility that plasma mRNA could be a useful biomarker for the risk assessment of obese subjects. Further longitudinal studies are required to examine if plasma miRNA could identify those at risk of developing DM.

Metabolic syndrome is a cluster of related biochemical and anthropometric features. There are various definitions of this syndrome [45]. However, it is well accepted that subjects with this syndrome are predisposed to develop T2DM. Various clinical and laboratory tests have been described to diagnose this syndrome [50]. Karolina et al. [30] examined the plasma miRNA profile of subjects with this syndrome and compared this with that from healthy subjects. They identified several miRNAs (miR-23a, miR-27a, miR-32a, miR-130a and miR-195, miR-197 and miR-509a) that were differentially expressed in subjects with this syndrome as compared to healthy individuals. This study highlights that plasma miRNA may be a useful biomarker of metabolic syndrome especially for those at risk of developing T2DM.

2.3 Diagnosis of DM

Although DM is a well recognized condition there is still controversy and discussion about the best test to diagnose DM. The current recommendation for diagnosing DM is based on fasting blood glucose, 2 h post prandial blood glucose or glycated hemoglobin (HbA1c). The cut off value of each of these tests to diagnose DM is based on the risk of developing complications. As plasma glucose is a continuous variable, the cut off value used is arbitrary and arrived as a consensus. These issues related to the diagnosis of DM have been recently discussed [46]. It is notable that the groups of people identified by these different tests are not identical. A significant number of subjects identified by one criterion is not picked up by the other tests [46]. For these and other limitations none of these tests is currently recommended as the preferred test [46]. Furthermore, large numbers of subjects remain undiagnosed [47] and up to 25 % of subjects have established complications by the time of diagnosis [48]. These issues have prompted the search for alternative diagnostic tests for DM and the pre-diabetic state. For example, leucocyte O-GlcNAcylation has been suggested as a possible biomarker for the early detection of T2DM [49]. Others have examined cfNAs as potential biomarkers. Kong et al. [31] measured seven miRNAs in serum (miR-9, miR-29a, miR-30d,

miR-34a, miR-124a, miR-146a and miR-375) as potential biomarkers of T2DM. These miRNAs were measured in patients with newly diagnosed T2DM, subjects either with impaired glucose tolerance or impaired fasting glucose (pre-diabetic state) or individuals who are susceptible for DM but with normal glycemia (those with family history and/or obesity). They found that in T2DM all of the above miRNAs were up regulated of which five were up- regulated in the pre-diabetic state.

Zampetaki et al. [32] analyzed the plasma miRNA profile of a large group of T2DM and healthy controls in a longitudinal follow-up study. They observed lower plasma miRNA (miR20b, miR21, miR24, miR15a, miR126, miR191, miR197, miR223, miR320 and miR486) and a higher miR-28-3p in T2DM. Importantly, these changes in plasma miRNA were seen before the diagnosis of DM. Furthermore, they identified miR-126 as a potential marker of endothelial function. These authors suggest that plasma miRNAs have the potential to identify those who are likely to develop DM and may be of value in the prediction of micro- and macro-vascular complications.

In a preliminary study of a small number of diabetic subjects and controls Moussa et al. (unpublished observations) measured circulating miR-192 and miR-215 and found that these were elevated 16.5 and 19.2 times, respectively, to that seen in healthy controls. Receiver operating characteristic curve (ROC) analyses showed that the area under curve (a measure of potential diagnostic value) were 0.980 and 0.991 for miR-192 and miR-215 respectively. This indicates that miRNAs are potentially useful in the diagnosis of DM.

2.4 Gestational DM (GDM)

GDM is a condition where women who have no previous DM develop high blood glucose during pregnancy and is associated with maternal (e.g. pre-eclampsia) and foetal (large babies and respiratory syndrome, etc.) complications. Early diagnosis of GDM is important in order to start treatment to normalize the blood glucose and so to reduce these complications. Many methods have been proposed to identify these subjects. Most of these are based on plasma glucose measurement after a glucose load at 24–28 weeks of gestation. This means treatment cannot be started until about 32 weeks of gestation. Circulating miRNAs have been suggested as possible early test for GDM. Zhao et al. [50] identified miR-132, miR-29a, and miR-222 from microarray studies and measured these in serum from pregnant women at 16–19 weeks of gestation and showed them to be lower in those women who went on to be diagnosed as GDM. If these results can be replicated it will be a major advantage in identifying women who are likely to develop GDM and to be able to start treatment early. In addition, placental specific miRNAs have been described in the maternal circulation [51] and these also may be additional markers of GDM.

3 Diabetic Complications

Long-term complications of diabetes may be divided into two general categories: macro- and microvascular complications. In the case of the latter, retinopathy, nephropathy and neuropathy are commonly observed after 10–20 years from the development of diabetes. Indeed, in T2DM, these are often the first symptoms to present when the initial diagnosis for diabetes is confirmed. In each of these diabetic complications the prognostic and diagnostic value of cfNAs are discussed further.

3.1 Diabetic Retinopathy

Diabetic Retinopathy (DR) is the leading cause of blindness in the working age group and is dependent on the duration and age of onset of diabetes. For instance, prevalence rates for DR are very low during the first 5 years in patients below 30 years of age. However, the rate increases to over 95 % after 15 years of diabetes [52]. There are also several risk factors for the development of DR, including: (a) duration of diabetes; (b) blood glucose control [53, 54], (c) hypertension and (d) dyslipidemia [55]. At the same time, there is good evidence from a number of studies suggesting that controlling these associated risk factors might significantly decelerate progression of DR. Untreated, loss of vision would ensue. Therefore, it is important that any changes in the retina are detected early so that appropriate treatment may be administered. Currently, classification of eye status is assessed according to a scale for the number of abnormalities observed and retinopathy categorized into four stages of severity, that is, no pathology, background, pre-proliferative and proliferative. The assessment involves retinal screening by digital fundus photography and is guided by criteria for classification based on the recommendations of The Diabetic Retinopathy Grading and Disease management Working Party [56]. However, such screening protocols are expensive and labor intensive, requiring the input of several different healthcare professionals. A further consideration is that the grading system itself is substantially subjective [57]. Thus, an objective, quantitative blood test may circumvent the limitations associated with the screening procedures currently in place. The additional benefit of such a blood test may be that it would be relatively inexpensive, not requiring expensive hardware and equipment or specialist healthcare personnel. The blood test could be performed anywhere and would not require the patient to attend specialist clinics for this purpose. The test would also overcome the problems associated with grading retinae occluded by media opacities.

One of the earliest investigations exploring the possibility of using a circulating retina specific molecular marker was reported by Hamaoui et al. [58]. The authors postulated that damage to the retina would promote cell death of rods and cones. This would in turn release retina specific mRNA species to enter the circulation detectable levels of which would be indicative of the extent of retinal cell damage.

The hypothesis was tested by measuring mRNA in whole blood samples from clinically proven cases of diabetic retinopathy. As mRNA had long been thought to be highly labile, blood samples were collected into Paxgene Blood RNA tubes designed to stabilise blood mRNA at the point of collection. Whole blood RNA was extracted and the mRNA therein reverse transcribed to cDNA. The latter was quantified by qRTPCR using rhodopsin sequence specific primers and probe. mRNA for the house keeping β actin was also measured at the same time and the results expressed as a ratio. This original pilot study demonstrated that retina specific mRNA was detectable in the circulation. In addition, the levels of rhodopsin mRNA were seen to increase with the severity of DR. Moreover, compared to healthy control subjects, diabetics without retinopathy appeared to show higher levels of rhodopsin mRNA, suggesting that the latter group may go on to develop retinopathy or have it sub-clinically (Fig. 3). The investigators concluded that circulating cell free retina specific mRNA had a potential prognostic and diagnostic role to play in the assessment of DR.

Further work from this group looked at additional retina specific markers, including RPE65 and retinoschisin [59]. As with rhodopsin (Rho), RPE65 mRNA showed increased levels with severity of DR (Fig. 4a). In contrast to these two markers, levels of retinoschisin mRNA decreased with DR severity (Fig. 4b). In an effort to enhance specificity and sensitivity, more rod, cone and retinal ganglion cell-specific markers were added to the panel of retinal markers, including retinal amine oxidase (RAO), phosphodiesterase 6C (PDE6C) and melanopsin. While mRNA for Rho and RAO were detected in 100 % of the subjects, PDE6C mRNA was only found in 60 % of the individuals and melanopsin mRNA was not detected. When diabetic subjects were divided according to their DR status, significant differences were observed – Rho increased while RAO tended to decrease. The area under the curve ROC for Rho (Fig. 5) and Rho/RAO ratio to differentiate mild or no DR from significant DR (pre-proliferative and proliferative stages) were 0.756 and 0.823, respectively [60]. This provided further evidence that circulating mRNA may be useful in assessing DR.

Some of the markers discussed above were also used in other related disorders. For instance, it was proposed recently that hypoxia, as seen in Obstructive Sleep Apnea (OSA), might be associated with DR [61]. To investigate this further, Wong et al. [62] used T2DM patients to determine the effect of oxygen desaturation on circulating retina-specific mRNA. Oxygen desaturation was defined as the number of times per hour the oxygen saturation decreased by 4 % or greater (number of dips h^{-1}) and percentage of sleep time with oxygen saturation (SpO_2) <90 %. The results indicated that in patients with >/=5 dips h^{-1}, mRNA values for rhodopsin and RPE65 were significantly higher than in patients with <5 dips h^{-1} (Fig. 6). Levels of retinoschisin mRNA remained unchanged. In patients with pre-proliferative or proliferative DR, median levels for rhodopsin mRNA and RPE65 mRNA were 30 and 80 % higher and retinoschisin mRNA was lower in patients with >/=5 dips hr^{-1} when compared to patients with <5 dips hr^{-1}. Thus, hypoxia may modulate expression of genes in the retina and this may be reflected in the variation of retina specific mRNA measured in the circulation.

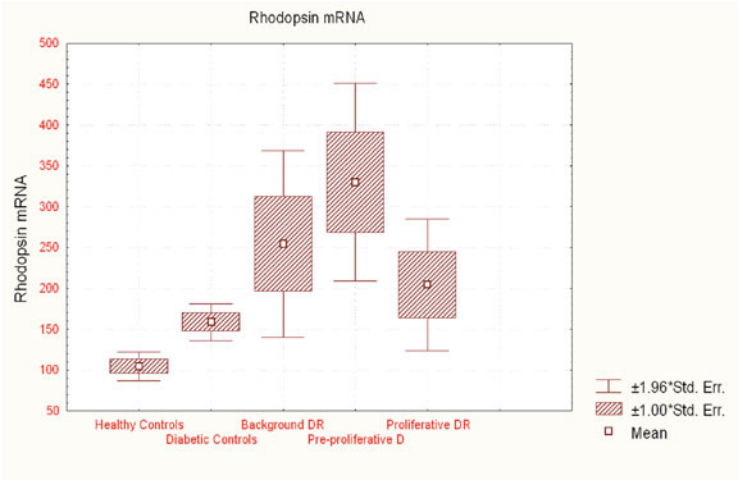

Fig. 3 mRNA for rhodopsin in healthy subjects and diabetic subjects with and without retinopathy. Results are expressed as a percentage of that in controls. Circulating mRNA for rhodopsin is increasing with increasing severity of retinopathy except for the severest grade

MicroRNA and Diabetic Retinopathy

Several studies have shown the role of miRNAs in DR [26]. With miR-146, miR-155, miR-132, and miR-21 being shown to be up regulated in the retina of diabetic animals [25]. To date there have been no studies on the possible use of serum/plasma miRNA in the detection of DR.

4 Diabetic Nephropathy

Diabetic nephropathy (DN) is the commonest cause of end-stage renal disease [63]. Approximately 40 % of all diabetic patients have DN with the incidence expected to rise alongside the escalating rates of DM worldwide. Patients with DM are screened regularly for DN, which is based on the measurement of urinary albumin excretion (UAE). Microalbuminuria, the presence of small amounts of albumin in the urine, is believed to be a strong predictor of DN [64]. However, despite its widespread use, the interpretation of UAE is subject to many sources of variation. For instance, many non-diabetic patients have microalbuminuria and not all patients with microalbuminuria go on to develop DN [65]. Sample collections are also time-consuming and prone to errors [66]. Nevertheless, even with these known limitations, current recommendations state that all diabetic patients should have an annual measurement of albumin in the urine [67]. As increased albumin excretion in diabetic patients arises from damage to the glomerular basement membrane, measurement of circulating nephron-specific mRNA may offer some

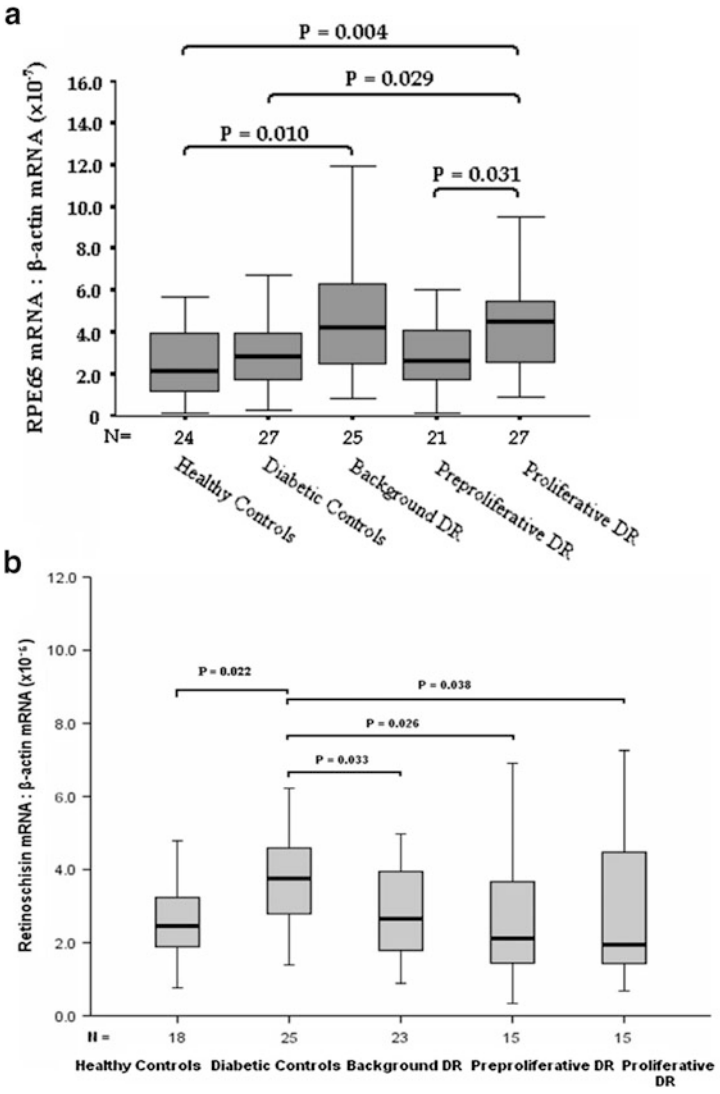

Fig. 4 (**a**) RPE65 and (**b**) retinoschisin mRNA levels in healthy controls and diabetic patients with and without retinopathy. (**a**) Circulating mRNA for RPE65 is higher in early and late stages of retinopathy. (**b**) Highest values for retinoschisin is seen diabetics without retinopathy. Levels decreased with increasing severity of retinopathy

potential in the diagnosis and monitoring of DN. This approach was found to be feasible in assessing diabetic retinopathy using circulating retina specific mRNA suggesting that renal specific mRNA may also serve a similar function in the case of renal status analysis.

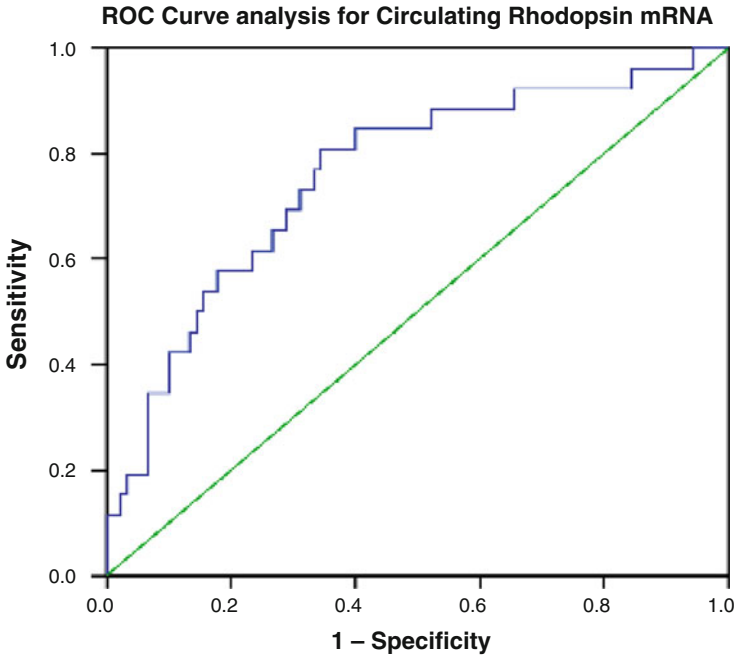

Fig. 5 Receiver operator characteristic curve to differentiate mild form severe retinopathy using mRNA for rhodopsin. The area under the curve for circulating rhodopsin mRNA is 0.756 – (values for the area under the ROC curve approaching 1.00 are better discriminatory tests)

Nephrin is a transmembrane protein [68] expressed by visceral epithelial cells (podocytes) in the slit diaphragm of the glomerulus and is crucial for the integrity of the slit diaphragm. Abnormalities in this protein can lead to proteinuria and eventually to nephrotic syndrome. Measurement of nephrin mRNA in blood offers the possibility of assessing renal function without the constraints associated with urinary albumin analysis. Additionally, such an approach may also provide a clue to the etiology of the renal disease. This was first illustrated by a study by Orlandi et al. [69] who measured nephrin mRNA in healthy individuals and in renal transplantation patients. They showed that nephrin mRNA in circulation is influenced by age and transplantation, both of which are associated with reduced renal mass. Nephrin mRNA was also higher in females, both in healthy individuals and in transplant patients, suggesting that the nephrin mRNA expression is influenced by estrogens or other female hormones (Table 2).

Extending this original finding, Butt et al. [70], measured nephrin mRNA in peripheral blood of healthy subjects and patients with diabetic nephropathy and found that nephrin mRNA levels were increased in DN (Fig. 7). However, whilst encouraging from a biomarker perspective, the interpretation of results was inconclusive as factors known to effect renal function, e.g. age, gender and ethnicity had not been accounted for. In a further proof-of-concept study Moussa et al. [71]

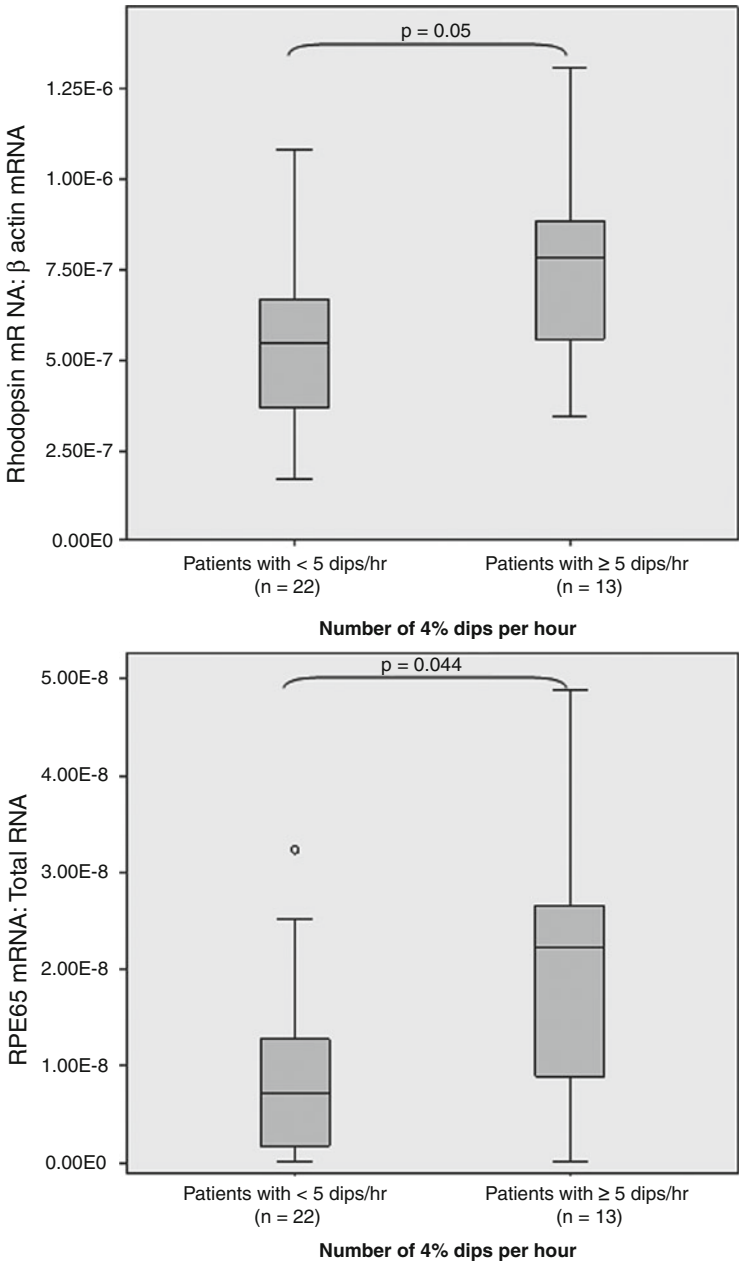

Fig. 6 Circulating rhodopsin (*upper panel*) and RPE65 (*lower panel*) mRNA levels in patients with <5 dips/h and patients with ≥5 dips/h. Circulating mRNA were higher in those with more severe sleep apnea

Table 2 Blood nephrin mRNA concentrations (ratio of nephrin mRNA to total blood β-actin RNA) in healthy persons and kidney transplant patients

	Median (range)		
	Females	Males	*P*, Mann–Whitney *U*-test
Healthy persons	0.076 (0.003–0.180)	0.0394(0.0064–0.1339)	0.04
Transplant patients	0.028(0.0023–1.38)[a]	0.0106(0.0013–0.492)[b]	0.022

[a] $P = 0.05$ compared with healthy males
[b] $P = 0.029$ compared with healthy females

measured the levels of a panel of kidney specific cfm RNAs (nephrin, paracellin, nyctalopin and podocin). In this study mRNA for nephrin, paracellin and nyctaloplin was detected in the circulation of all the subjects studied while podocin mRNA was only detected in 53 % of subjects. In contrast to the study by Butt et al. [70], there was no significant difference in nephrin mRNA between healthy subject and DN patients. By way of explanation for the lack of reproducibility of the nephrin levels, Moussa et al. [71] noted that they used a much smaller sample of individuals which was different to the group studied by Butt et al. [70]. Nyctalopin mRNA levels also remained unchanged between the groups studied. As nyctalopin is expressed by tissues other than the kidney, the differential contribution of renal mRNA for nyctalopin may have been masked by the total circulating mRNA for this protein. Paracellin mRNA was significantly higher in the microalbuminuric group compared to healthy subjects, normoalbuminuric or macroalbuminuric groups (Fig. 8). However, there was no difference in paracellin mRNA between groups within the diabetic population based on the renal status.

MicroRNA and diabetic nephropathy: MicroRNAs may play a role in the pathogenesis of DN. In *in vitro* studies and in experimental studies miR-192, miR-200b/c, miR-216a, and miR-217 were altered in DN [20]. In a recent study we measured miR192, miR-215, and miR-377 in the circulation of diabetic subjects and controls and showed that miR-192 and miRNA-215 but not miR-377 were detectable in the circulation [71]. In the diabetic patients group miRNA-192 levels were 26.5 fold greater than that seen in healthy subjects. However, there was no difference in miR-192 within the diabetic group based on renal status. A similar profile was seen with miR-215 measurement between and within the groups under study (Fig. 9). While the mechanisms for this observed increase in microRNAs in the circulation remains unknown, there is a suggestion that over-expression of microRNAs in the cells leads to this increased release. It is further proposed that microRNAs are released into exosomes, which maintain their stability and resistance to endogenous RNAse breakdown [72]. These two findings may explain the altered and increased levels of miR-192/mir-215 in the circulation of patients with DN. Interestingly, and unlike ACR measurement, circulating levels of microRNA were not related to age, gender, BP, duration of diabetes or renal function. Independence from factors that would otherwise require microRNAs levels to be corrected offers considerable advantage over ACR measurement when making direct comparisons between individuals or defined groups.

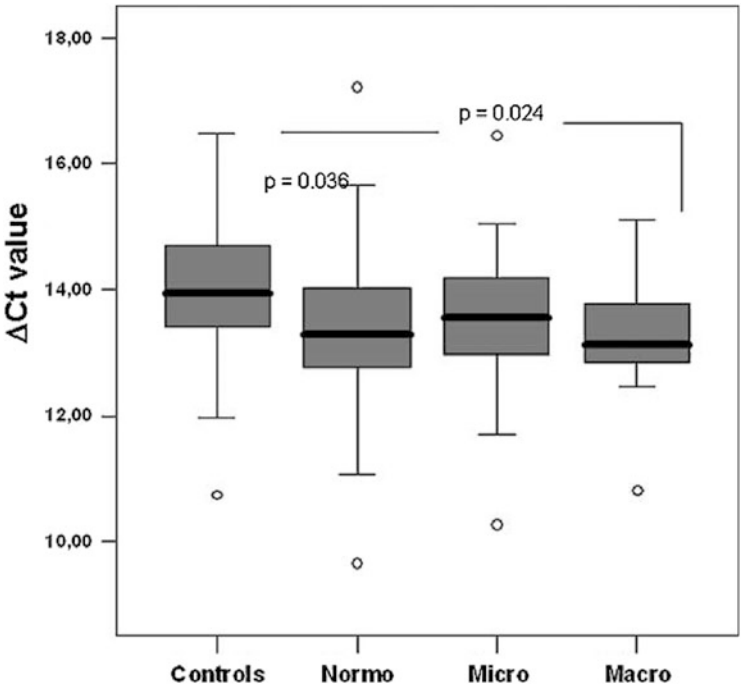

Fig. 7 Ct values for mRNA for nephrin in healthy subjects and diabetic patients with different degrees of albumin excretion. mRNA for nephrin was higher (hence lower Ct value) in subjects with diabetes

4.1 Diabetic Neuropathy

Approximately 131 million individuals are affected by diabetic neuropathy (DNeu) [73]. It is estimated that the prevalence of neuropathy in diabetes patients is approximately 20 %. However, this may vary depending on the diagnostic criteria [74], e.g. prevalence of DNeu after 25 years was 45 % [75]. Although duration of diabetes, age, smoking, hypertension and hyperlipidemia are important, the main risk factor for DNeu is hyperglycemia. In the DCCT [54] study, the annual incidence of neuropathy was 2 % per year, but dropped to 0.56 % with intensive treatment of Type 1 diabetics. DNeu is also implicated in 50–75 % of non-traumatic amputations [76, 77]. Despite a range of diagnostic tools being available, under diagnosis or misdiagnosis of DNeu remains a problem in clinical practice. As a viable alternative, Sandhu et al. [78] postulated that in diabetic neuropathy, neuron specific nucleic acids may appear in the circulation and these may form a blood test for the early detection of this disorder. The marker proposed was neuron-specific enolase (NSE), which is a highly soluble intracellular protein principally located in neuronal cytoplasm and in neuroendocrine cells [79, 80].

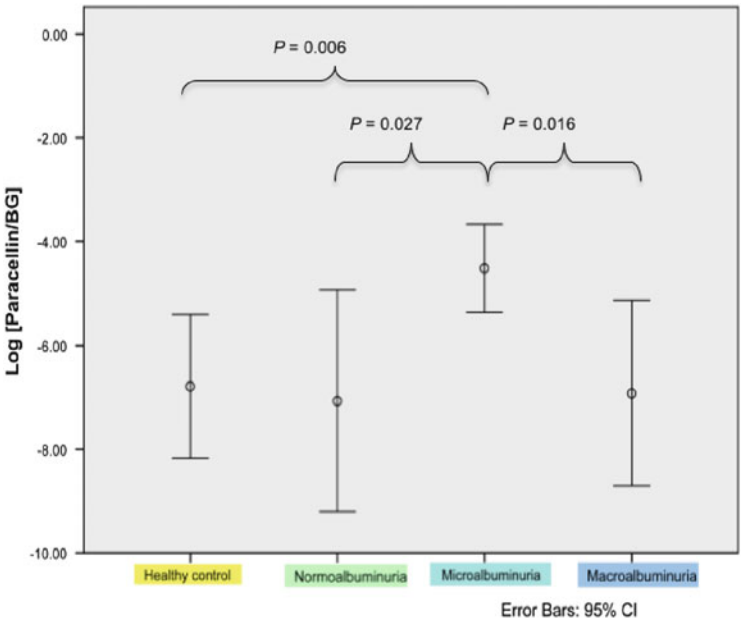

Fig. 8 Circulating levels of paracellin mRNA in healthy control subjects and patients with diabetes (data are expressed as mean and 95 % confidence interval). Circulating pracellin mRNA was higher in diabetic nephropathy

In this pilot study 72 individuals consisting of 26 healthy subjects and 46 diabetic patients, of whom 24 were diagnosed with DNeu, were tested for circulating NSE mRNA. Of the 24, all but one were also diagnosed with diabetic retinopathy. The results of this original study revealed that diabetic controls had significantly higher levels of circulating enolase mRNA than healthy subjects (Fig. 10), indicating that hyperglycemic insult, even before clinical manifestation of DN, could lead to increased release of NSE mRNA into the circulation. However, levels of NSE mRNA were lower in the diabetic neuropathy group relative to the diabetic controls and healthy subjects. One possible explanation for this outcome is that the widespread cellular damage, due to persistent hyperglycemic insult, in the years before the diagnosis of DNeu may affect the ability of neurons to transcribe NSE mRNA. When the DNeu patients were examined according to their eye status, patients with pre-proliferative retinopathy had significantly higher levels of circulating enolase mRNA compared to those with background retinopathy (Fig. 11). This was not surprising as DR is known to lead to ischemic changes in the inner retina, resulting in atrophy of the neuronal layer. Furthermore, extensive loss of retinal neurons may lead to release of NSE mRNA into the circulation, which may rise with increasing severity of DR. The investigators concluded that levels of enolase mRNA are decreased in diabetic neuropathy and this molecular marker may also be useful in differentiating early from advanced eye disease in those diabetics diagnosed with neuropathy.

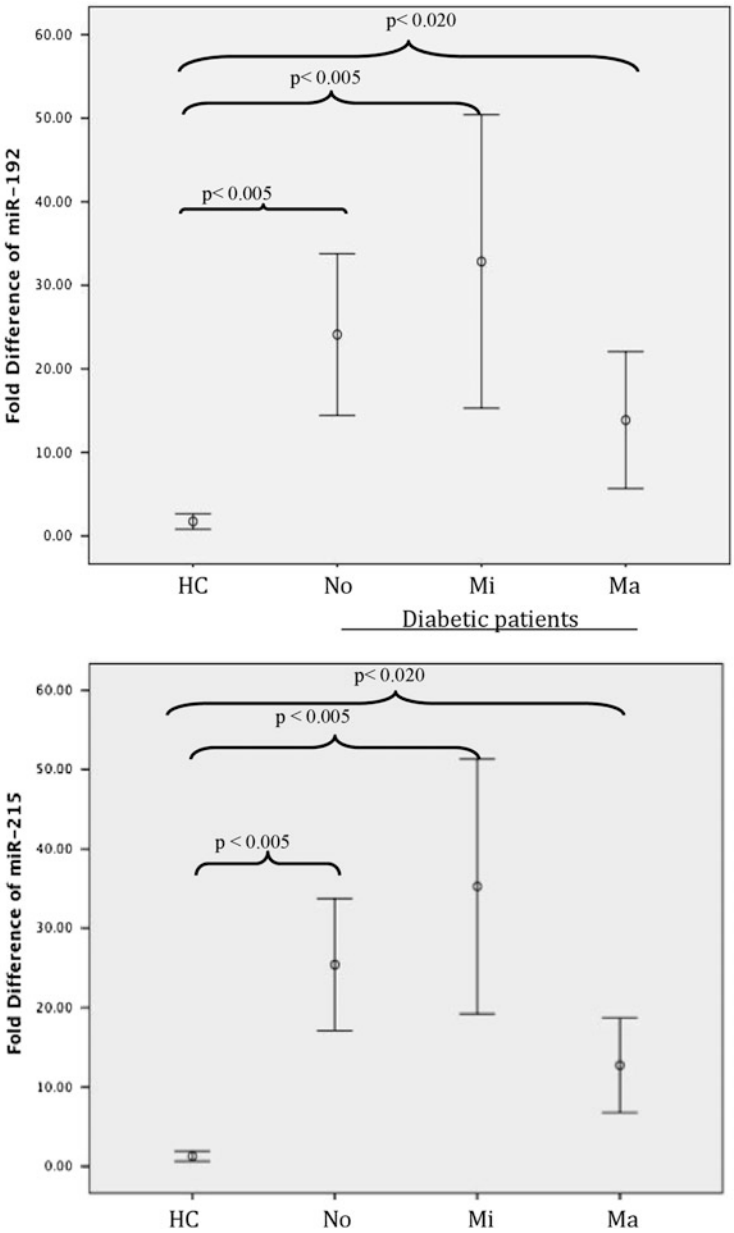

Fig. 9 Circulating levels of microRNA-192 (**a**) and microRNA-215 (**b**) in healthy control and diabetic subjects (Data is expressed as mean and 95 % confidence interval). *HC* Healthy controls, *No* Normoalbuminuria, *Mi* Microalbuminuria, *Ma* Macroalbuminuria. miR-192 and mi-215e were higher in microalbuminuric subjects

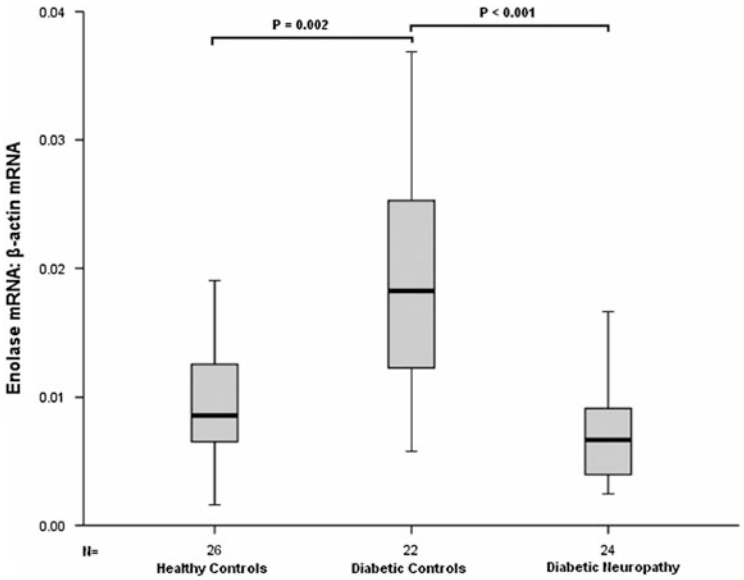

Fig. 10 Circulating enolase mRNA levels in healthy subjects, diabetic patients, and patients with diabetic neuropathy. mRNA for enolase was higher in albuminuric subjects

4.2 Macrovascular Complications

Cardiovascular disease is the leading cause of death in DM. Early detection of those who are likely to develop cardiovascular disease and peripheral vascular disease will greatly improve the management of these conditions. As discussed earlier Zametaki et al. [32] identified miR-126 to be associated with vascular complications. Caporali et al. [40] have shown that miR-503 in muscle biopsy samples and in plasma from patients with limb ischemia were higher compared to those with these complications. Several studies have shown the potential of microRNAs in the diagnosis of heart failure and acute myocardial infarction ([11]; see also chapter "CNAPS and General Medicine"). These are discussed elsewhere in the book in greater detail. These studies illustrate the potential of cfNAs in the detection of macrovascular complications.

There is presently very little or no specific and sensitive non-invasive test for the assessment of macrovascular complications associated with diabetes. cfNAs represent a potential prognostic and diagnostic utility to overcome this deficiency. It is of the utmost importance that these early stage pilot studies are expanded and verified by other independent workers in the field.

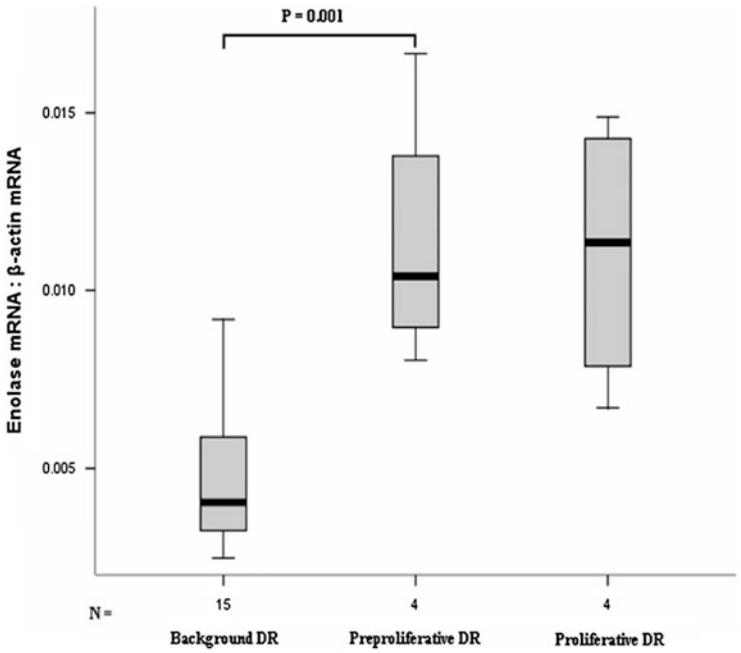

Fig. 11 Circulating enolase mRNA levels in patients with diabetic retinopathy and neuropathy. mRNA for enolase was higher in patients with diabetic retinopathy

Concluding Remarks

In this chapter we have described the potential of cfNAs (cfDNA, cfmRNA and cfmiRNA) in the early detection of diabetes and its complication. Most of the studies described here are early studies and require much larger, well-designed trials to confirm the applicability of circulating nucleic acids as biomarkers in diabetes. These are likely to be of great value as they could predict who will get clinical diabetes or its complications. However several issues need to be addressed before full clinical application.

As discussed, above and as shown in Table 1, different papers identify different nucleic acids as of potential value. There is no consensus as to the best markers. The differences in the markers identified so far may be due to the different approaches used by these investigators. Some have used systematic profiling while others have adopted a candidate marker approach. Larger, well-designed studies are needed to identify the best biomarkers. Some researchers have used plasma, others have used serum and yet others have used whole blood. Standard protocols for sample collection, preparation, storage, nucleic acid extraction and analysis are required before embarking on large-scale studies (see chapter "Pre-Analytical Requirements for Analyzing Nucleic Acids from Blood"). Some of the markers described, for

example miR-15b and miR-16 are affected by hemolysis and contamination of cellular material [81].

The origin of cfNAs is not fully understood (see chapter "The Biology of CNAPS"). A better understanding of this process may help to improve the diagnostic utility of these markers. Transport of nucleic acids in the circulation is complex. Some are carried in exosomes, others as lipoprotein or protein complexes. Knowledge of the transport process of specific nucleic acids may help to design better methods to isolate these particles before analysis. This will help to identify small amount of organ specific nucleic aids from the sometimes large amount of other nucleic acids in circulation.

Diabetes is a complex disorder affecting almost all of the organs of the body. Changes described in this chapter need to identified as specific for diabetes. Studies examining these cfNAs in other related diseases are required in order to identify more sensitive and specific biomarkers (see chapters "CNAPS and General Medicine", "Fetal CNAPS – DNA/RNA" and "Extracellular Nucleic Acids and Cancer").

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Extracellular Nucleic Acids and Cancer

Michael Fleischhacker and Bernd Schmidt

Abstract Since the clear proof of the presence of tumor-associated genetic alterations in extracellular nucleic acids almost 20 years ago this field gained has attracted much interest. According to our current knowledge it seems as if all tumor-associated alterations found in tumor cells are also found in the extracellular environment. The isolation of extracellular nucleic acids from tumor patients and its genetic characterization with very sensitive and highly specific methods led to the concept of "liquid biopsy". This means that for follow-up analysis of tumor patients, the physicians no longer depend exclusively on a single examination of tissue biopsies (usually at the time of diagnosis) but are able by longitudinally analyzing the extracellular nucleic acids to follow the reaction of a tumor to e.g. a given therapy, the development of resistance mechanisms. In this chapter we will discuss the detection and characterization of different genetic (e.g. mutation analysis and structural variations as seen in microsatellites), epigenetic (e.g. hypermethylation of selected sequences) and regulatory alterations (as in different miRNA expression patterns found in tumor patients). We will also touch on some confounding factors that have to be taken into consideration as well as the functional and biological aspects of extracellular nucleic acids.

Keywords Circulating DNA/mRNA/microRNA • Inflammation • Cancer • Plasma • Serum • Stool • Urine • Cerebrospinal fluid • Epigenetics • Microsatellites • Study design • Predictive diagnosis • Patient stratification • Personalized treatment

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1 Introduction

Cancer has been seen for many years as a purely genetic disease and was even enthroned as "The Emperor Of All Maladies" [1]. This view has been changed recently up to the point where scientists are arguing that only 5–10 % of cancers are associated with genetic defects whereas the majority of malignant diseases are connected with our environment and lifestyle like cigarette smoking, diet, alcohol, sun exposure, environmental pollutants, infections, stress, obesity and physical inactivity [2]. Apart from the fact that our modern lifestyle is part of the problem, the life expectancy of humans is rising [3] leading to an increase on the global cancer burden [4]. Contributing to the increased number of humans suffering from a malignant disease is not only the age-associated enhanced risk of developing cancer [4] but also the huge improvement of the diagnostic tools in the last decades. This is demonstrated by the reduced mortality from breast cancer due to the mammographic screening and improved therapies [5]. On the other hand there are highly sophisticated techniques available for the detection and isolation of a single tumor cell (obtained by laser micro-dissection or by capturing CTC from the cancer patients blood) and their molecular genetic characterization by next generation sequencing analysis, dPCR.

Recently, the analysis and characterization of cfNAs gained much popularity. While in our exhaustive overview in 2007 443 references were cited [6], there are now far more than 1,000 papers published dealing with this subject. The aim of this chapter is not to give a complete compilation of work devoted to the analysis of cfNAs but to point out successful and failed approaches in this field, recent method and technical improvements together with different aspects not previously covered [6, 7].

2 General Remarks

cfNAs can be isolated from a variety of different body fluids such as plasma/serum, tears, urine, breast milk/colostrum, seminal fluid, saliva, amniotic fluid, bronchial lavage, cerebrospinal fluid, pleural fluid and perioneal fluid ([6, 8, 9]; chapter "Other Body Fluids as Non-invasive Sources of Cell-Free DNA/RNA"). All of these body fluids have been used for the isolation and characterization (qualitatively and/or quantitatively) of cfNAs.

The last years have seen an impressive progress in our understanding of cellular processes, regulatory circuits and the myriad of factors involved, leading to the so-called systems biology approaches [10]. Also, the development of new methods such as NGS analysis, the improvement of high-throughput expression analysis systems and methods for the detection of a few mutated sequences in a background of millions of wild type DNA (dPCR) broadened our view on the "cancer problem" (see chapter "Genomic Approaches to the Analysis of Cell Free Nucleic Acids").

3 DNA Quantification/DNA Integrity

Leon and coworkers [11] were the first to demonstrate an increase in cfDNA in tumor patients compared to a control population. In the late 1990s (after the landmark papers by Chen et al. [12] and Nawroz et al. [13] were published) this observation initiated a surge of papers confirming and extending the results described by Leon et al. Unfortunately, it became clear very soon that the increase in cfDNA in tumor patients is not specific and that many factors/diseases lead to the higher quantity of cfDNA (see chapter "The Biology of CNAPS"). When cfDNA had been quantified it was shown that the amount is increased in a variety of different conditions such as myocardial infarction [14], cardiac arrest [15], exhaustive exercise [16–18], in patients with systemic lupus erythematosis [19], in older humans [20], in febrile patients [21], in children on peritoneal dialysis [22], in patients with obstructive sleep apnea [23], patients with chronic kidney disease [24], patients with severe sepsis or septic shock [25], in trauma [26] and burn patients [27] (chapter "CNAPS and General Medicine"). In an attempt to differentiate lung cancer patients from a control population according to their sputum cfDNA it was demonstrated that the amount of cfDNA was related to the severity of the inflammatory processes but not the presence of lung cancer [28]. When a capillary electrophoresis (CE) method and qPCR were used to determine the amount of plasma cfDNA in non-small cell lung cancer (NSCLC) patients and healthy controls, the quantity of cfDNA obtained with CE was almost twice as high as with qPCR and with both methods, almost twice as high in NSCLC patients when compared with a group of healthy subjects. This led the authors to conclude that this method "is an effective diagnostic tool to discriminate NSCLC patients from healthy individuals" [29]. According to our opinion, this statement will not stand the test of time. We strongly believe that the lack of an association between an increased amount of cfDNA and a malignant disease makes a quantitative assessment of cfDNA for diagnostic purposes completely useless. This very likely also holds for a combination of a quantitative cfDNA measurement plus a clinical assay (including but not limited to imaging methods) or any other biomarker. Whether an increased amount of plasma cfDNA might be useful for an early detection of disease recurrence in NSCLC patients [30] has to be seen in future trials. In contrast, there was no association between post-surgery cfDNA levels and disease recurrence in patients with renal-cell carcinoma [31]. A group of 15 patients suffering from a variety of different malignancies (all solid tumors) who were treated with radiation therapy were followed over several months and their plasma cfDNA was quantified [32]. In 13/15 patients a decrease in plasma cfDNA concentration was seen over time. Two of these patients had local tumor recurrence, three other patients had distant metastasis and eight patients were in complete remission. From their experiments, the authors concluded that there was no obvious association between patient outcome and plasma cfDNA concentration before treatment and during therapy. In an animal system. it was shown that there is a positive correlation between the amount of plasma tumor cfDNA and tumor size and that

the level of WT and mutated plasma cfDNA fluctuated among individual animals (rats) depending on individual specific factors [33]. As long as we do not understand the factors regulating the release of cfDNA into the environment a quantitative analysis would appear to have no meaning.

Considering the facts mentioned above and the observation that there is a large overlap in the amount of circulating cfDNA in cancer patients vs. controls (as discussed in detail in a previous overview; [6]), several groups tried to differentiate patients from controls with a cfDNA integrity index [34]. The basic assumption for this approach is the observation that tumor-derived cfDNA is released by mechanisms different to those for cfDNA from non-tumor cells together with the different sizes of these fragments. The index is determined by a qPCR in which two amplicons targeting the same sequence, but of different lengths are generated. The Ct values are used to calculate the cfDNA integrity index with several formulas [35]. In the first paper reporting on this approach, a different integrity index was found in patients with gynecological malignancies compared to healthy women [34]. Several other groups tried to replicate this observation with varying success in patients with prostate cancer [36–38] renal cell and bladder cancer [39, 40], head and neck cancer [41], gastric cancer [42] and lung cancer [43, 44]. In the report mentioned before, it was shown that the plasma cfDNA integrity index in tumor patients was higher than in healthy subjects, but no data were supplied for cfDNA integrity during radiation therapy [32]. In a slightly modified approach, several groups used the integrity index as a marker for therapy monitoring but these data are very preliminary [45].

3.1 Synopsis

Clinical relevance of cfDNA quantification of extracellular nucleic acids

	Diagnosis	Therapy response	Prognosis	Clinical utility
DNA quantification	None	Probably not	None	None
DNA integrity index	None	Maybe	Maybe	Maybe

4 Number Crunching with dPCR and BEAMing

dPCR and its further development i.e. BEAMing were only established a few years ago but are already on their way to replace qPCR as a standard for nucleic acid quantification (Table 1; [46–48]; chapters "Detection of Genetic Alterations by Nucleic Acid Analysis: Use of PCR and Mass Spectroscopy-Based Methods" and "Genomic Approaches to the Analysis of Cell Free Nucleic Acids"). It had been demonstrated that dPCR/BEAMing is not only more precise than qPCR, but also

Table 1 Summary of papers using digital PCR, BEAMing and NGS for in depth genetic characterization of extracellular DNA

Method	Body fluid	Tumor	Approach	Target sequence	Sensitivity/ specificity	Clinical relevance	Remarks	Reference
Sequencing	Plasma	NSCLC	Therapy monitoring	EGFR, T790M mutation		Pats $\leq$ 65 years with partial response + T790M mutation had shorter PFS	Detection of T790M mutation	[49]
Sequencing	Plasma	N/A	Optimization of method	Mutation detection	1 variant/5,000 WT molecules			[50]
Amplification refractory mutation system (ARMS), Sanger sequencing	Malignant pleural effusion (MPE) and plasma	NSCLC		EGFR	63 % tissue vs. cell-free MPE, 67 % tissue vs. plasma		MPE and plasma are valid surrogates for NSCLC tumor EGFR mutation detection when tissue is not available	[51]
Cycleave PCR	Sputum	NSCLC	Comparison of methods	EGFR	3/10	High specifity since sputa from 10 COPD pats tested negative	Data in column 6: sputum/tumor tissue	[52][a]
Cold PCR					5/10			
PangeaBiotech SL Technology					5/10			
HRM					3/10			

(continued)

Table 1 (continued)

Method	Body fluid	Tumor	Approach	Target sequence	Sensitivity/ specificity	Clinical relevance	Remarks	Reference
ARMS WAVE/Serveyor	Plasma	NSCLC	Comparison of methods, proof of principle study	EGFR	21/30 EGFR activating mutations in plasma 5/7 T790M mutations in plasma	Useful for T790M mutation detection in plasma in resistant patients		[53]
BEAming	Plasma	Metastatic breast cancer	Feasibility study	PIK3CA	Retrospective analysis: 100 % concordance (tumor vs. plasma) prospective analysis: 73 %	Loss or gain of PID3CA mutations over time occurs in 20 % of the patients		[54]
Affymetrix Genechip Human Mitochondrial Resequencing Array + capillary sequencing	Blood (plasma?), bronchial lavage, sputum	Early stage lung, bladder, and kidney cancer	Feasibility study	Any mutation of mitochondrial DNA	36 % of the body fluids from cancer patients with mtDNA mutation in the tumor had mtDNA mutation/s		2/12 sputa from heavy smokers w/o cancer had mtDNA mutations	[55]
BEAming	Plasma	Stage IV CRC, metastatic breast cancer	Feasibility study	Copy number alterations		Are the patients with or w/o "long" plasma DNA clinically different?	In CRC patients with higher plasma DNA conc. there is more "long" DNA (≥240 bp)	[56][b]

Whoe genome analysis by NGS	Plasma	CRC	Feasibility study	Copy number alterations, chromosomal rearrangements		Amplification of ERBB2 + CDK6 was found	Percentage of plasma tumor DNA varied from 1.4 to 48 %	[57]
ARMS, direct sequencing	Pleura fluid, plasma	NSCLC	Prediction for therapy response	EGFR		Positive result = good indicator for TKI therapy		[58][c]
Whole genome NGS	Plasma	Breast cancer		Identification of cancer-related sequences from patients	Diagnostic specifity 95 % and sensitivity of 90 %			[59]
Sanger sequencing and NGS	Bronchial lavage and pleura fluid	Lung cancer	Comparison of methods	EGFR	Patients with 0.3–9 % malignant cells: Sanger = mutation in 16 % cases, NGS = 81 % Patients w/o tumor evidence: Sanger = mutation in 0 % cases, NGS = 42 %	Sensitive NGS technology can detect patients elegible for TKI treatment		[60]
NGS	Plasma	Non-Hodgkin lymphoma	Feasibility study	IgH gene rearrangements				[61]

(continued)

Table 1 (continued)

Method	Body fluid	Tumor	Approach	Target sequence	Sensitivity/ specificity	Clinical relevance	Remarks	Reference
Digital PCR	Plasma	Breast cancer		HER2 amplification	Sensitivity 64 % specifity 94 %		PPV 70 % NPV 92 %	[62]
NGS and PCR	Plasma	Breast cancer, osteosarcoma	Proof-of-principle study	genomic rearrangements		Rising level of tumor DNA are an indicator for disease progression		[63]
Tagged-amplicon deep sequencing	Plasma	Advanced ovarian cancer	Proof-of-principle study	Sequencing of entire genes for mutation detection	Sensitivity and specificity $\geq$ 97 %	Frequency of mutant p53 mutation reflected course of disease	Mutations were found as low as 2 % allele frequency	[64]
Whole exome sequencing	Plasma	Breast cancer, ovarian cancer, lung cancer	Proof-of-principle study	PIK3CA, RB1, MED1, GAS6, EGFR		Therapy resistence was mirrored by increased mutant alleles	Sequential analysis for up to 2 years	[65]
Methyl-BEAming	Plasma	CRC	Proof-of-principle study	Vimentin	59 % sensitivity 93 % specificity	52 % sensitivity in Duke A + B patients	Level of methylated vimentin DNA decreased after surgery	[66]
Digital MethyLight	Plasma	Breast cancer	Proof-of-principle study	RUNX3A, CLDN5, FOXE1			DNA from 100 µL serum was enough for digital MethyLight	[67]

Digital PCR	Plasma	CRC	Proof-of-principle study	KRAS	14/19 plasma mutations matched tumor mutations		Two pats with WT KRAS in the tumor had mutant plasma DNA	[68]
Digital PCR	Plasma	NSCLC	Proof-of-principle study	EGFR	92 % sensitivity 100 % specifity	Quantity of mutant DNA correlated with clinical response		[69]

Abbreviation: *HRM* high resolution melting curve analysis

[a](Hubers et al. 2013): in four pats no mutation was found in sputum with any method and in three patients sputum mutation was found with one of the four methods only

[b](Heitzer et al. 2013): the appearance of "long" plasma DNA was associated with a higher percentage of tumor DNA and with an increased number of CTCs, in these samples a direct analysis of genome-wide tumor-specific copy number alterations directly from plasma DNA is possible, a similar observation was made in patients with metastatic breast cancer

[c](Liu et al. 2011): the test only works reliably if there is enough extracellular tumor-derived DNA in the sample

very robust and obviates the need for a calibrator since the quantity of nucleic acid molecules (DNA, cDNA, RNA in its different forms) are counted as absolute numbers [70–72]. The first studies using these methods nicely demonstrated that there is a correlation between the number of mutant cfDNA in the plasma of colorectal cancer patients before and during therapy [73], the possibility to detect tumor-associated mutations and methylated genes in plasma and stool samples from cancer patients [66, 74], the detection of copy number alterations [56], an amplification of the *HER2* gene in the plasma of breast cancer patients [62] and the quantification of mRNA transcripts in serum and cerebrospinal fluid from glioma patients [75]. A sensitive and specific detection of EGFR mutations in the plasma of lung cancer patients was shown by Taniguchi et al. [76].

In a retrospective BEAMing analysis of plasma and tumor samples from metastatic breast cancer patients, the concordance of *PIK3CA* mutations was found to be 100 % while in the prospective cohort, the concordance between plasma and tissue was only 72 % [54]. The authors explain this discrepancy by the fact that the data from plasma samples of patients with recurrent disease were compared to archived tissue obtained at least 3 years before the actual blood draw and point out that it is important to use contemporary and not archived material (chapter "Pre-analytical Requirements for Analyzing Nucleic Acids from Blood"). On the other hand, it might be possible that recurrent tumors demonstrate a different mutational spectrum than the original tumor analyzed at the time of diagnosis though this is not discussed by the authors.

In a sequential BEAMing analysis of plasma cfDNA for the detection of *KRAS* mutations in colorectal cancer patients undergoing an anti-EGFR therapy, Misale et al. [77] demonstrated the sensitivity of this assay by showing that *KRAS* variants leading to a resistance for cetuximab were detectable in plasma 10 months before a disease progression was found by radiological evidence.

All these data demonstrate the technical feasibility for a sensitive and quantifiable detection of molecular alterations in different body fluids, but before these methods can be applied in a routine clinical setting, large patient populations have to be analyzed in prospective trials to demonstrate their clinical benefit for the patients and for setting cut-off values.

4.1 Synopsis

Counting of cfNA molecules

	Diagnosis	Therapy response	Prognosis	Clinical utility
Digital PCR and BEAMing	Not any time soon	Yes	Yes	Yes

5 Mutation Analysis

The power of newly developed methods such as NGS and dPCR for a quantitative mutation analysis was recently demonstrated in several papers in which serial plasma cfDNA samples were analyzed. In a prospective study, 30 women with metastatic breast cancer receiving systemic therapy over 2 years were examined for CA 15-3 level, the number of CTCs, mutations and structural variants like deletions in their plasma cfDNA [78]. It was demonstrated that in the majority of patients (19/20 women) the fluctuation of circulating tumor cfDNA generally correlated with treatment response. A progressive disease was seen during follow-up and an increase in cfDNA reflected the CT data. Although the number of CTC showed some fluctuation as well, this was observed to a somewhat lesser extent than the amount of tumor cfDNA. The tumor marker CA 15.3 was the marker with the least changes over time. In addition, increasing levels of circulating tumor cfDNA was associated with inferior overall survival, whereas CA 15-3 had no prognostic power. MPS of exomes from circulating tumor cfDNA was used for the detection and enumeration of plasma cfDNA mutations leading to acquired resistance to cancer therapy in patients with advanced breast, ovarian and lung cancers who were followed over 1–2 years [65]. The quantification of allele fractions in plasma identified increased representation of mutant alleles in association with the emergence of therapy resistance such as an activating mutation in *PIK3CA* following treatment with paclitaxel, a truncating mutation in *RB1* following treatment with cisplatin, a truncating mutation in *MED1* following treatment with tamoxifen and trastuzumab, a splicing mutation in the *GAS6* gene following subsequent treatment with lapatinib and a resistance-conferring mutation in *EGFR* following treatment with gefitinib.

The detection of *EGFR* mutations in the plasma of lung cancer patients by dPCR has been demonstrated and the authors showed a very high concordance rate of mutations found in tumor tissue as compared to plasma [69]. The analysis of plasma cfDNA from colorectal cancer patients for the detection of *KRAS* mutations with a multiplex dPCR demonstrated that in 14/19 patients the same mutation was present in tumor tissue and plasma whereas 1 patient had a different *KRAS* plasma mutation and four patients had no detectable plasma mutations [68]. Equally, there are also reports in which it was shown that the detection rate of *EGFR* mutations in cfDNA is far less sensitive than the analysis of tissue DNA [79]. So far, it is not completely understood as to whether the discordance between mutation frequency found in tumor tissue vs. plasma is due to either techniques with insufficient sensitivity or a low amount of tumor derived plasma cfDNA or tumor heterogeneity, i.e. the existence of tumor subclones harbouring different mutational spectra.

Using a variety of different methods, activating mutations of the *BRAF* gene are found in cfDNA from patients with melanoma [80], hairy-cell leukemia [81], papillary cancer of the thyroid [81, 82], colorectal cancer [83], ovarian cancer [84] and NSCLC [50]. Therefore, a quantitative analysis, i.e. an enumeration of

cfDNA molecules (from plasma or any other body fluid) harbouring a mutation might be useful for therapy monitoring and prognosis, but not as a diagnostic tool.

5.1 Synopsis

Mutation detection and quantification of cfDNA in body fluids

	Diagnosis	Therapy response	Prognosis	Clinical utility
Mutation analysis	Not before organ specific alterations are found	Yes	Maybe	Yes

6 miRNA

Since the first description of miRNA in *Caenorhabditis elegans* [85] the analysis of these nucleic acids has been evolving as a very hot topic due to several factors. cfmiRNAs are very stable even under harsh conditions and survive storage at low temperature (−80 °C and less) for a long time. In contrast to isolated total RNA, the RNA integrity number (RIN value) of which decreased considerably after dilution in water and treatment at 80 °C for up to 4 h; this had almost no influence on the stability of cfmiRNA when measured by qPCR [7, 86]. Also repetitive freezing and thawing of miRNA samples [87] or of urine samples which were either subjected to seven freeze/thaw cycles or were incubated at room temperature for up to 3 days did not change the amount of miRNA detectable by qPCR [88]. It was also shown that stool derived miRNAs that are either bound to cell membranes or are inside exosomes are rather stable [89]. In addition to the high stability of miRNAs, they are lower in numbers as compared to mRNA (approx. 2,500 mature forms in *Homo sapiens* listed in www.mirbase.org as of September 2013), they can be isolated and quantified easily with a number of different commercially available kits and they play important roles in tumor development – and might be even therapeutic targets. The number of papers dealing with cfmiRNA and their exploration for diagnosis, prognosis and staging is increasing almost exponentially.

The papers recording the cfmiRNA expression in either plasma or serum or sputum was analyzed with the aim of (early) diagnosing lung cancer patients are given in Table 2. Upon reviewing these data, it can be concluded that (i) the reported cfmiRNA expression levels are very heterogenous (even papers originating from the same laboratory report different results) and that only very few cfmiRNAs are confirmed in several publications as up - or down-regulated; (ii) the possibility cannot be excluded that different techniques (sequencing vs. qPCR) yield different results; and (iii) most groups used healthy subjects

Table 2 Analysis of cell-free miRNA expression in plasma/serum or sputum aiming at the (early) detection of lung cancer

Sample	Reduced miRNA	Increased miRNA	Method	Reference	Remarks
Serum		25, 223	Solexa sequencing	[90]	Pool of 11 sera from lung cancer patients, controls: healthy subjects
Plasma	126, 486-5p	21, 210, 182	qRT-PCR	[91]	Controls: healthy individuals
Plasma		Deregulated miRNA; 17, 660, 92a, 106a, 19b	TLDA applied biosystems, qRT-PCR	[92]	These miRNA were most frequently deregulated at the time of lung cancer diagnosis
Serum		20a, 24, 25, 145, 152, 199a-5p, 221, 222, 223, 320	qRT-PCR	[93]	Controls: healthy subjects
Plasma or serum		1268, 574-5p, 1254, 1228	GenoExplorer miRNA expression system, qRT-PCR	[94]	Controls: healthy subjects
Plasma		21	qRT-PCR	[95]	Controls: healthy subjects
Serum	625 361-3p		Microfluid biochip Febit Biomed, qRT-PCR	[96]	miRNA-625 differentiated between lung cancer and benign lung disease miRNA-361-3p did not differentiate between lung cancer and benign lung disease
Serum	92a, 484, 486, 328, 191, 376a, 342, 331, 30c, 28, 98,17, 26b, 374, 30b, 26a, 142, 103, 126, let-7a, let-7d, let-7b, 22, 148b, 139	32, 133b, 566, 432, 223, 29a, 148a, 142, 140,	TLDA applied biosystems	[97]	Controls: healthy subjects matched by age, gender and smoking history

(continued)

Table 2 (continued)

Sample	Reduced miRNA	Increased miRNA	Method	Reference	Remarks
Serum	Differential miRNA expression pairs: 15b/27b, 15a/27b, 142-3p/27b, 15b/301, 27b/301		qRT-PCR	[98]	Controls: healthy subjects
Plasma		155, 197, 182	qRT-PCR	[99]	Controls: age-matched healthy subjects
Plasma	486-5p	21, 210	qRT-PCR	[100]	Controls: healthy subjects + patients with benign solitary pulmonary nodules
Serum		125b	qRT-PCR	[101]	Controls: non-pregnant women + non-tumor donors
Serum	485, 30d	1, 499	Solexa sequencing, qRT-PCR	[102]	Detection of these miRNAs is significantly associated with survival
Serum		21, 155, 197 increased in cancer + pneumonia, 182, 197 increased in cancer + tuberculosis	qRT-PCR	[103]	miRNA-21, 155, 197 differentiated cancer + pneumonia patients from controls but not from each other, miRNA-182, 197 differentiated cancer + tuberculosis from controls but not from each other, controls: matched healthy population
Serum		21	qRT-PCR	[104]	Controls: healthy subjects increased expression was also found in other tumors (breast, colorectal, esophagus, gastric)
Sputum	126, 139, 429	205, 210, 708	Gene-ChipR miRNA array, qRT-PCR	[105]	Controls: healthy subjects matched by age, gender and smoking history
Sputum	486	21, 200b, 375	TLDA applied biosystems, qRT-PCR	[106]	Controls: healthy individuals
Sputum		21	qRT-PCR	[107]	Controls: cancer-free subjects

Sputum	Optimized miRNA panel: 21, 155, 210, 143, 372	qRT-PCR	[108]	Controls: patients w/o previous malignancy but history of non-malignant pulmonary disease (asthma, emphysema, etc.)
Plasma or serum	miR-146b, miR-221, let-7a, miR-155, miR-17-5p, miR-27a and miR-106a miR-29c	qRT-PCR	[109]	Controls: healthy smokers, there were significant differences but the data suggest that circulating miR levels may not provide sufficient predictive accuracy to be used for screening, differences seen in serum did not show up in plasma

Abbreviation: *TLDA* Taqman low density array

Table 3 Summary of different miRNAs aberrantly expressed miRNAs in more than one organ

miRNA	Lung cancer	Different malignancy	Remarks
342	Underexpressed [97]	Underexpressed in AML [112]	
let-7a	Underexpressed [97]	Underexpressin in gastric cancer [113]	
let-7b	Underexpressed [97]	Overexpressed in AML [112]	
374	Underexpressed [97]	Overexpressed in AML [112]	
148a	Overexpressed [97]	Overexpressed in multiple myeloma [114]	
21	Overexpressed in several papers, see Table 2	Overexpressed in: colorectal cancer [115] pancreatic cancer [116]	Overexpressed in: hepatitis C patients with/ without hepatocellular
		Tong squamous cell carcinoma [117]	Carcinoma [119]
		Osteosarcoma [118]	Students performing acute exhaustive excercise [120]
		Gastric cancer [113]	
155	Overexpressed in several papers, see Table 2	Overexpressed in: pancreatic cancer [116]	
		Breast cancer [121]	
372	Differentially expressed in lung cancer [108]	Overexpressed in tong squamous cell carcinoma [117]	
92a	Underexpressed [97] Overexpressed [92]	Overexpressed in colorectal cancer [122],	
200b	Overexpressed [106]	Overexpressed in metastatic breast cancer patients [123]	
143	Differentially expressed [108]	Underexpressed in osteosarcoma [118]	
106a	Overexpressed [92]	Overexpressed in gastric cancer [113]	

(in some cases poorly defined) which is not an adequate control population. In many papers the authors state that the panel of cfmiRNA they discovered to be aberrantly expressed in lung cancer patients are potentially useful biomarkers for diagnosis, an overly optimistic view. One of the genes which has been recurrently described as being over-expressed in lung cancer, miR-21, is not organ specific at all but over-expressed in glioma, cancer of the breast, ovary, stomach, liver, prostate, pancreas, head and neck, thyroid, cervix, leukemia and lymphomas [110]. The same holds true for miR-155 that is not only over-expressed in different malignant tumors but also plays a role in a variety of physiological and pathophysiological conditions such as immunity, inflammation and cardiovascular diseases (more examples of aberrantly regulated cfmiRNAs in more than one organ are summarized in Table 3) [111]. In a recently published meta-analysis, it was demonstrated that cfmiR-21 is not suitable as a diagnostic marker for cancer [124]. This statement does not come as a surprise given the facts that in most studies cancer patients (i) were compared

with healthy subjects, (ii) miR-21 is one of the key players in inflammation and (iii) inflammatory processes are not only present in cancer but also play a significant role in premalignant conditions [125, 126]. The fact that we are still far away from the point where we can apply our knowledge and turn it into clinical routine becomes very obvious upon reviewing some of the papers in which cfmiRNA expression profiles from plasma were compared [127]. In a meta-analysis of 13 publications, almost no congruence in cfmiRNA profiles from different groups was found. In 10/13 papers selected for their meta-analysis, healthy subjects served as controls and only one group used an appropriate control population (comparing plasma cfmiRNA expression in patients with malignant solitary pulmonary nodules (SNPs) with patients having benign SNPs). Another example involves studies in which cfmiR-342, which was originally described as being down-regulated in colon cancer [128], was later was found to be down-regulated in patients with acute leukemia [129], acute myeloid leukemia [112], malignant peripheral nerve sheath tumors [130], malignant glioma [131], in cancer-associated fibroblasts from breast cancer patients [132], breast cancer [133] and lung adenocarcinoma [134] but up-regulated in melanoma patients [135] and patients with Sézary syndrome [136].

That there is only partial overlap between the circulating cfmiRNA species identified in the three studies of CRC has been shown by Ng et al. [137] and Huang et al. [138]. In two studies, qPCR analysis was applied while one relied upon Solexa sequencing of cfmiRNA from serum [90]. Although both qPCR studies used similar RNA extraction methods and the same qPCR approach, one of the studies found miR-17-3p to be higher in patient plasma [137] whereas the other reported plasma levels to be too low to be accurately quantifiable [138]. A similar conclusion can be drawn when comparing the results of cfmiRNA expression analysis in patients with ovarian cancer. The quantity of circulating exosome-derived miRNA from sera of ovarian adenocarcinoma patients was compared to sera from age-matched women with benign ovarian adenoma and women without ovarian disease [139]. No exosomal miRNA was found in normal controls and the level of eight cfmiRNAs (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205 and miR-214, all of them were over-expressed) was different in cancer patients compared to patients with benign disease. In contrast to these data, another group also examined sera from ovarian cancer patients and compared them to sera from healthy women. They described a panel of five cfmiRNAs as being over-expressed (miR-21, miR-92, miR-93, miR-126 and miR-29a) and three under-expressed (miR155, miR-129 and miR-99b) [140]. It is not too surprising that, apart from miR-21 that was found to be over-expressed in both studies, there is basically no concordance between these studies so reflecting the current state of affairs. Additionally, the latter group states that their panel might be useful as early detection markers for ovarian cancer that is doubtful given the fact that their control population comprised healthy individuals which is not necessarily an appropriate control group. Discordant results generated in the same laboratory for the expression of cfmiRNA were also published. In the first study, Solexa sequencing data showed an increase in cfmiR-25 and cfmiR-223 in plasma of lung cancer patients compared to controls, while let-7a which had been described as a diagnostic marker

for lung cancer in other papers did not demonstrate any changes in expression in plasma from lung cancer patients as compared to the controls [90]. In a second paper by the same group, they performed Taqman probe-based qPCR assays to find differentially expressed serum cfmiRNAs from NSCLC patients and healthy subjects. None of the ten cfmiRNAs detected were described in their earlier paper [93].

6.1 cfmiRNA Quantification for Prognosis, Diagnosis, Follow-Up and Treatment Monitoring

In a few studies, the quantity of cfmiRNA before and after treatment was analyzed. In patients with squamous cell carcinoma of the tongue, the level of plasma cfmiR-184 before and after surgery was analyzed and a significant reduction was observed after surgical removal of the primary tumor [117]. Unfortunately, the authors did not specify the time between surgery and the post-surgery examination and did not report on the outcome of the patients in whom increased post-surgery cfmiR-184 levels were seen. A decrease of plasma derived cfmiR-17-3p and cfmiR-92 was also observed in colorectal cancer patients, the levels of which were determined before and after surgery [137]. A similar observation was reported in gastric cancer patients in whom an increased level of plasma cfmiR-17-5p, cfmiR-21, cfmiR-106a, cfmiR-106b was followed by a decline after surgery [113]. The reduction of cfmiR-21 and cfmiR-26b plasma levels post-surgery in head and neck squamous cell carcinoma patients is associated with a better survival [141]. A decrease of serum cfmiR-21 and cfmiR-24 levels after surgery was also seen in lung cancer patients leading the authors to conclude that this method might be useful for disease recurrence assessment [142]. When resectable NSCLC patients (stage I-IIIA) were followed for at least 18 months, an increased cfmiR-155-5p and cfmiR-223-3p and a decreased cfmiR-126-3p plasma level was found to be associated with a higher risk for progression in adenocarcinoma patients [143]. A decreased plasma level of cflet-7f and cfmiR-30e-3p in NSCLC patients was associated with a shortened disease-free survival and overall survival in a long-term study in which patients were followed for several years [144]. A similar observation was made in HCC patients in whom a deregulated serum cfmiR-1 level is associated with longer overall survival [145]. A possible clinical usefulness was demonstrated in prostate cancer patients who experienced a relapse or disease progression that was associated with an elevated serum cfmiR-146b-3p and cfmiR-194 level [146]. A different set of circulating cfmiRNAs, the serum levels of which were up-regulated i.e. cfmiR-375 and cfmiR-141, was correlated with advanced cancer disease in prostate cancer patients [147]. The quantity of plasma cfmiR-141 in patients with metastatic prostate cancer was followed during therapy for several months when it was demonstrated that the quantification of cfmiR-141 was able to predict outcome (i.e. progression vs. non-progression) with an almost 80 % sensitivity [148]. When plasma cfmiRNA was profiled from 12 multiple myeloma patients and eight

healthy controls using TaqMan low density arrays (TLDAs) in a different set of patients, it was found that high levels of cfmiR-20a and cfmiR-148a were related to shorter relapse-free survival [149]. All these data show some promise but they need to be confirmed in studies including higher patient numbers and longer follow-up times.

6.2 *Why Is cfmiRNA Quantification Not Yet a Clinically Useful Tool?*

Our knowledge on the relationship between biogenesis, passive release or active secretion and the stability of different cfmiRNAs is still limited [150]. We need to know what influence different pre-analytical factors might have on the yield of cfmiRNA expression levels. Therefore, it is mandatory to develop standard protocols for collection and processing the starting material, i.e. different body fluids. We do not know the reasons for the contradictory results in expression levels when different platforms (sequencing vs. qPCR) are used. Of utmost importance is the normalization of data. So far in almost all of the papers a different house-keeping/reference gene(s) was/were used, making it impossible to compare the data. We do not have any data on (i) the cfmiRNA expression levels in healthy and diseased people over time (no longitudinal studies), (ii) the baseline concentration of cfmiRNAs in healthy and diseased people and (iii) the biological significance of the changes over the time.

A recently published paper exemplifies many of the mistakes and pitfalls one should avoid [95]. For the detection of lung cancer patients, the authors compared this group to age and sex matched healthy controls. The plasma was obtained by centrifugation (only once!) at a rather low speed that is very unlikely to remove all cells. Only cfmiR-21 was analyzed (i) which is not organ specific, (ii) the expression of which is strongly influenced by inflammatory processes and (iii) inflammatory processes were neither considered nor controlled. The possibility that plasma cfmiR-21 quantification might have a value for predicting the response to chemotherapy cannot be dismissed, but it is very unlikely that this marker will have any diagnostic value.

An example of the difficulty to reproduce results obtained in one laboratory by a different group concern the data reported by Song et al. [151] and Shiotani et al. [152]. Both groups aimed to identify a panel of serum cfmiRNAs for the early detection of gastric cancer. The first step in the paper by Song et al. consisted of a TLDA analysis of pooled samples from gastric cancer patients and controls (patients with superficial gastritis or mild chronic atrophic gastritis). From the large panel of prospective candidates, 16 cfmiRNA were selected and their usefulness confirmed by qPCR. Nine cfmiRNAs from this panel showed a good performance in discriminating between gastric cancer patients and controls as well as patients with dysplasia vs. controls. It is not clear from the published data whether or not this cfmiRNA panel can also differentiate between cancer patients and dysplasia

patients. In an additional analysis, it was demonstrated that cfmiR-221, cfmiR-376c and (to a lesser degree) cfmiR-744 showed an increased expression level over time (starting with ≥15 years to 2–5 years before diagnosis) and that the serum levels of the same cfmiRNA panel also increased in gastric cancer patients (starting 1989 till 1999/2003). From these data the authors concluded that their cfmiRNA panel might provide potential biomarkers for an early detection of gastric cancer patients. Shiotani et al. [152] analyzed serum samples from patients with a recent medical history of endoscopic submucosal dissection (which are considered to be a high-risk group for gastric cancer) and a control population of subjects who had been previously diagnosed as *H. pylori*-positive gastric ulcer or atrophic gastritis. They used a panel of 20 cfmiRNAs plus the afore-mentioned cfmiR-221 and cfmiR-744 and found that cfmiR-106b and cfmiR-21 were the best for indicating an increased risk for early gastric cancer.

Some of the problems associated with the use of cfmiRNA in cancer research that are neglected in most papers dealing with this topic are touched on in the work published by Cookson and coworkers [153] on breast cancer patients. They failed to confirm a significant association between the level of specific cfmiRNAs and the disease and found a different panel of cfmiRNAs than published before. They were also unable to use cfmiR-16 as a reference gene for quantification as the expression of this cfmiRNA varied substantially in plasma samples. Instead, they used a mean cfmiRNA level as a normalizer that worked much better. In contrast to other reports, they observed only small reductions in circulating cfmiRNA levels after surgery though not one cfmiRNA was consistently reduced. There was almost no relationship between high expression levels of cfmiRNAs in tumor cells and their presence in the circulation leading them to conclude that the release of cfmiRNA into the circulation is caused by an active and very selective secretion. The analysis of 15 reports on circulating cfmiRNA in breast cancer patients also revealed a very low reproducibility between the datasets published before [154]. Recently, two papers by Kanaan et al. were published describing plasma cfmiR-21 as a potential diagnostic marker for CRC and a panel of eight plasma cfmiRNAs for the discrimination of patients with colorectal adenomas from subjects without polyps or sporadic CRC [115, 155]. There is no concordance in the cfmiRNA panels given in both papers. If the assumption is correct that genetic alteration seen in premalignant conditions plays a role in the process of cancer development, one should expect to see at least some of these alterations in tumor cells as well. The fact that none of the aberrantly expressed cfmiRNAs found in adenoma patients is seen in cancer patients and that none of the dysregulated cfmiRNAs seen in CRC can be detected in adenoma patients leads to the conclusion that either these processes have nothing to do with each other or that the plasma cfmiRNA expression panel in adenoma patients is not indicative of a tumor development. In contrast to these observations, Brase et al. [147] observed a very good correlation between up-regulated cfmiR375 and cfmiR141 levels in serum samples and tumor tissue from the same prostate cancer patients. Similar findings were also reported in gastric cancer patients in which cfmiR-106b was over-expressed and cflet-7a under-expressed in tumor tissue and plasma from the same patients [113].

In a recently published review on the use of extracellular cfmiRNAs as biomarkers for patients with lung cancer, the authors analyzed 13 studies and found a sensitivity of 70–100 % and a specificity of 66–100 %, concluding that the analysis of cfmiRNAs in body fluids could be used as a screening tool for the detection of NSCLC patients [156].

In many of the papers published on profiling of cfmiRNA the results are very inconsistent and this also applies to publications coming from the same laboratory. This is exemplified in the profiling of breast cancer patients [157, 158] and patients with lung cancer whose sputum was examined [105, 106]. In addition, there are only a few data on the age-related expression changes of cfmiRNAs in healthy subjects underscoring the fact that our knowledge on the biology of cfmiRNAs is still very rudimentary [159] (see Sect. 10).

The problems we still face in making use of cfmiRNA quantification as a clinical tool are illustrated in one of the first papers on this topic [138]. The authors show that plasma cfmiR-29a and cfmiR-92a are over-expressed in colorectal cancer patients and to a lower degree in adenoma patients. Both markers can discriminate a patient population (i.e. CRC and adenoma patients) from healthy controls but neither can differentiate between CRC and adenoma patients. Also, there is a large overlap of single patients in all groups compared to healthy subjects making a differentiation based on a single patient almost impossible. The data summarized on the diagnostic power of circulating cfmiRNAs in patients with a variety of haematologic or solid tumor diseases look quite impressive, but as long as they cannot be confirmed in different laboratories they are only a promise for the future [160].

6.3 Synopsis

Expression analysis of cfmiRNA isolated from body fluids

	Diagnosis	Therapy response	Prognosis	Clinical utility
Profiling of cfmiRNA	Not any time soon	Maybe	Maybe	Yes, in the long run

7 cfNAs in Stool and Urine (See also Sect. 4)

The gold standard for detecting colorectal neoplasia is still a colonoscopy, although it is known that up to 27 % of neoplastic lesions $\leq$5 mm can be missed with this procedure [161]. In order to enhance the compliance for this examination and as an alternative/complement to colonoscopy, large efforts were undertaken to develop non-invasive methods for an early detection of CRC [162, 163].

Miller and Steele [164] and Hong and Ahuja [165] together with dozens of other papers were published in the recent years in which tumor-associated genetic alterations found in either plasma cfNAs or in stool samples were described.

There are different approaches to tackle this problem; the detection and characterization of fecal cfDNA, the demonstration of hypermethylated cfDNA in plasma/serum and stool, the presence of cfRNA (particularly cfmiRNA) and mutation analysis in blood or stool samples.

The first description of the presence of *K-RAS* mutations in stool samples from patients with CRC [166] was followed by a broader method which included the use of cfDNA from feces for the detection of point mutations in three genes (*K-RAS, p53* and *APC*), a microsatellite marker (BAT-26) and the generation of PCR products of different length to generate a DNA integrity index [167]. The methylation of the *SFRP2* gene in fecal cfDNA was the first assay to be used as a marker for the detection of colorectal cancer [168] and colorectal polyps [169]. The leftovers of fecal occult blood rests were useful for RNA isolation, cfDNA synthesis and cfmiRNA analysis but only when the stool samples had been stored at 4 °C (compared to either room temperature or 37 °C) and an increase of cfmiR-106a was detectable in CRC patients (as compared to healthy volunteers) [170]. Not only cfmiRNA, but even cfmRNA (which is much more fragile) was successfully amplified from stool samples of CRC patients, but with a lower sensitivity compared to the detection of methylated cfDNA [171]. By now there are many papers in which new and old markers have been used for the detection of CRC and other gastro-intestinal tumors. Some of them are listed to demonstrate their usefulness and limitations.

The analysis of a panel of six methylated genes from stool DNA was sufficient to discriminate cancer from non-cancer patients (adenoma patients + disease-free subjects without large bowel pathology) with a sensitivity of 65 % and a specificity of 81 %, but the methylation profile for stool DNA did not reflect the one found in colonic mucosa [172]. The authors explained this difference on the assumption that the DNA isolated from stool is not representative for the multitude of alterations found in colorectal mucosal cells. If this explanation holds true, the immediate question is: which markers are clinically relevant for the (early) detection of CRC and do they necessarily have to reflect cellular alterations? In a recent paper, a panel of three hypermethylated genes (*AGTR1*, *WNT2* and *SLIT2*) detected colorectal cancer in stool samples with a sensitivity of 78 % [173]. The control population for this study consisted of healthy subjects so leaving the question open as to whether or not this marker panel will be able to differentiate tumor patients from adenoma/hyperplasia or inflammatory bowel disease patients. The detection of the methylated spastic *paraplegia-20* gene which was heralded as a biomarker for the non-invasive detection of adenomas and colorectal cancer [174, 175] had also been found to be hypermethylated in ovarian [176] and prostate cancer [177] i.e. this marker very likely lacks an organ specificity. A similar observation was made for the methylation of the *integrin alpha 4* gene which was described as a good biomarker for the early detection of colonic neoplasms. A fecal DNA based assay found 69 % of individuals with adenomas with a sensitivity of 79 % [178] but this marker was later described as methylated in cholangiocarcinoma patients as well [179]. When four hypermethylated genes (i.e. *BMP3*, *EYA4*, *vimentin* and *NDRG4*) were analyzed in stool samples from IBD associated CRC patients, those

with a tumor could be differentiated from the controls, but there was no significant difference between tumor patients and patients with high-grade or low-grade dysplasia [180]. In contrast, a promoter methylation of miR-34b/c was found in 75 % of fecal DNA samples from CRC patients but in only 16 % in high-grade dysplasia patients [181]. The combination of an assay for the analysis of *TFPI2* methylation plus the amplification of "long DNA" (245 bp amplicon) differentiated between controls and CRC patients, but not between CRC and adenoma patients [182].

Apart from the sDNA test developed for the detection of CRC in stool samples (encompassing the qPCR quantification of four methylated genes and *K-RAS* mutation), there is only one commercial test available which quantifies the amount of methylated *Septin9* cfDNA in plasma. In a head-to-head comparison, the sDNA test demonstrated 82 % sensitivity for adenomas compared to 14 % for the plasma-based *Sept9* assay while for stage I-III CRC the values were 91 % and 50 % and for stage IV CRC 75 % and 88 %, respectively [183]. The authors explained the large difference (especially for adenomas and early stage tumors) with a model in which epithelial cells are constantly exfoliated into the lumen of the gastro-intestinal tract forming finally the stool. In contrast, plasma-based markers have to be transported/released into the vascular system to be detectable. This group also showed that the size of a neoplasm or an adenoma affects the detection rate, which for CRC with their sDNA test is about 100 % for tumors larger than 3 cm, whereas only 50–60 % of tumors with a size of 1–3 cm can be found. For adenomas, there is a linear increase from 30 % detection rate (size 1 cm) to 90 % for adenomas $\geq$4 cm [184].

Apart from CRC there is an urgent need for methods for an early detection of other tumors originating in the gastro-intestinal tract, the first being pancreatic cancer. The analysis of sDNA seems to be able to discriminate pancreatic cancer patients from sex-, age- and smoking-matched control populations using methylated *BMP3* and *KRAS* [185]. Unfortunately, no patients with precursor lesions of pancreatic cancer or benign diseases were included in this study. Such a control group seems to be even more appropriate as it has been demonstrated that *KRAS* mutations are detectable in plasma cfDNA of healthy subjects who might develop bladder cancer later in life [186]. Recently the question was asked as to whether or not it would be of advantage if there would be a fecal DNA based "pan-detection" assay for gastro-intestinal tract cancers [187]. The answer is "yes", provided that the marker (panel) would target the originating organ e.g. colon, pancreas, bladder, stomach, gall bladder.

The isolation and characterization of cfNAs from urine is seen as a non-invasive method for detecting patients suffering from malignancies of the urogenital tract.

In most of the reports published on this topic the cellular fraction of urine was used for the analysis that cannot be viewed as cell-free in sensu stricto. In one of the first reports, microsatellite analysis was used for the detection of renal cell carcinoma patients [188]. Interestingly, the authors demonstrated that the examination of urine (from the paper it is not clear whether the cellular sediment or cell-free urine

supernatant was used) is better suited than serum (52 LOH + MA in urine vs. 23 LOH + MA in serum). That the analysis of cell-free urine is better suited for microsatellite alterations than urine sediments was shown by several groups and the reason for this difference might be a "contamination" of the sediment with normal epithelial cells leading to a high noise vs. signal ratio [189, 190]. The total amount of cell-free DNA and the relative quantity of long DNA (measured as 400 bp amplicons in q PCR) in urine supernatant is seen as a potential biomarker for bladder cancer [191]. Since neither tumor stage nor grade is associated with the amount of long DNA and the amount of cfDNA is not tumor associated at all (see above) it might well be that the observed increase in cfDNA is related to inflammatory processes in the patient population. The possibility to detect aberrantly methylated plasma cfDNA sequences from breast cancer patients in their cell-free urine as well is interesting but unfortunately the authors supply no data on the methylation level in urine from cancer-free women [192]. This leaves the question open as to whether the assay might have any clinical utility. It had been demonstrated that the increased integrity of cfDNA from urine can be used as a marker for an early detection of prostate or bladder cancer, but since in both papers the control group consisted of healthy volunteers instead of patients suffering from prostatitis or cystitis or any other benign prostate/bladder disease we can not be sure whether these findings might have any clinical relevance [193, 194].

In summary, it can be said that there has been much progress in the field of fecal DNA testing but so far none of the assays are validated up to the point that they can be used as a primary test for the detection of CRC [195, 196]. Additionally, the optimal DNA marker panel of markers is unknown. A recently performed meta-analysis on the detection of aberrant gene methylation in stool samples from colorectal cancer or adenoma patients included 19 studies with 2,356 patients [197]. The overall sensitivity and specificity for the detection of patients with CRC was 64 % and 90 % for hypermethylated genes in stool samples and in studies with adenoma patients, these values were 54 % and 88 %, respectively. The authors concluded from their results that the diagnostic accuracy of hypermethylated sDNA for the detection of CRC or adenoma was better than a fecal occult blood test but "none of the gene panels tested are currently accurate enough to be used alone for colo-rectal neoplasia screening," Before stool-based DNA assays are introduced, there are a couple of problems that have to be tackled: the sample collection, storage and handling have to be standardized since these factors might have a large influence on the performance of a specific test, a single marker or (more likely) a marker panel has to be generated and validated in prospective studies in many patients, the diagnostic accuracy to screen for colorectal cancer in asymptomatic, average-risk patients, and soforth ([198, 199]; see chapter "Quality Assurance").

7.1 Synopsis

Detection and characterization of cfNAs from stool and urine

	Early detection, Diagnosis	Therapy response	Prognosis	Prospective clinical utility
Extracellular nucleic acids in plasma	Maybe, but not any time soon	Good idea but no data so far	Good idea but no data so far	Yes
Extracellular nucleic acids in stool/urine	Promising, but not any time soon	Good idea but no data so far	Good idea but no data so far	Yes

8 Microsatellite Alterations

By the turn of the millennium, the analysis of microsatellite alterations i.e. loss of LOH and microsatellite instability (MI) in cfDNA was very popular, but in the last few years few papers were published on this topic. Among them is one by the group of Schwarzenbach and colleagues who demonstrated that the detection of LOH at D12S1725 (mapping to the cyclin D2 gene) in plasma cfDNA of breast cancer patients is associated with shorter survival [200], an association between the presence of allelic imbalances in cfDNA and the detection of CTCs in prostate cancer patients [201] and a possible clinical value for the molecular staging of prostate cancer patients since their analysis showed a higher allelic imbalance in the plasma of cancer patients compared to benign prostatic hyperplasia patients [202]. The authors explain the low incidence of allelic imbalances in the plasma of cancer patients compared to the paired tumor cell DNA with the presence of normal cfDNA in the plasma. This could be due to the fact that they applied only one centrifugation for the plasma preparation that has been shown to be insufficient for a complete cell removal (see chapters "Pre-analytical Requirements for Analyzing Nucleic Acids from Blood" and "Circulating DNA and miRNA Isolation"). Field et al. [203] used a panel of 12 microsatellite markers for the analysis of cfDNA from bronchial lavage and found genetic alterations in 35 % of the lung cancer patients, but also in 23 % of patients without cytological or radiological evidence of bronchial neoplasia (see Sect. 6 bronchial lavage).

There are several factors contributing to the decreasing interest in microsatellite analysis in cfDNA such as (i) no high-throughput techniques for microsatellite analysis, (ii) problems with the reproducibility of the results, (iii) drop-out experiments due to low and highly degraded DNA and (iv) the introduction of PCR artifacts due to low DNA concentration i.e. false positive results due polymerase slippage.

8.1 Synopsis

Microsatellite alterations in plasma cfDNA

	Early detection, Diagnosis	Therapy response	Prognosis	Prospective clinical utility
Microsatellite alterations in plasma	Probably not	Maybe	Maybe	Maybe

9 Epigenetic Modifications

So far, we have only a rudimentary knowledge of the influence diet, lifestyle [204] and other environmental factors have on epigenetic modifications resulting in aberrant expression of tumor suppressor genes and oncogenes leading to increased cancer susceptibility [205]. Also, we still do not fully understand the relationship between age-related and tumor-related methylation processes and how methylation changes the biology of a tumor cell [206, 207]. Some of the markers assumed to be tumor-associated, such as *CDH1*, *HIC1*, *TWIST1*, *DAPK1*, *APC* and *RARß*, have been described as being hypermethylated in mononuclear cells which very likely contribute to the cfDNA in plasma [208]. Thus, before establishing an epigenetic marker panel able to specifically detect precancerous lesions or signs of malignancy at an early stage, much more information is needed [209]. A good example is given by Hauser et al. [210] who found that the methylation level of nine genes isolated from serum is variable and able to differentiate between healthy individuals and patients with bladder cancer but not between the latter and patients with benign bladder diseases. Nevertheless, there are quite a few papers published in which several hypermethylated sequences isolated from cell free body fluids were analyzed for their diagnostic value. For the early detection of lung cancer alone there are some 20 papers published in which methylated genes isolated from bronchial lavage, bronchial washings, sputum and plasma were described as being useful [211]. Similar approaches have been published for an early detection of malignancies of the breast, stomach, prostate, colon, the urogenital system, liver, pancreas and thyroid, but so far none of them made its way into clinical routine.

A promoter hypermethylation of the *CST6* gene was found in plasma of breast cancer patients that could be clearly distinguished from a group of healthy subjects, but there was no difference in the groups of patients who relapsed or died [212]. In contrast, the quantitative analysis of methylated cfDNA for either staging or prognosis or the differentiation between patients with local disease vs. patients with metastases seems to be a very promising approach. This was demonstrated in a study in which patients with locally confined prostate cancer and patients with metastases were examined. The authors showed that patients with metastatic disease had a higher amount of BMP6 mRNA and a decreased quantity of methylated *H3K27* gene in their plasma cfDNA [213]. A similar observation was made

on patients with metastatic melanoma that is associated with the presence of methylated tissue factor pathway inhibitor 2 (TFPI2) cfDNA in serum [214]. In lung cancer patients treated with tyrosine kinase inhibitors the presence of unmethylated CHFR serum cfDNA is a predictor of increased survival [215].

9.1 Synopsis

Detection and quantification of epigenetic modifications of cfDNA

	Early detection, Diagnosis	Therapy response	Prognosis	Prospective clinical utility
Epigenetic alterations	Maybe, if organ specific changes are found	Maybe	Maybe	Maybe

10 Inflammation and Cancer

There are strong links between inflammation and cancer and recently cancer-related inflammatory processes were included as another "hallmark of cancer" [216]. The tight junctions between inflammatory processes and the development of a tumor embrace both extrinsic inflammatory pathways causing/promoting cancer and intrinsic pathways in which cancer causing genetic events lead to inflammation [217–219]. Frequently, tumor patients demonstrate an elevated C-reactive protein level [220, 221] and in Table 4 some of the premalignant conditions often associated with inflammatory processes are summarized. This observation has been neglected in many studies searching for cancer-specific biomarkers. The examination of sputum in cancer-free patients with idiopathic pulmonary fibrosis demonstrated changes in markers previously associated with lung cancer [239]. No microsatellite alterations were found in the sputum of smoking, non-chronic obstructive pulmonary disease patients whereas chronic obstructive pulmonary disease patients demonstrated microsatellite alterations and/or LOS leading to the assumption that chronic inflammation is the reason for the detected DNA damage [240]. In addition, microsatellite instability in non-neoplastic mucosa from patients with chronic, ulcerative colitis was described by Brentnall et al. [241] and in plasma cfDNA as well [242]. It is possible that the alterations in these patients are not indicative of a tumor but the result of inflammatory processes. This can also be applied to the relationship of miRNAs in inflammatory processes and their association with cancer [243]. An over-expression of miR-21 has been demonstrated in inflammatory processes but at the same time in malignant diseases. This miRNA species is found to be over-expressed in sputum and plasma of lung cancer patients, in feces from CRC patients [244] and is seen as a marker for early tumor detection

Table 4 Cancer and inflammation, modified from [222]

Malignancy	Inflammatory processes	Reference
Colorectal	Inflammatory bowel disease, adenomas, polyps	[223–225]
Gastric	Helicobacter pylori gastritis	[226]
Esophagus	Barrett's esophagus	[227]
Pancreas	Chronic pancreatitis	[228–230]
Lung	Smoking, silica, benign lung diseases	[231]
Mesothelioma	Asbestosis	[232]
Bladder	Cystitis, schistosomiasis	[233]
Hepatocellular	Hepatitis B and C, cirrhosis	[234]
Cervix and anus	Papillomavirus	[235]
Ovary	Pelvic inflammatory disease	[236]
Kaposi's sarcoma	Human herpesvirus type 8	[237]
Leukemia	Essential thrombocythemia, myelofibrosis	[238]

[106, 245]. Thus, inflammation is a very important confounding factor and has to be considered when searching for biomarkers in plasma and other body fluids [246].

11 Functional and Biological Aspects

The debate about the mechanisms by which cfNAs are released into the cellular environment is still ongoing as well as the discussion on the question as to whether or not cfNAs are only "leftovers" from dead cells or are actively released. That the latter mechanism might be responsible for the presence of cfmiRNA was demonstrated by Ohshima and coworkers who showed that cultivated cells with a high intracellular level of let-7 miRNA secreted this miRNA via exosomes into the extracellular environment [247]. Since it had been demonstrated previously that *let-7* has a tumor-suppressive role (by targeting oncogenes like *RAS* and *HMGA2*), the authors speculated that the highly metastatic cell line producing this miRNA generated a "metastatic niche" and therefore maintained the oncogenesis. Mature cfmiRNAs come in different forms, i.e. bound to proteins belonging to the Argonaute family or encapsulated in microvesicles, apoptotic bodies or high-density lipoprotein particles [248]. The question as to whether the majority of cfmiRNA is bound to Ago2 protein making them highly nuclease-resistant [249] or is particle-associated as reported by Garcia et al. [250] is open for discussion.

It had been shown that cfRNA secreted from human tumor cell lines promotes tumor cell trafficking and progression via TNF-ά [251]. Also a unidirectional intercellular transfer of miRNA from T cells to antigen presenting cells and a gene expression in recipient cells has been described [252]. miRNAs which are released into the cellular environment can be transferred and perform a function in recipient cells [253, 254]. Tumor-derived melanoma exosomes might be mediators of tumorigenesis and able to reprogram bone marrow progenitor cells

toward a pro-vasculogenic phenotype [255]. Additionally it has been shown that microvesicles and exosomes are not only able to transport miRNAs but deliver these miRNAs to recipient cells and alter their gene expression pattern [248, 256]. Multiple myeloma bone-marrow mesenchymal stromal cells release exosomes that are transferred to multiple myeloma cells resulting in a modulation of their tumor growth *in vivo*. In addition, these multiple myeloma bone-marrow mesenchymal stromal cells (BM-MSC) derived exosomes had higher levels of oncogenic proteins, cytokines and adhesion molecules compared with exosomes from the cells of origin and promoted the growth of multiple myeloma cells whereas exosomes from normal BM-MSC inhibited the growth of myeloma cells [257].

12 Technical Issues and Study Design

12.1 Choice of Material

Either plasma or serum is frequently the first choice for the analysis of cfNAs. So far, it is not known if one of them is preferable over the other, but there are a number of reports comparing plasma vs. serum as starting material (see chapter "Pre-analytical Requirements for Analyzing Nucleic Acids from Blood"). These include the observation that the amount of cfDNA in serum is higher (3–20-fold) as compared with plasma from the same individual. This seems to be due to the release of nucleic acids from white blood cells during clotting [258]. In one report it was demonstrated that plasma is better than serum for the detection of *EGFR* mutations (95 % in plasma vs 72 % in serum) and this was explained by a higher amount of DNA from healthy cells in serum due to clotting [259]. The comparison of miRNA profiles between plasma and serum samples from the same donors showed that (i) the total number of detectable miRNA species obtained with Taqman cards and Exiqon panels varied, (ii) the two platforms differed very much in the amount of miRNA detected, (iii) there was no correlation between the number of detected miRNAs and the RNA concentration and (iv) the higher concentration of RNA/miRNA in serum than plasma might be the result of the coagulation process and the release of cellular RNA/miRNA into the environment [131]. A degree of correlation between the levels of circulating microRNA biomarkers in plasma and serum using qRT-PCR was demonstrated by Kroh et al. [260].

Nevertheless it is worth thinking carefully about the starting material. When cfmiRNAs from serum and cerebrospinal fluid from patients with neurological diseases were profiled by NGS, there were (as one could expect) substantial differences found [261]. A similar observation was made when miRNA signatures from plasma and bronchial lavage fluid from the same patients were compared [262]. For the detection of genetic alterations associated with tumors of the

gastro-intestinal tract gastric juice, pancreatic juice and/or feces/lavage fluid obtained during colonoscopy might be better suited than either plasma or serum. Other sources might be nipple aspirate for the detection of malignant alterations of the breast, urine for the urogenital system (kidney, bladder), either saliva or sputum for head and neck and lung cancer, vitreus fluid for ocular diseases and pleural fluid or bronchial lavage for lung diseases. In a direct comparison, a panel of four methylated genes was quantified from prostate cancer patients when all of them showed a greater sensitivity on analysis from urine as compared to plasma [263].

12.2 Pre-analytic Variables

A variety of different factors such as blood sampling and processing, the use or non-use of anticoagulants, the time interval between blood sampling and processing, the storage temperature before and after processing, freezing of plasma samples vs. isolated DNA have an impact on the amount and quality of cfNAs that can be isolated [7, 86, 264, 265]. Nevertheless, there is so far no agreement on a standard operational procedure for any of the steps mentioned (discussed in chapter "Pre-analytical Requirements for Analyzing Nucleic Acids from Blood").

The influence of storage of blood samples on the amount of cell-free DNA was examined by Lee et al who demonstrated that the concentration of extracellular DNA increased in serum samples during storage at 4°C over a period of up to 5 days, whereas the amount of cell-free DNA in citrate plasma increased only to a small degree [258] and similar results were made by others as well [266]. Recently cell-free DNA blood collection tubes (BCT) were introduced containing a stabilizing substance that prevents the extracellular DNA from being diluted by cellular DNA [267]. The use of these tubes might be a good choice when samples have to be shipped for processing to another laboratory but when the blood samples are processed in a reasonable time (several hours to 1 day) EDTA collection tubes are not inferior [268]. In a head-to-head analysis the BCT tubes and blood drawing tubes containing CPDA (citrate, phosphate, dextrose, adenine) were stored for up to 48 hrs at room temperature before plasma was isolated. While in cell-free plasma DNA from CPDA tubes the methylated SEPT9 gene was consistently detected the BCT system failed in 35% of the samples [269].

Importantly, Madisen et al. [270] demonstrated that isolated DNA can be stored either in a dried form or in solution at −20 °C for several years without an increase in degradation. In contrast, when circulating nucleosomes in serum were stored at −70 °C and the DNA concentration was measured shortly after sampling and at a later time (≥5 years), there was found to be a significant drop in the amount of nucleosomes detectable [271].

The generation of plasma or other body fluids that are cell-free is of the utmost importance since an incomplete cell removal leads to misleading and/or nonreproducible results [272]. It is known that a one step low speed centrifugation of EDTA blood for 10 min at 1,700 g produces a "cloudy supernatant" in which

cfmiRNAs are heavily "contaminated" with cellular miRNAs, mostly of hematopoietic origin. Only a second spin for 10 min at 2,000× g produced a plasma fraction containing mainly circulating cfmiRNA species [273, 274]. The latter group also demonstrated, in a thorough analysis, that the processing of plasma samples had a big influence on cfmiRNA levels [273]. Using a different approach, it was demonstrated that a "contamination" of plasma DNA with as low as 5 % of normal (WBC) DNA prevents LOH from being detected in prostate cancer patients [275]. Therefore, either high-speed centrifugation or filtration of the plasma is highly recommended [276] and it is clear that, once started, one has to stay with one of the two methods. That the use of haemolytic blood samples can lead to a change in the plasma cfmiRNA expression pattern was illustrated for miR-16 and miR-451, an important observation since both genes are frequently used as reference genes [277].

12.3 Technical/Biological Pitfalls

Blood Preparations and RNAs

Some reports concern the use of *PAX* gene blood collection tubes for RNA stabilization [278, 279]. In these experiments, the resulting RNA is mostly of cellular origin as the stabilization reagent in these tubes lyses all blood cells and, therefore, this system is not suited for the analysis of extracellular RNAs. Pritchard et al. [280] analyzed the expression of 79 cfmiRNAs that were reported to be good tumor biomarkers for a variety of different solid tumors. Of these, 58 % (46/79) were shown to be highly expressed in blood cells and, therefore, the number of blood cell counts as well as hemolysis can alter the amount of some of these plasma cfmiRNA by up to 50-fold. A similar observation was published by Kirschner et al. [281] who demonstrated that some of the cfmiRNA that had been proposed as tumor markers e.g. miR-21, miR-106a, miR-92a, miR-17 and miR-16, are increased in hemolytic plasma samples. The storage of sera at 4 °C for up to 4 days, followed by the isolation of exosomes by EpCAM antibodies bound to magnetic beads, did not change the quantity of exosome-associated miR-21, miR-200b and miR-205 [139] This group also demonstrated that all analyzed miRNAs (i.e. miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, miR-214) were elevated in exosomes from stage I-III ovarian cancer patients compared to women with benign ovarian disease [139].

The cfNAs found in body fluids are always a mix the origin of which is not easy to trace. Chen and coworkers [90] observed a considerable overlap of cfmiRNA profiles in serum as compared to blood cells in healthy subjects, leaving the possibility that in papers in which healthy subjects are used as controls, not cfNAs but rather a blood cell derived signature is analyzed. The cfmiRNA composition in different body fluids (amniotic fluid, breast milk, bronchial lavage, cerebrospinal fluid, colostrum, peritoneal fluid, plasma, pleural fluid, saliva,

seminal fluid, tears and urine) from healthy subjects is distinct for each fluid, a reflection of the surrounding tissues confirming this observation [9]. Another point for consideration is the observation that in most examinations there was no complete concordance between the alterations found between tumor tissue and body fluids. We can only speculate on the different reasons for this observation such as access of the tumor cells to the vascular system, differences of the tumor subpopulations for shedding nucleic acids into the environment, size of the tumor and so forth. In a recently published study it was shown that the concordance for a methylation of the protocadherin 10 promoter region in tissue and plasma was higher in early stage colorectal cancer patients compared to patients with a later stage of the disease [282]. A similar lack of concordance for the detection of a methylated sequence (CpG island of the *TFPI2* gene) in tumor cell DNA and serum of 20, paired cases of metastatic melanoma patients was recently reported [214]. Interestingly, in 9/20 cases, the mean % *TFPI2* methylation was higher in serum than in the tumor. Unfortunately, the authors did not supply the clinical details of these patients e.g. TNM, Breslow thickness, so leaving the question open as to whether or not there is any correlation between higher serum methylation and clinical data. When the status of the *EGFR* gene was analyzed in tumor tissue and matched plasma from NSCLC patients using the sensitive mutant-enriched PCR method, the concordance was only 71 % (79/111) [283].

In three recently published papers, the authors used different platforms, i.e. NGS, qPCR and microarrays for the quantification of cfmiRNA and concluded that each of the methods had its pros and cons and that it is necessary to critically evaluate the results obtained [284–287].

Isolation Methods

cfmiRNAs are very stable and can be isolated from a variety of different body fluids (see above). Nevertheless the quantity is very low and, therefore, it is crucial to rely on very efficient isolation methods. In an effort to maximize the cfmiRNA yield obtained from human cerebrospinal fluid, ten commercially available RNA isolation kits were compared and the isolated cfmiRNAs analyzed by NGS [288]. The authors not only demonstrated that the yield of total RNA obtained from 200 µL plasma varied by a factor of 15 between the different methods, but that a simple re-extraction (even when kits were used) almost doubled the yield of miRNA.

Since the amount of cfDNA in body fluids is also rather small (not to mention the fact that it is highly degraded) it is important to maximize the recovery rate for these nucleic acids as well [289]. When the amount of plasma as starting material for the isolation and quantification of methylated cfDNA was increased from 500 µL to 2 mL and at the same time the bisulfite treatment was optimized, this resulted not only in a higher DNA yield (1.5–5-fold) but also in a 25-fold increase in sensitivity for the detection of methylated sequences [290]. An increase in the amount of starting material can have a negative effect on the quantification of cfmiRNA isolated from plasma or serum leading to the conclusion that a titration of the

starting material is necessary and yields a higher quantification accuracy [291]. In most papers, authors use a method for cfNA isolation from body fluids involving strong chaotropic salts such as guanidium or phenol/chloroform purification to remove proteins. In an approach described by Ho et al. [292], 100 µL plasma were boiled for 10 min at 100 °C, the samples spun at 10 min at 13,000 g and 1 µL supernatant used for miRNA detection [292]. They found an increased cfmiR-210 level in cancer patients as compared to age-matched non-cancer controls but did not report the Ct values. According to our experience, such a crude purification method is likely to yield unreliable results.

When plasma DNA was isolated from breast cancer patients using the QIamp DNA mini kit (Qiagen) and the Wizard Plus SV columns (Promega) to obtain high molecular weight (>1,000 bp) and low molecular weight DNA (<1,000 bp), respectively, 38 % of the patients showed LOH in all eight markers when short DNA was analyzed as compared to 28 % in the fraction of long DNA [200].

In a study reported by Kumar et al. [293], it was demonstrated that the results obtained in microarray experiments using saliva derived cfmRNA were not influenced by treatment of the samples with either RNase or DNase so leading to the conclusion that the generated RT-PCR signal is not RNA-based but DNA. There are some reports on the use of cfNAs from saliva for the detection of different types of cancer (e.g. lung, head and neck) and the cautionary note expressed by Kumar et al. [293] has to be taken very seriously.

So far there are no published data on the influence improved isolation protocols might have on gene expression patterns in either miRNA or mRNA analysis, in the sensitivity of mutation detection systems or the quantification of methylated sequences. Therefore, standardized isolation systems are clearly warranted.

Real-Time Quantification

Apart from apoptosis and necrosis, i.e. cell death in its different forms leading to the liberation of cellular DNA and RNA, there is also the possibility of "extrusion" by living cells. These different mechanisms could lead to DNA fragments of different sizes. cfNAs in general are highly degraded (no matter by which mechanism they were liberated) and therefore qPCR methods should amplify short targets, i.e. ≤100 bp, to work reliably [294]. This is a very important point since it is known that a reduction in amplicon size results in an increase amount of cfDNA [295, 296]. Inhibition of PCR by various inhibitors is also an important point for consideration. Recently, it was demonstrated that dPCR is less susceptible to inhibitory substances such as SDS, heparin or EDTA than qPCR ([297]; chapter "Circulating DNA and miRNA Isolation").

The lack of rigorously tested reference genes used in gene expression analysis, resulting in the difficulty to compare findings from different laboratories, is still one of the major problems. In an attempt to solve this problem, cfmiRNA profiling (TLDA arrays and Solexa sequencing) was performed from pools of sera from patients with lung, breast, cervical, gastric cancers and two control groups (pooled

sera from male/female healthy subjects). When two miRNAs (miR-191 and miR-484) were chosen as endogenous controls, the authors discovered a panel of four miRNAs able to differentiate between breast cancer patients and controls [157]. Whether or not the authors' conclusion that these two miRNAs might be useful as endogenous controls for serum cfmiRNA detection in most (!) cancers will prove to be correct has to be demonstrated in future experiments.

Choice of Appropriate Controls

The majority of papers in which cfNAs were used for the characterization of tumor-associated alterations, compare tumor patients with healthy subjects matched in age, sex, life-style [140, 261, 274, 298–300]. In most cases this is an inappropriate control group since factors like inflammatory processes and alterations associated with premalignant conditions are not taken into account [246]. In one of the very few studies using a proper control group, the levels of circulating cfmiRNAs were analyzed in breast cancer patients and patients with a benign breast disease [301]. With a panel of 4 cfmiRNAs only miR-214 could discriminate between the two patient populations whereas the others failed. Interestingly, this is the only cfmiRNA concentration which decreased after tumor removal by surgery whereas the other three cfmiRNA levels did not change. In another study, miR-155 and miR-196a were used for the examination of chronic pancreatitis (CP) and pancreatic ductal carcinoma patients (PDAC) demonstrating that these two miRNAs discriminated between healthy controls and both patient groups, but were not able to differentiate between CP and PDAC [116]. A proper control group of patients with cervical intraepithelial neoplasia was used for the analysis of patients with uterine cervical cancer and the over-expression of circulating Bmi-1 cfmRNA described as a useful marker for discriminating the two groups [302]. The analysis of plasma cfmiRNA from patients with colorectal cancer, advanced adenomas and healthy controls demonstrated that a panel of six cfmiRNAs was significantly up-regulated in CRC patients compared to the control population but that these markers did not discriminate CRC patients from adenoma patients [303]. This paper demonstrates the problems being faced in the search for biomarkers useful for diagnostic purposes.

The data published by Wang et al. [304] must be seen critically for several reasons. The authors used heparin stabilized plasma which is known to be a very powerful polymerase inhibitor, the plasma being obtained by spinning the tube only once at rather low speed (leading to a sample which is very unlikely to be cell-free, see above), the pancreatic cancer patients were compared to healthy subjects and the panel of four cfmiRNAs discriminating the two groups was based mainly on a few cases in the patients cohort demonstrating exceptionally high quantities of plasma cfmiRNA [304].

13 Early Tumor Detection

The ultimate goal of cancer research is the detection of a tumor at an early stage in which a curative treatment is not only possible, but successful in the majority of patients (we are not even mentioning screening of an asymptomatic population which increases the demands even more). In one of the earliest reports on the development of lung cancer, Saccomanno et al. [305] described the possibility of detecting lung cancer by dysplasia and carcinoma *in situ* approximately 4–5 years before the time of diagnosis. Since then, substantial efforts have been made to establish a marker panel able to, not only detect cancer at a very early stage, but to discriminate premalignant stages from early cancers. Unfortunately, none of the immunocytochemistry markers tested so far has been able to detect premalignant changes in peripheral blood. Also, the cancer model depicting the increased amount of genetic alterations over time and the association with morphological changes of the lung clearly illustrates the stepwise nature of tumor pathogenesis, though so far has not been demonstrated as clinically helpful [306]. In an attempt to combine imaging diagnostics with molecular genetic analysis for an early detection of lung cancer, subjects enrolled in a screening trial were additionally analyzed for the presence of microsatellite alterations, *K-RAS* and *p53* mutations and the amount of cfDNA in plasma and sputum. In this study, a statistically significant difference in the frequency of allelic imbalances (sputum + plasma) in subjects with a negative computerised tomography scan (17 alterations) and positive computerised tomography scan (people with non-calcified nodules, 44 alterations) was observed [307].

When Diehl et al. [308] looked for mutations in the plasma of colorectal cancer patients, they wrote that "it is unlikely that circulating mutant DNA could be used to detect premalignant tumors, based on the fact that we were unable to detect such DNA even in very large adenomas". A similar statement was made by Berger and Ahlquist [309]: "It is biologically implausible that DNA markers will be present in plasma of patients with precancers irrespective of the assay method employed". Whether or not these statements are true has to be seen in the future. In the case of colorectal cancer patients it could be that a more sensitive stool-based assay might yield better results. When the frequencies of *SFRP2* methylation in tumor tissue, stool and serum DNA were compared, the sensitivity of the assay was 88 % and 84 % for tissue and stool DNA, but dropped to 67 % for serum DNA. On the other hand, there was an increase in the specificity (number of benign adenomas without *SFRP2* methylation) from 35 % (tissue) and 54 % (stool DNA) to 94 % in serum cfDNA [310]. As for other tumor locations, it may be useful to look for genetic alterations in body fluids closer to the scene of the action e.g. bronchial lavage in lung cancer, sputum in head and neck cancer, nipple fluid in breast cancer. Their observation that the amount of tumor cfDNA released into circulation is independent of tumour size is confirmed by results from Garcia-Olmo et al. [311], but contrasts with reports in which a correlation between tumour size and the amount of cfDNA was demonstrated [294, 312] leaving this question open to debate.

14 Literature Search

For the majority of researchers, the most obvious and straightforward approach to find the relevant papers is a search in NCBI's Pubmed (http://www.ncbi.nlm.nih.gov/pubmed/?otool=idehallib). Recently, quite a few very useful key words such as cell free, cell free DNA, circulating cell free DNA, cell free DNA cancer, cell free circulating DNA, cell free RNA, cell free DNA plasma, circulating tumor nDNA, circulating miRNA, circulating nucleic, circulating free DNA and so forth, have been included. Unfortunately, these terms are not yet included in the MeSH database that results in many but frequently not relevant "hits" when searching for papers dealing with these topics.

Possible supplementary sources and alternatives to Pubmed are the web of knowledge (http://apps.webofknowledge.com/UA_GeneralSearch_input.do?product=UA&search_mode=GeneralSearch&SID=R2bUyJUav2wOccVFZ8i&preferencesSaved=), quertle (http://www.quertle.info/) or recently (http://www.recentlyapp.com/).

15 Summary

In the last few years, cfNAs have become a very hot topic and it is almost impossible to keep pace with the number of new papers published basically every day. Nevertheless there are quite a few fundamental questions and problems for which we do not have an answer.

1. Our basic knowledge on cfNAs is far from being complete. We still do not know all of the mechanisms leading to the liberation of nucleic acids into the extracellular environment. There is some evidence that these cfNAs are not just artifacts but may serve a biological function (see chapter "The Biology of CNAPS"). These and other problems have to be tackled before we can move on to establish new clinically relevant and useful biomarkers [313].
2. There are technical and methodological issues that have to be solved. Once obtained, cfNAs are highly degraded and generally in a poor shape, no matter what is done to protect them from further degradation. Some species e.g. miRNA, which are tiny and very resistant to harsh conditions, can be isolated as intact molecules whereas mRNA and DNA are more susceptible to nuclease attacks and can only be isolated as highly degraded fragments. There is some evidence that the size of nucleic acid fragments from normal healthy cells is different from the ones released from tumor cells, i.e. the latter one seems to be smaller. This should be kept in mind when designing methods and assays (such as qPCR, dPCR) for the detection of tumor-associated alterations.
3. So far there is no consensus on a "gold standard" for the isolation of cfNAs (chapter "Circulating DNA and miRNA Isolation"). There are many bead-based or column-based manual and robot-based methods available (homebrew as well

as commercially produced kits) but none of them is seen as "the winner". Only a streamlined, robust procedure which starts with blood drawing (or obtaining other body fluids), preparation of cell-free body fluids, to storage conditions at low temperatures, isolation of nucleic acids up to measurement of different parameters which relies upon rigid SOPs and the application of certified ingredients for all steps will ultimately lead to success.

4. The choice of an appropriate control population for the search of new biomarkers is of utmost importance. In many papers healthy individuals have been used as controls making the results obtained at least questionable. It is likely that these biomarkers will be unable to differentiate between tumor patients and patients with premalignant precursor lesions or patients with benign diseases affecting the target organ.
5. It might be useful to think about future approaches in which the genetic analysis of cfNAs in combination with the isolation and characterization of CTCs is combined. This combination may lead to deeper insights into tumor biology and to improved strategies for the care of cancer patients [314].
6. The use of plasma/serum or any other body fluid for the isolation and detection of tumor-associated genetic alterations has many advantages (as compared to the difficulty of obtaining (repeat) biopsies) in that it is only minimally invasive, opens the possibility for kinetic studies, but has also disadvantages (e.g. the "dilution" of tumor-associated cfNAs with genetic material from different cell types of hematopoietic and non-hematopoietic origin).

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Other Body Fluids as Non-invasive Sources of Cell-Free DNA/RNA

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Abstract In addition to plasma and serum as sources of nucleic acids circulating in the whole body, amniotic fluid, saliva, urine, pleural effusion, bronchial lavage, bronchial aspirates, breast milk, colostrums, tears, seminal fluid, peritoneal fluid, pleural effusion and stools are all available for minimally invasive analysis of nucleic acids. This chapter introduces the possibilities of using nucleic acids from amniotic fluid, saliva, urine, cerebrospinal fluid and bronchial lavage/aspirates in attempts to produce reliable early markers for diagnosis, prognosis and treatment monitoring using minimally invasive methodology. Moreover, the data from amniotic fluid can be used also to further the understanding of normal and abnormal fetal development *in utero*. In addition, the data from saliva can be employed for monitoring the progress of premature born infants.

Keywords cffDNA/cffmRNA/cffmicroRNA • Amniotic fluid • Saliva • Urine • Cerebrospinal fluid • Bronchial lavage • Fetal sex • Human fetal development • Aneuploidy • Preterm birth • Cancer • Predictive and preventive medicine

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1 Introduction

Although the first circulating DNA and RNA were found to be present in peripheral blood plasma and serum, and hence the development of studies to find early diagnostic and prognostic disease markers, other body fluids have also been analyzed for the possibility of new approaches to diagnosis, prognosis and treatment monitoring. These include saliva, urine, amniotic fluid and bronchial lavage/aspirates. Breast milk, tears, seminal fluid, peritoneal fluid and colostrums have all been mentioned as possible sources [1] together with fresh stools [2, 3]. Some examples will be given concerning amniotic fluid, saliva, urine, cerebrospinal fluid and bronchial lavage/aspirates in order to show how these alternative sources of DNA/RNA have been exploited and for what purposes. Emphasis has been placed upon the sources most likely to have broader applications in diagnosis, prognosis and treatment monitoring.

2 Amniotic Fluid (AF)

AF is a complex fluid that surrounds the fetus throughout gestation and is involved in diverse functions, including physical protection and organ development [4]. The composition, volume and circulation of AF alters during pregnancy with the maturation of fetal skin, progressive increases in urine production, swallowing and respiratory movements. AF can be obtained during pregnancy via amniocentesis where an ultrasound-guided, trans-abdominal needle puncture of the uterus allows aspiration of AF from around live fetuses. The volume of AF withdrawn depends upon the gestational age and the indication for testing but typically 10–30 mL of AF can be removed safely from the amniotic sac from 15 weeks gestational age. Amniocentesis is most commonly offered to women at increased risk for bearing a child with a chromosome abnormality such as trisomy 21, but can also be performed for the diagnosis of single gene disorders and for fetal Rhesus. After amniocytes are removed for diagnostic testing, the remaining 10–15 mL of AF supernatant, which contains cffNAs, can be retained for other purposes. Although the obtention of amniotic fluid is an invasive procedure with a small risk of causing pregnancy loss, this approach does permit the study of a number of aspects of both normal and abnormal development of the fetus, including the identification of fetal defects for which clinical management could commence *in utero*. The following examples can give an idea of the range of problems that can be tackled using AF cffNAs.

2.1 *cffDNA in AF*

Most studies of cffDNA have been made on plasma and serum. However, with the availability of residual AF supernatant after clinical testing, Bianchi et al. [5] were able to demonstrate that its cffDNA content was present at concentrations much higher (100–200-fold mL^{-1}) than that observed in plasma. Subsequently, Lapaire et al. [6] developed methodological improvements in cffDNA extraction from AF that increased the median yield of GAPDH cffDNA from 10 mL of AF from 246 genomic equivalents (GE) mL^{-1} to 1,700 GE mL^{-1}.

Methylation studies have demonstrated that DNA in AF is comprised essentially of fetal, rather than placental, DNA. Using the *RASSFIA* gene, which is hypermethylated in the placenta but hypomethylated in the fetal tissues and the maternal blood cells, several investigators have shown that the placenta does not appear to be the primary source of cffDNA in AF supernatant [7, 8]. Thus, AF appears to contain a separate pool of cffDNA to that of the maternal plasma [9].

Aneuploidy

The first study of potential clinical applications of AF cffDNA involved comparative genomic hybridization (CGH) microarray analysis. Using CGH array analysis of AF cffDNA from fetuses with congenital abnormalities, it was possible to detect fetal sex and whole chromosome gains or losses, (e.g. trisomy 21 and monosomy X). In this work, Miura et al. [10] were able to correctly identify 12 fetuses with chromosomal losses or gains using AF cffDNA, rather than the conventional metaphase karyotype from intact amniocytes. Only one false-negative result occurred with a fetus having a balanced translocation, 45, XY, der(14;21)(q10; q10), which is a recognized limitation of CGH technology.

A further study of the molecular karyotype approach using AF cffDNA and array CGH detected whole-chromosome differences between AF cffDNA samples for chromosomes X, Y and 21 in both female and male, and euploid and aneuploid fetuses [11]. The microarray approach appeared to provide a higher resolution and sensitivity as well as a more specific localization (within 100–200 kb) of abnormalities in the fetal genome than those found in the standard metaphase karyotype using cultured amniocytes (generally limited to pattern recognition of ~450 Giemsa-stained bands). Of 17 AF cffDNA samples there were four euploid females, nine euploid males, two male trisomy 21, one female trisomy 21 and one female single X (Turner syndrome).

Fetal Sex

The method used above for the detection of fetal chromosomal abnormalities can also be used for the determination of fetal sex [11].

2.2 *cffRNA in AF*

Although most studies of cffRNA have been made on plasma and serum, cffRNA has also been shown to be present in AF [12, 13]. AF contains a heterogeneous population of suspended cells that originate from all three germ layers of the embryo. These range from unspecified progenitors and pluripotent stem cells to mature differentiated cells, including those of the renal, heart, lung, liver and haematopoietic cell lineages [14]. Subcellular fragments from different organs are also released into the AF, including kidney-derived exosomes containing cffmRNA [15, 16].

These features of AF indicate that it can provide gene expression information from multiple cell types in a manner analogous to plasma. At least a proportion of AF cffRNA is contained within secreted micro-particles such as exosomes [15]. Fetal kidney-derived exosomes have been demonstrated in AF and their RNA contents have been used to perform fetal sexing, suggesting future applications in clinical diagnostics [16]. Other studies have shown real differences according to gestation for several genes expressed in lung, intestine and skin epithelial cells, all of which are in contact with the AF. Data obtained at different gestational ages have documented the appearance and concentration of a variety of genes for surfactants, mucins and keratin that mirror known developmental physiology [17].

To date, published global gene expression studies of AF cffRNA have been almost exclusively performed using whole genome microarrays. The application of newer techniques such as RNA sequencing are currently being explored for their potential to provide information on novel fetal RNA transcripts and transcript isoforms in AF [18].

Human Fetal Development

Due to ethical considerations, gene expression data from live human pregnancies is extremely limited. AF cffRNA thus represents a potential source of biological information about live human fetuses that can be obtained without the risks associated with fetal blood or tissue sampling. Two studies in particular have investigated the AF RNA from normal pregnancies ranging from mid-gestation to delivery at term [19, 20]. The first systematic description of the AF transcriptome was an in-silico study by Hui et al. [19]. These investigators identified 476 well-annotated genes that were consistently present in the AF from 12 euploid mid-trimester AF samples. The three most common types of molecules represented by transcripts in the AF core transcriptome were enzymes, ribosomal proteins and transcription regulators. Functional analysis identified six statistically significant physiological systems enriched in the AF transcriptome (skeletal and muscular system development and function, tissue development, hematological system development and function, nervous system development and function, embryonic

development and organismal development). A key canonical pathway identified was that of the mammalian target of rapamycin (mTOR) signalling. Using a gene expression atlas, AF core transcriptome was found to contain 23 highly organ-specific transcripts, six of which were highly expressed in the fetal brain. These findings indicated that developmental information from multiple organs including fetal brain could be obtained from AF.

There are relatively few AF studies aiming to characterize normal third trimester fetal physiology using high dimensional biology techniques such as functional genomics or proteomics. Existing studies fail to provide a detailed biological interpretation of the proteins differentially expressed in the third compared with the second trimester [21]. However, Hui et al. [20] made a further study on global gene expression analysis comparing AF cffRNA from eight normal-term pregnancies with eight second-trimester controls. Using bioinformatics tools for functional analysis and tissue expression profiles, they sought differences between the relative representation of specific organs in term and second-trimester AF cffRNA. The methodologies for the biological interpretation of the gene data are given in Table 1.

The average microarray hybridization rate for all samples was 41 % (range 33.0–50.1 %). In total, there were 2,871 genes that were significantly differentially regulated in term compared with mid-trimester AF. Of these genes, 1,307 were up-regulated and 1,564 were down-regulated.

Overall, the results demonstrated changes in tissue expression profiles according to gestation and up-regulation of fetal maturation processes at term. In term AF supernatant, tissue expression analysis showed enrichment of salivary gland, tracheal and renal transcripts, as compared to brain and embryonic neural cells in mid-trimester AF. Furthermore, in the term group, all five pulmonary surfactant protein genes (*SFTPA1*, *SFTPA2*, *SFTPB*, *SFTPC*, *SFTPD*) were significantly up regulated compared with mid-trimester, reflecting known patterns of increasing pulmonary maturation.

A comparison between term and mid-trimester AF showed 609 well-annotated genes that were up-regulated by at least fourfold in the term group. Core pathways analysis of these genes indicated enhancement of physiological systems involved in newborn functions e.g. immune defense, eating and respiration. As might be predicted for term fetuses, statistically significant up-regulation of molecular and cellular functions was observed for 41 genes for carbohydrate metabolism, 139 genes for cellular movement and 93 genes for lipid metabolism. In the mid-trimester there were six upstream regulators that were statistically significantly predicted to be activated including genes related to thyroid function, oxidative stress and liver development.

One of the limitations of these data is the sample size. Nevertheless, even with these small numbers, the authors claim to have demonstrated significant differential gene expression in 7.44 % of the total probe sets at a very stringent false discovery rate (Benjamini-Hochberg $P < 01$).

Table 1 Methodologies employed for the biological interpretation of whole transcriptome data from mid-trimester and term samples after identification of significantly regulated genes (After Hui et al. [20])

Methodology	Identification
BioGPS mapping of top 10 upregulated genes	Inferred tissue sources and gene functions
DAVID tissue expression profile analysis	Identified tissues that highly express the same genes
IPA analysis of genes up-regulated at term	Enriched physiological systems and cellular functions in term AF cffRNA
IPA upstream regulator analysis	Up-regulated transcription factors with significant downstream effects

Aneuploidies

Functional analysis of AF cffmRNA from fetuses with trisomies 21 or 18 using global gene expression microarrays has provided new insights into the pathophysiology of aneuploidy. In the first of these reports, Slonim et al. [13] performed a paired analysis of seven fetuses with trisomy 21 matched for gestational age and fetal sex with euploid controls. There were 311 differentially expressed genes of which only five were physically located on chromosome 21. Pathways analyses showed that trisomy 21 fetuses had an altered response to oxidative stress as well as disruptions of ion transport, G protein signalling, immune and stress response, circulatory system function and sensory perception [13].

In a subsequent work using AF from five second trimester fetuses with trisomy 18, 251 differentially-expressed genes were identified of which only seven genes were physically located on chromosome 18. Functional analysis of the trisomy 18 fetuses indicated abnormalities in ion transport, immunity, DNA repair and G protein signaling and dysregulation of adrenal development-associated pathways [22].

One initial conclusion to draw from these results is that each fetal aneuploidy has multiple dysregulated genes that are not physically located on the trisomic chromosome, suggesting that the abnormal phenotype is not solely due to a gene dosage effect but rather a global downstream effect on gene expression [23].

Another important result of these transcriptomic studies of AF cffRNA is the generation of hypotheses for future studies of potential treatments for aneuploidy. The finding that fetuses with Down syndrome experience oxidative stress in the second trimester by Slonim et al. [13] has led to further studies assessing whether or not treating oxidative stress *in utero* could improve brain development in a mouse model of Down syndrome [24]. In this way, studies utilizing AF cffRNA have the potential to translate into new candidate therapies and future improvements in clinical care.

The novel finding by Hui et al. [19] that nervous system development and function is enriched in the euploid AF transcriptome led to a more detailed analysis of fetal neurodevelopment in aneuploid pregnancies using AF cffRNA [25].

Gene expression data from mid-trimester aneuploid AF supernatants were reanalyzed [13, 22] focussing on the transcripts associated with nervous system development. This permitted the development of the trisomy 18 and 21 transcriptomes consisting of universally detected transcripts from mid-trimester fetuses with the respective aneuploidies. Multiple bioinformatics resources were involved in the analysis, including DAVID, Ingenuity Pathway Analysis (IPA), and the BioGPS Gene Expression Atlas.

The trisomy 21 transcriptome was comprised of 1,184 individual genes that were consistently present in seven of seven samples. The trisomy 18 dataset comprised 746 genes present in all five samples. The euploid dataset contained 536 genes present in twelve of twelve AF samples. Nervous system gene expression was consistently enriched in all of these datasets. The trisomy 21 AF transcriptome included four genes physically present on chromosome 21 (*APP*, *SOD1*, *DYRK1A*, and *RCAN1*). The functional analysis of the transcriptomes of the aneuploid fetuses indicated that neurological disease highly enriched in both the trisomy 21 and 18 datasets (Table 2).

Differentially regulated genes that were specifically expressed by the nervous system were identified in the AF supernatants of trisomy 18 and 21 fetuses. In trisomy 18, *PTPRD*, *SOBP* and *NEUROD2* were down-regulated while *PLEKHA4*, *GPM6A* and *PRPH2* were up-regulated. In trisomy 21, the down-regulated genes included *SOX11* and *DAAM2* while *MEF2C* and *CELSR2* were up-regulated. Notably, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway for Alzheimer's disease was found to be significantly enriched in both the trisomy 21 AF transcriptome and an independent Down syndrome meta analysis based on a variety of tissues sources [26]. This is consistent with the known neuropathology of Down syndrome, where >25 % of Down syndrome individuals aged over 35 years show the signs and symptoms of Alzheimer's-type dementia. Thus, the data obtained from AF supernatant is consistent with findings from cellular RNA including brain tissue, confirming that the approach using AF supernatant can have a real biological relevance for studies of human development in live fetuses.

Twin-to-Twin Transfusion Syndrome (TTTS)

TTTS is a serious perinatal complication unique to monochorionic twins. Due to the presence of a shared single placenta, monochorionic twins always have some placental vascular anastomoses resulting in a shared blood circulation. In approximately 15 % of cases, however, the placental anastomoses are unbalanced leading to blood flowing disproportionately from one twin (known as the "donor") to the other (the "recipient"). The resulting hypovolemia in the donor twin leads to growth restriction, anaemia and oliguria, producing a clinical result of a small fetus with oligohydramnios. In contrast, the blood volume of the recipient twin increases, which can lead to fetal heart dysfunction, hydrops fetalis and polyhydramnios. TTTS is historically associated with a very high perinatal mortality rate, but

Table 2 Trisomy 18 and 21 significant diseases and disorders using IPA (After Hui et al. [25])

Category	No of genes Trisomy 18	No of genes Trisomy 21
Neurological disease	107	201
Dermatological diseases and conditions	45	114
Infectious disease	119	177
Reproductive system disease	–	96
Connective tissue disorders	5	6
Respiratory disease	–	6
Cardiovascular disease	–	4
Skeletal and muscular disorders	9	–

significant improvements in survival have been achieved with prenatal laser ablation of the placental anastomoses [27, 28].

TTTS is unusual in that there is no suitable animal model with which to study the pathophysiology *in vivo*. AF has been explored as a potential biofluid in which to obtain gene expression data on fetuses with active disease. Larrabee et al. [17] first compared gene expression in a small number of TTTS cases compared with pooled normal singleton controls. They identified significant up-regulation of an aquaporin water transport gene in the TTTS cases, indicating a possible role for this gene in the transport of water out of the fluid-overloaded recipient compartment.

In a subsequent, larger prospective study of TTTS, Hui et al. [20] provided the first transcriptome-wide data on the impact of TTTS on fetal development. AF samples were obtained from recipient fetuses at 17–22 weeks gestational age at the time of clinically-indicated laser therapy for TTTS. AF controls were obtained from normal singleton fetuses matched for gestational age and fetal sex. Total RNA was extracted from 15 to 30 mL of AF from TTTS cases and compared with 5 mL AF from singleton controls. Five micrograms of cDNA prepared from each sample were biotinylated, fragmented and hybridized to a whole human genome expression array (Affymetrix GeneChip Human Genome U133 Plus 2.0). Analysis of paired data (TTTS cases versus controls) showed differential expression for 801 genes. Neurological disease and cardiovascular system pathways were specifically enriched whilst 13 molecular and cellular pathways were dysregulated in the TTTS cases. The gene expression profiles of five recipient fetuses with ultrasound evidence of abnormal fetal blood flow were also compared with five TTTS cases at a less critical clinical stage. Cardiovascular genes and pathways associated with the presence of critically abnormal Doppler measurements in TTTS recipients were thus identified, providing new molecular evidence for the haemodynamic deterioration seen in severe disease. This approach confirmed that gene expression involving neurological and cardiovascular pathways are already altered in recipient fetuses prior to surgical treatment, so raising potential explanations for the origins of long-term complications seen in treated survivors and suggesting the utility of AF cffmRNA for the development of future fetal biomarkers for staging or prognosis in TTTS.

3 Saliva

Saliva, produced primarily by the parotid, submandibular and sublingual salivary glands, aids chewing and swallowing as well as the initial digestion of food. Many of the compounds found in blood are also present in saliva and appear to mirror a range of states of bodily health. Comparative analyses between plasma and saliva in both adult and neonatal subjects have shown a 27 % concordance between proteins found in saliva and plasma of adults [29]; and a 37 % concordance between gene transcripts found in neonatal saliva and blood [30].

3.1 Saliva cfRNA

Saliva provides a non-invasive alternative for obtaining samples for clinical analysis. Amongst the components of saliva are cfRNAs both cfmRNA and cfmiRNA as well as proteins. Historically, there has been some debate regarding the source of RNAs in salivary samples. Following gene expression microarray analyses, an initial study by Kumar et al. [31] concluded that saliva arose from genomic DNA, not RNA. This was in contrast to the studies of Li et al. [32], and others, demonstrating the presence of RNAs in cell-free saliva supernatant using RT-qPCR and microarray analysis. Park and colleagues [33] provided further proof for the presence of salivary cfRNA when the integrity of ß-actin cfmRNA found in cell-free saliva supernatant from the three major salivary glands and gingival crevice fluid was assessed. After passing the supernatant through filters of either 0.22 or 0.45 μm pore-size and incubating with Triton X-100 prior to RT-qPCR, the authors concluded that both full-length and partially degraded forms of mRNA were present. The authors went on to further demonstrate through RT-qPCR and microarray analyses the presence of RNA in both whole saliva and saliva supernatant taken from the three major salivary glands (the parotid, submandibular and sublingual glands), minor glands and gingival crevice fluid. They found over 6,000 gene sequences and identified what is now considered as the 183 normal 'salivary core transcriptome' in human salivary supernatant. More recently, Spielmann et al. [34] applied transcriptome profiling to RNA isolated from both whole and cell-free saliva from healthy individuals using MPS. This study not only confirmed the preservation of the structural integrity of the RNA but also demonstrated that ~20–25 % of the sequenced reads from cell-free saliva aligned to the human genome. Over 4,000 coding and non-coding genes appeared to be expressed in both whole and cell-free saliva. Thus, it is now believed that salivary cfRNAs are plentiful and can be readily analyzed for a broad range of physiological and pathological conditions.

Research on the characteristics of salivary cfRNAs is not limited to the adult population. Neonatal studies conducted by Deitz et al. [35] compared gene expression differences between whole saliva with cell-free salivary supernatant through

microarray and RT-qPCR analysis. Total cfRNA was successfully extracted from all neonatal salivary supernatant samples, even from volumes as low as 10 μl (range 10–100 μl). Paired whole saliva and cell-free salivary supernatant samples showed a 92.5 % gene expression concordance following microarray analysis. Interestingly, 16 unique biological systems were identified in the cell-free supernatant layer but were absent from whole saliva. These data indicate that the salivary supernatant more readily detects rare gene transcripts on the microarray platform once cellular material is removed.

Since these original studies classifying and describing salivary cfRNAs, many reports have been published linking distinct salivary transcriptomic profiles to disease states. Diagnostic platforms have been described for adult patients with oral cancer [32, 36], breast cancer [37], Sjögren's disease [38], pancreatic cancer [37], melanoma [39], lung cancer [39, 40] acute myocardial infarction [41], diabetes [42] and ovarian cancer [43]. In addition, in a study by Maron and colleagues [44] it was found that as little 50 μL of whole saliva provided diverse developmental information that could be monitored in real-time in the developing premature neonate. As technology continues to advance, there is great diagnostic potential to not only identify gene expression differences in diseases and development, but to further our understanding of transcriptional regulation driving these processes.

Salivary proteomic studies are equally robust and are contributing significantly to our understanding of human health and disease. Over the past decade, multiple efforts have been made to describe and characterize the human salivary proteome. To date, more than 3,000 different protein species have been identified [45] High-throughput screening tools and advances in mass-spectrometry have largely contributed to the success of this work. Combined with ongoing research on cfNAs, the field of salivary diagnostics is poised to improve non-invasive diagnostics for a breadth of disease states across the human lifespan.

Premature-Born Infants

Preterm birth is considered as birth before 37 completed weeks of gestation (WHO guidelines), its rate having risen dramatically during the past 20 years e.g. the preterm birth rate in the USA was 12.2 % in 2009 [46] being even higher among African Americans, 17.5 % in 2009. Other developed countries have had similar rising preterm birth rates over the past decade [47–49]. A number of possible, though not certain, explanations have been cited including the increases in multiple births, older maternal age, elective caesarean sections before 37 weeks of gestation and the use of assisted reproductive technologies such as *in vitro* fertilization. However, the rise in preterm birth can only be partially attributed to these factors [50] and the high level of such preterm births results in very high annual health care costs.

The International Preterm Birth Genome Project plans to publish a large genome wide association study (GWAS) in early preterm birth. The GWAS should be able to identify common genetic variants influencing health and disease including those

that are associated with preterm birth [51]. Clearly, such preterm infants need to be handled with extreme care and the development of a method to identify biomarkers for the detection of their disorders at a very early stage would permit the initiation of treatment. To this end, Maron et al. [44] developed a method for the isolation and identification of cfRNAs from the saliva of premature infants, cfDNA not being present. They used saliva cfRNA amplification together with hybridization onto whole genomic microarrays and bioinformatic analyses. The big advantage of such an approach concerns the screening of premature infants employing only a small volume of saliva (10–200 μL) taken by a minimally invasive approach. Since the saliva samples can be taken at successive times from any one infant, this will allow not only possible treatment to be instigated but also have its effect monitored over a time period. Using this approach, Maron et al. [44] were able to identify 9,286 gene transcripts showing statistically significant gene expression changes across individuals over time. Thirty seven point nine percentage of such genes were down-regulated and 62.1 % genes up-regulated with gene expression changes being closely linked to developmental pathways. As might be predicted, the down-regulated expression was related to embryonic development (e.g. connective tissue and hematological system development and function). Interestingly, the significantly up-regulated genes involved those linked to behaviour and the development of the nervous system, tissue, organ and digestive system.

Cancer

A major study of the use of saliva cfRNA in the early detection of cancers has emanated from Wong's group. Working with both whole saliva and the supernatant, they were able to identify a number of mRNAs and miRNAs associated with various cancers that could possibly be exploited as early markers. RT-qPCR and microarray were used to analyze cfRNA isolated from saliva from both healthy and oral squamous cell carcinoma (OSCC) patients. Some 1,679 genes exhibited significantly different expression levels with seven cancer-related cfmRNA biomarkers showing, minimally, a 3.5-fold increase in the OSCC saliva namely, cfRNA transcripts of IL8, IL1B, DUSP1, HA3, OAZ1, S100P, and SAT. Various combinations of these biomarkers gave a sensitivity of 91 % and a specificity of 91 % in separating the OSCC patients from the controls [32]. An analysis of cfmiRNAs [52] using RT-preamp-qPCR was performed on whole and cell-free saliva samples from 50 OSCC patients of whom ten were at tumor stage I, 14 at stage II, 16 at stage III and ten at stage IV versus 50 matched control patients. A total of 314 cfmiRNAs were analyzed of which 71 were found to be present in at least two participants. An initial analysis of a subset of 12 control and 12 OSCC patient datasets identified four possible cfmiRNAs as being present at statistically significant levels between the groups ($P < 0.05$) namely, miR-93, miR-125a, miR-142-3p and miR-200a. A further analysis of these cfmiRNAs in all patients

and controls gave significantly different average p-values for miR-200a and miR-125a between the two groups i.e. 0.01 and 0.03, respectively. In contrast, p-values for miR-142-3p and miR-93 were not significantly different between the two groups. The combined AUC between miR-200a and miR-125a was 0.66 whilst the AUCs for miR-142-3p and miR-93 were lower i.e. 0.58 and 0.57, respectively. It is possible that both miR-200a and miR-125a could be used as potential early markers for OSCC [52].

Liu et al. [53] examined 43 OSCC patients and 21 controls for the presence of miR-31 in serum both before and 6 weeks after tumor resection. A similar study was made with saliva supernatant when eight out of nine patients exhibited decreased saliva miR-31 following tumor resection. However, the importance of saliva miR-31 for use as an early marker was diminished by the limited number of samples employed. Interestingly, the saliva miR-31 level was higher than that of the plasma miR-31 in both patients and controls. Zhang et al. [37] employed Affymetrix HG U133 Plus 2.0 Array in the identification and validation of 12 cfmRNA biomarkers to distinguish between pancreatic cancer patients and both chronic pancreatitis and healthy controls. A combination of four of the cfmRNA biomarkers (*KRAS*, *MBD3L2*, *ACRV1* and *DPM1*) permitted the differentiation of pancreatic cancer patients from the two controls (90.0 % sensitivity and 95.0 % specificity). In contrast, no significant differences were found in the level of four saliva internal reference genes (*GAPDH*, *ANXA2*, *RPL37* and *RPS16*) between pancreatic cancer and healthy controls.

4 Urine

Urine is a relatively easily obtained, non-invasive source of cfDNA/cfRNA for the detection of disorders and pathologies. Although there were many problems concerning cfNA isolation from urine, these seem to have been overcome with cfNA extraction kits now available. Much of the DNA so obtained would appear to be genomic DNA (>1 kb) from exfoliate cells present in urine and only low levels of small M Wt fragments (150–250 bp) represent the actual cfNA [54]. Such DNA was considered to be transrenal and to pass from the circulatory system through the kidney barrier to enter the urinary tract, producing so-called transrenal DNA [55]. Nevertheless, although a number of workers have indicated that the DNA in the urine was transrenal (reviewed in Umansky and Tomei [56, 57]), other researchers disagreed. Thus, cffDNA was not detectable in maternal urine including the urine from pregnant women with a compromised kidney barrier function due to hemolysis, increased liver enzymes and low platelet count syndrome [58–60].

4.1 Urine cfNAs (See also Sect. 7)

Hung et al. [56] re-examined the topic using blood and urine samples from 22 hematopoietic stem cell transplant (HSCT) patients all of whom exhibited high amounts of donor-derived DNA in plasma samples. In particular, five female sex mismatched HSCT recipients had male donor-derived DNA in the urine. Interestingly, although the urine cfDNA contained fragments that were >350 bp, such fragments were absent from the plasma. The data may be interpreted as demonstrating that much of the cfDNA was donor-derived as opposed to being transrenal from plasma cfDNA [58].

Many of the successful attempts to determine early tumor markers tend to extract DNA/RNA from cellular material present in the urine. An example concerns the isolation of mRNAs for *TMPRSS2:ERG* fusion transcripts [61] which, together with prostate cancer antigen 3 (PCA3) enhances the standard serum PSA test [62]. A fraction of the mRNAs of the *TMPRSS2:ERG* fusion transcripts would appear to be derived from exosomes [63].

Nevertheless, Melkonyan et al. [64] developed a method for the isolation of 10–150 bp cfDNA fragments as well as cfmiRNAs that could open up an alternative form of diagnosis. Such small DNA fragments from peripheral blood have proved valuable in the early detection of colorectal cancer [65].

In the examples given below, urine supernatant cfNAs have been analyzed so demonstrating their possible uses for a number of different situations. There is clearly more work needed in this promising area.

Bladder Tumors

Bladder tumors represent about 3 % of all tumors and are the second most frequent urinary tract tumor after prostate cancer, being most common in men. Of the various types of bladder cancers, urothelial or transitional cancers form about 90 % in advanced countries being derived from the transitional cells lining the bladder. Recent studies have indicated a number of DNA sequences that may be useful as markers for bladder cancer [66–68] whilst an earlier study indicated that the DNA circulating in urine supernatant increased in content in the presence of a tumor whilst that of the urine sediment after centrifugation of the urine did not [69]. Blood, urine and tumor samples (20 control and 44 patients with bladder cancer plus16 healthy volunteers) were analyzed using 12 microsatellite markers mapped on six chromosomes. Alterations in the latter were found in both the sediment and supernatant (86 % of cancer cases) with urine sediment alone having 68 % and urine supernatant 80 % of the tumor markers. A loss of the 16q24 chromosomal region gave a significant correlation with the tumor stage ($p = 0.02$). The most effective marker was found to be *IFNA* (9p21 chromosomal region) showing LOH in 46 % of the cases or 57 % if combined with *D4S243* (4q32). *D16S476* (16q24) used as a third marker increased the sensitivity to 64 %.

The authors proposed the chromosomal region 16q24 as a marker for its prognostic value [69].

A study of micro-satellite analysis was performed using cfDNA from urine in parallel with serum and plasma cfDNA and in comparison with DNA from normal lymphocytes and tumors of 40 patients with conspicuous bladder lesions [70]. Six micro-satellite markers were used for the detection of alterations on chromosomes 4, 9 and 17. Twenty-six of the 36 micro-dissected bladder tumor tissue samples showed alterations. Micro-satellite changes matching those in the tumor tissues were detected in at least one of the body fluids in 23 cases. However, the amount of urine cfDNA was very low and the fact that alterations were detectable in just 29 % of the all tumor tissue samples indicated that the results are very dependent upon the markers selected for the analysis. It is suggested that the results could be improved by using many multiple markers from different chromosomes, their specificity being crucial for bladder tumor diagnosis.

This preliminary study indicated that simultaneous and multiple investigations of micro-satellite markers on cfDNA from urine and blood could have clinical relevance as a minimally invasive method for both diagnosis and screening for bladder cancer [70].

Prostate Cancer

A recent approach has involved a comparative study of the integrity of urine cfDNA with that isolated from a human bladder cancer cell line (MCR) [71]. Total cfDNA (6 ng μL^{-1} – range 2–36 ng μL^{-1}) was not statistically significantly different between urine cfDNA in cancer patients and healthy individuals. However, an analysis of the diagnostic accuracy for the cfDNA integrity of three oncogenes (*c-MYC*, *HER2*, and *BCAS1*) showed *c-MYC* having the highest AUC. These genes are known to be involved in bladder cancer development with *c-MYC* involved in prostate tumorigenesis [72]. The diagnostic accuracy for the three genes was *c-MYC* > *HER2* > *BCAS1* [71].

A novel approach involved isolating exosomes from the urine of patients after removal of cells and cell debris by centrifugation. The exosomes can then be removed by filtration through a 0.45 μm filter device prior to pelleting on ultracentrifugation [63]. Total exosomal RNA was isolated, purified and subjected to nested qPCR analysis. Patients were grouped into untreated prostate cancer (4), treated with androgen deprivation therapy/medical castration (2) and patients with verified bone metastases, either medically castrated or prostatectomised (3). mRNA transcripts for the fusion gene *TMPRSS2:ERG* were detected in two out of the four patients with a high Gleason score and PSA levels though not in two low-risk tumors (untreated), whereas *PCA-3* transcripts were detected in all of the patients after mild prostate massage, This pilot study shows the possibility of utilizing exosomal mRNA and miRNA in the search for tumor markers [63].

Colorectal Cancer (CRC)

Human urine contains, sub-microgram per milliliter amounts of cfDNA of between 150 and 250 bp [54]. A comparison was made of the mutated K-RAS sequences present in DNA isolated from tumor, blood and urine obtained from a CRC patient with a mutation in codon 12 of the *K-RAS* proto-oncogene. There was an abundance of the low molecular weight mutated *K-RAS* sequences in the urine. A blinded study determined the correlation between mutant *K-RAS* sequences found in the urine, the diagnosis of CRC and polyps containing the mutant *K-RAS*. When DNA from paired urine and tissue sections (20 patients with either CRC or adenomatous polyps) were analyzed for the K-RAS mutation, an 83 % concurrence of mutated urine cfDNA and its corresponding disease tissue was obtained. The authors proposed that apoptotic cells were the source of the cfDNA [54]. Moulière and Thierry [65] demonstrated that CRC K-RAS fragments isolated from peripheral blood tended to be smaller than 100 bp, and given that the study of Su et al. was based upon fragments of 150–250, it would be of interest to determine if smaller fragments could be present in urine and in high abundance.

Transplant Rejection Markers

Since the first kidney transplant in 1950, thousands of people across the world have had such transplants, an increased success rate occurring on the development and application of immuno-suppressors. Nevertheless, transplant rejection is still a possibility for a variety of reasons including immune rejection. More recently, studies have been performed on urine cfDNA and cfRNA in order to determine if they may be used as markers for the rejection of allograft kidneys.

Initial studies were made exploiting cfDNA as a possible marker of kidney graft rejection. Thus, Zhang et al. [73] examined 35 females with kidney grafts, 17 of these having male kidney donors. This permitted the determination of the Y-chromosome *SRY* gene which was found to be present in the urine of all such patients, but absent from the urine of all patients with kidneys from female donors. Urinary concentrations of the ß-globulin gene were markedly increased during the acute rejection period, but returned to lower levels on anti-rejection treatment so offering a possible means of the early detection of rejection. Using both nested and qPCR, Zhong et al. [60] were able to confirm these results.

mRNA has been exploited in assessing rejection and its successful reversal. However these studies were based upon the use of mRNA extracted from the cell pellets isolated from the urine samples and not by using cfmRNA. Muthukuma et al. [74] assayed mRNA for *FOXP3, CD25*, *CD3*, perforin and 18S rRNA from urine specimens of 36 subjects with acute rejection, 18 subjects with chronic allograft nephropathy and 29 subjects with normal biopsy results. However, only *FOXP3* proved a useful marker of acute rejection, the levels of *FOXP3* diminishing on the reversal of rejection. Mas et al. [75] also studied mRNA levels of AGT,

TGF-ß1, EGFR, IFN- *gamma*, TSP-1 and IL-10 in urine cell pellets using RT-qPCR. Only AGT, EGFR, and TGF-ß1 were identified as predictors of chronic allograft nephropathy (CAN) and stable kidney function (SKF) with or without proteinuria. AGT, EGFR and TGF-ß1 appeared to be usable as predictors of CAN, SKF ± proteinuria. Many other studies also use the pelleted cells and fragments from urine e.g. studies on mRNA FOXP3, CD25, CD3ε, perforin and 18S rRNA of which the authors found that FOXP3 mRNA could offer a non-invasive means predicting acute rejection of renal transplants [74].

Clearly, the majority of workers are those used to using the pellets for other investigations and have discarded the supernatant. Whether or not the supernatant has also been investigated is not given in these papers. However, it could be interesting to examine the supernatant for some of the markers proposed from the pellets.

Although the following two situations are based upon urine sediment analyses they offer examples of analyses that might be further extended via cell/sediment free urine studies. For this reason they are included here.

Diabetic Nephropathy (DN) (see also Chapter "Circulating Nucleic Acids and Diabetes Mellitus")

Szeto et al. [76] demonstrated that the mRNA expressions of nephrin (NephRNA), podocin (PodRNA) and synaptopodin (SynRNA) in urinary sediment gave significant differences for those of NephRNA and PodRNA between patients with acquired proteinuric diseases including nephropathy. Furthermore, after a median follow-up over a period of 2 years, there was a significant correlation between the rate of decline in renal function for both NephRNA ($r = 0.559$, $p = 0.001$) and PodRNA ($r = 0.530$, $p = 0.002$), but not SynRNA ($r = 0.054$, $p = NS$) mRNAs. Wang et al. [77, 78] then extended the study to 21 patients with biopsy-proven DN when significant expression differences were observed for nephrin, podocin, synaptopodin, alpha-actinin-4 and WT-1 mRNAs when compared to results from healthy controls. In particular, nephrin expression was significantly correlated with proteinuria ($r = 0.502$, $p = 0.020$); urinary synaptopodin was significantly correlated with proteinuria ($r = 0.585$, $p = 0.005$), serum creatinine ($r = 0.516$, $p = 0.017$) and estimated glomerular filtration rate (GFR) ($r = -0.560$, $p = 0.008$) while urinary WT-1 expression was significantly correlated with the degree of tubulointerstitial fibrosis ($r = 0.558$, $p = 0.009$. In a further study, Wang et al. [79] showed that in patients treated with ACE1 + angiotensin receptor blocker as opposed to treatment only with ACE1 during a period of 12 weeks, the combination group had a significantly lower urinary synaptopodin expression (7.49 (95 % confidence interval (CI), 0.62–115.29) vs 14.83 (95 % CI, 1.03–241.43), $P = 0.026$) than the control group. The percentage change in urinary podocin expression over the 12- week treatment period had a small correlation with the rate of GFR decline in 1 year ($r = -0.243$, $P = 0.041$) [80].

In more recent experiments Zheng et al. [81] separated DN patients (N = 51) into a normoalbuminuria group, a microalbuminuria group and a macroalbuminuria group according to their urinary albumin excretion. Relative mRNA abundance of synaptopodin, podocalyxin, CD2-AP, α-actin4, and podocin were quantified. All mRNA levels studied were significantly higher in the DN group compared with controls ($p < 0.05$), mRNA levels increasing with DN progression. Urinary mRNA levels of all target genes positively correlated with both UAE and BUN. The expression of podocalyxin, CD2-AP, α-actin4, and podocin mRNA correlated with serum creatinine ($r = 0.457$, $p = 0.001$; $r = 0.329$, $p = 0.01$; $r = 0.286$, $p = 0.021$; $r = 0.357$, $p = 0.006$, respectively). Furthermore, podocalyxin mRNA was found to negatively correlate with eGFR ($r = -0.349$, $p = 0.01$). They concluded that the quantification of podocyte-associated molecules will be useful biomarkers of DN.

Once again, as with mRNAs in renal transplant rejection, only pelleted material was used as the source of the mRNAs. It will be of interest to determine if such mRNAs occur in the urine supernatant and their possible use as DNA markers.

Lupus Nephritis (LN)

The following analyses have been performed on urinary pellets but are included as examples that may give some directions for studies on urine cfNAs.

Wang et al. [77] examined the urinary expression of podocyte-associated molecules in patients with LN. mRNA expression of nephrin, podocin and synaptopodin in urinary sediment was determined for 32 patients with active LN (Active group) and 17 patients with inactive lupus (Silent group). Although there was no relation between urinary gene expression and the histological class of LN, urinary nephrin expression correlated with proteinuria ($r = 0.480$, $p < 0.01$) and the score of the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) ($r = 0.578$, $p < 0.01$). In addition, urinary podocin expression also correlated with the SLEDAI score ($r = 0.389$, $p = 0.006$). All patients were followed for about a year after the initiation of immunosuppressive treatment, the declining of the glomerular filtration rate (GFR) correlating with urinary expression of podocin ($r = 0.406$, $p = 0.005$) and synaptopodin ($r = 0.337$, $p = 0.021$). Thus, the concentration of podocyte-associated molecules in urinary sediment correlated with lupus activity and GFR declined.

5 Cerebrospinal Fluid (CSF)

The CSF is a colorless fluid derived primarily from arterial blood by the choroid plexuses of the lateral and fourth ventricles with a small amount being produced by the ependymal cells. It is present in the subarachnoid space, the ventricular system and the spinal cord. In adults, there is a total CSF volume of 140–270 mL with a

production of 600–700 ml per day. CSF passes into the venous circulation although a significant amount could drain into the lymphatic vessels around the cranial cavity and spinal canal. It acts as a cushion that protects the brain from shocks and supports the venous sinuses as well as playing an important role in the homeostasis and metabolism of the central nervous system. In addition to ions, protein, glucose and albumin, CSF also contains lactate, creatine, urea, CO and 0–5 white blood cells μL^{-1}.

More recently, DNA and RNA have been identified in the CSF and so permitted studies on their possible use in early diagnosis and prognosis. Initial studies included the development of methods for viral nucleic acids ranging from enteroviral RNA, herpes simplex virus and Varicella-zoster virus using qPCR following a single extraction with a guanidinium thiocyanate acid buffer, so eliminating the use of organic solvents [82]. Subsequently, van Harten et al. [83] proposed a method based upon cfmiRNA extraction from the CSF of either at risk of or Alzheimer disease (AD) patients (compared with healthy controls) using acidified phenol: chloroform. The cfmiRNAs were q-PCR treated and quantitatively analyzed using the Megaplex protocol with Taqman Array MicroRNA cards on small RNAs permitting analysis of 667 different cfmiRNAs and six endogenous controls. MiR-16 was used as a control since it is a housekeeping gene that is relatively uniformly expressed in all tissues [84]. This sensitive approach was used for a number of reasons including speed of a quantitative analysis (2d for a complete analysis), the small volumes necessary and the stability of cfmiRNAs [83]. Preliminary results using MiR-802 differed significantly between AD patients and the controls whilst there was little variation for MiR-16. NGS of cfmiRNA from the CSF of patients with neurological diseases, CNS tumors and traumatic brain injury has been developed [85]. This approach exploited small RNA sequencing libraries using Illumina's TruSeq sample preparation kit followed by sequencing the samples on the HiSeq 2000.

5.1 Aneurysmal Subarachnoid Hemorrhage

Other examples of the use of CFS nucleic acids in early diagnosis include the studies of Wang et al. [86]. Released DNA from nuclear and mitochondrial origins in CSF, in parallel with those from plasma, of patients with aneurysmal subarachnoid hemorrhage were examined with a view to predicting the treatment outcomes for such patients. Healthy donors were used as controls. Patients with a worse outcome presented with higher CSF cfDNA ($>$85.1 ng ml^{-1}) and mitochondrial cfmitDNA levels ($>$31.4 ng ml^{-1}). The authors concluded that higher CSF cfDNA levels, rather than plasma cfDNA levels, are associated with worse outcomes in patients presenting with acute spontaneous aneurysmal subarachnoid hemorrhage.

5.2 *Primary Central Nervous System Lymphoma (PCNSL)*

Primary central nervous system lymphoma (PCNSL) is a form of extranodal, high-grade non-Hodgkin B-cell neoplasm, usually a large cell or immunoblastic type originating in either the brain or leptomeninges or spinal cord or eyes. About 90 % of PCNSLs are diffuse large B-cell lymphomas, the remaining 10 % being poorly characterized low-grade lymphomas, Burkitt lymphomas and T-cell lymphomas [87]. The diagnosis of PCNSL depends on the histopathology of brain biopsies because disease markers in the CSF with sufficient diagnostic accuracy are not yet available. Central nervous system (CNS) biopsies do not always give a definitive histopathological diagnosis and can be associated with a risk of haemorrhage and neurological damage. Moreover, the less invasive analysis of cells from the CSF by genetic, cyto-pathological and immuno-phenotypical analyses appear to be much less sensitive than the biopsies [88–90].

Baraninskin et al. [91] considered the exploitation of cfmiRNAs in the CSF as possible primary markers to distinguish PCNSLs from other healthy individuals. A candidate miRNA approach assessing cfmiRNA expression using RT-qPCR of the cfRNA isolated from the CSF of PCNSL patients and control subjects with different neurological disorders was selected for the evaluation of the cfmiRNA potential. Six candidate cfmiRNAs (miR-15b, miR-19b, miR-21, miR-92a, miR-106b, miR-204) were selected for further investigation after compiling a list of published miRNA expression data in diffuse large B-cell lymphomas and primary CNS lymphomas based upon published miRNA expression data in lymphoma tissues [92]. Four cfmiRNAs (miR-24, RNU48, RNU6b, RNU44) were selected as controls due to likely uniform expression levels and an adequate amount in the CSF for potential normalization.

The initial screening indicated that three cfmiRNAs (miR-21, miR-19b, miR-92a) gave significantly increased levels in the CSF of PCNSL patients compared with those of control patients. Of these, miR-21 appears to be expressed in a number of tumors and is associated with the down-regulation of bcl-2 and phosphatase and tensin homologue whilst miR-19b and miR-92a are present in the polycistronic miRNA-17 ~ 92 cluster located on human chromosome 13.13 [93]. Interestingly, the miRNA-17 ~ 92 cluster is often over-expressed in B-cell lymphoma cell lines, the majority of diffuse large B-cell lymphomas [94] and also in PCNSL [95].

miR-15b, miR-106b, and miR-204 were detected by RT – qPCR but the expression levels were similar in both patients with PCNSL and control patients. Control cfmiRNAs, RNU48, RNU6b and RNU44, were undetected in the CSF though low-abundant expression of miR-24 was present in all PCNSL and control patient samples. Thus, it was used to normalize miRNA expression levels in subsequent analyses of CSF cfmiRNA expression in individual CSF specimens. To further distinguish PCNSL from other diseases using CSF cfmiRNAs, miR – 21, miR-19b, and miR-92a RELs were combined leading to the correct identification of 22 from 23 PCNSL patients (95.7 %) and 96.7 % of the control patients [91].

Popsipil et al. [95] investigated the abundance of cfmiRNAs in the CSF and sera in order to determine their levels during the diagnosis and treatment of PCNSLs. The patients studied included three primary PCNSL, three cases of systemic lymphomas with CNS dissemination and 11 cases without. Histologically, 13 cases were of diffuse large B cell lymphoma (DLBCL). Total cfRNA was isolated from 200 μL of cell free CSF and sera followed by RT-qPCR, the data being adjusted to levels of control miRNAs (either miR-let-7a or miR-24). miR-19a, miR-20a, miR-92a of the miR-17–92 cluster, miR-106b, miR-25 of the miR-106b – 25 cluster and miR-155 were detected in both the CSF and sera, but not miR-106a and miR-18a. In addition, miR-17–92 and miR-106b-25 were increased in the CSF of the PCNSLs and the systemic lymphomas with CNS involvement when compared with the systemic lymphomas without CNS involvement. The analysis at nine different time points within 3 months were made for one case of systemic DLBCL with CNS dissemination. During the treatment (R-CHOP alternating with RMPV) resulting in complete remission, a gradual decrease (~sixfold) of the levels of miR-19a, miR-20a, miR-92a, miR-106b and miR-25 were observed.

Both of the above studies show that the measurement of CSF cfmiRNAs could offer a sensitive tool for PCNSL tumor detection as well as for estimating the monitoring of therapy efficiency.

5.3 Detection of Glioma

A study by Chen et al. [96] involved the use of BEAming RT-qPCR as well as ddPCR to identify nucleic acid components of vesicles present in both the CSF and serum of glioma patients. Using these approaches, it was possible to reliably detect and quantify both mutant IDH1 and wild-type IDH1 RNA transcripts in the CSF of these patients. It was also possible to show that extracellular vesicles derived from the CSF patients with tumors have higher levels of IDH1 mRNA than CSF EVs from controls i.e. suspected non-malignant, neurological diseased individuals later shown to have normal CSF profiles.

Monitoring Treatment of Glioblastoma

An alternative approach concerns a study of nucleosomes present in CSF and their use in monitoring treatment. Holdenrieder et al. [97] compared the CSF nucleosome levels, pre- and post-operative in ten glioblastoma patients versus 20 with non-acute neurological disorders (ten with subarachnoid haemorrhage and five with non-ruptured aneurysms).

Therapy can include surgical resection of the visible tumor plus radiotherapy with chemotherapy both during and after radiotherapy [98, 99]. Nevertheless, glioblastoma often progresses despite initial tumor response, often with complications e.g. epileptic seizures and brain edema development [98, 99]. Thus, the study

involved nucleosomal levels in the CSF of patients with glioblastoma receiving intra-cerebral chemotherapy and their correlation with the appearance of complications. Nucleosome levels did not differ in pre-therapeutic CSF samples of patients with glioblastoma (median 13.7 ng mL^{-1}), control groups of patients with non-acute neurological disorders (16.6 ng mL^{-1}), subarachnoid haemorrhage (24.6 ng mL^{-1}) and non-ruptured aneurysms (10.3 ng mL^{-1}). Patients with recurrent glioblastoma and receiving tumor resection plus local, intra-cavity chemotherapy showed a constant increase of nucleosome levels in CSF from 13.7 ng mL^{-1} (day 0) to 24.9 ng mL^{-1} (day 4).

During the first postsurgical week, a significant cerebral oedema occurred in three out of the ten patients with treated glioblastoma. Only a slight increase of nucleosomes in the CSF occurred for the seven patients without oedema during the observation period (pre-operatively 16.6 ng mL^{-1} to reach a maximum level of 22.3 ng mL^{-1} on day 3). Those patients who developed postoperative oedema started pre-therapeutically from similar levels (11.4 ng mL^{-1} in CSF) followed by a dramatic increase to reach a maximum of 2,051 ng mL^{-1} (day 3). Such results led the authors to suggest that the preliminary data may be valuable in the monitoring of complications during intra-cerebral cytotoxic treatment of glioblastoma and that further studies would be beneficial [97].

5.4 Fetal DNA from Pregnant and Post Delivery Women

A further involvement of cfNAs in CSF concerns the presence of fetal DNA in the CSF of pregnant and post delivery women. In an initial study, Angert et al. [100] considered that since CSF is secreted by the choroid plexus and, therefore, is protected by the blood-CSF barrier, a mechanism similar to the blood-brain barrier. Normally, only hydrophobic and non-polar molecules of a molecular weight of $<$500 Da are permitted to enter and only during e.g. either infection or inflammation are other molecules or cells are permitted to breech the blood-CSF barrier. Although peptide nucleic acids can cross the blood-brain barrier and exhibit gene expression [101], DNA is a large, negatively charged molecule that would not be expected to be present in the CSF. However, if cffDNA can act similarly to tumor DNA, then it may also breech the blood-CSF barrier.

Hence, CSF was collected from nine woman of whom 26 carried male fetuses, the rest carrying female fetuses and the DNA isolated subjected to qPCR. ß-globin DNA was found to be present in all CSF samples whilst DYS-1 gene sequences were found in four samples from male fetus carriers, three during pregnancy and one after delivery; none was present in CSF from female fetus carriers.

6 Bronchial Lavage & Aspirates

The isolation of DNA/RNA from cell-free bronchial lavage supernatant (BLS) was first demonstrated by Schmidt et al. [102] and an improved methodology followed based upon the use of the Nucleo-Spin Plasma XS Kit for the isolation of cfDNA [103]. In a study of 33 lung cancer and 27 benign lung disease patients, no difference in cfDNA levels was observed for that isolated from serum and plasma though there was a difference between the two groups using BLS with a greater amount in the BLS from the lung cancer group. The lack of difference overall may have been due to the fact that there is a severe inflammatory reaction present in most of the control patients, severe inflammation being known to be associated with higher cfDNA levels in serum and plasma [104]. Kneip et al. [105] developed a novel technique for the detection of DNA methylation biomarkers, based on qPCR of bisulfite-treated template with enzymatic digestion of background DNA during amplification using the heat-stable enzyme Tsp509I. The lung cancer methylation biomarker BARHL2 assay was compared with the methylation-specific PCR technology to indicate the clinical and analytical performance of the new method. Both methods gave comparable results when analyzing both cfDNA mixtures and BLS samples from 75 suspected lung cancer patients. This approach was found to be useful especially when there are few copies of methylated cfDNA present in samples having a high background of unmethylated cfDNA, as is found with body fluid clinical samples. Kneip et al. [105] extended this approach for use with plasma *SHOX2* cfDNA methylation.

Schmidt et al. [106] were able to show that *SHOX2* methylation could be used as a biomarker for lung cancer based upon bronchial aspirate samples that were either fresh-frozen or Saccomanno-fixed samples. A study of 281 lung cancer cases and 242 control, benign lung disease patients employed the use of differential methylation hybridization and qPCR based on HeavyMethyl technology. The results showed the ability to distinguish between malignant and benign lung disease with a sensitivity of 68 % and a specificity of 95 %.

As already seen to be a useful biomarker for lung cancer using BLS, a new method to measure the methylation of the SHOX2 gene locus was developed and applied especially when there was no clear-cut outcome from standard cytological, histological and bronchoscopy results [107]. This new method was based on (a) generation of bisulfite converted template DNA from patient bronchial aspirate samples and (b) qPCR determination of SHOX2 biomarker methylation. The application of the method to 125 lung cancer patients and 125 controls resulted in a diagnosis for the cancer with an AUC 78 % sensitivity and a 96 % specificity [107].

Early studies on the presence of cfRNA in BLS using RT-qPCR permitted the identification of intact cfRNA in the BLS from 126/129 patients. In parallel, the cfRNA content of 64 serum samples was also determined when cfRNA levels were higher in the BLS than in the serum. Moreover, higher BLS cfRNA concentrations were found in tumor patients than in patients with benign lung disease. Although

quantification of intact cfRNA present in BLF could be used as a tool for distinguishing between tumor and non-tumor patients, there has been little follow-up to this work.

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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)

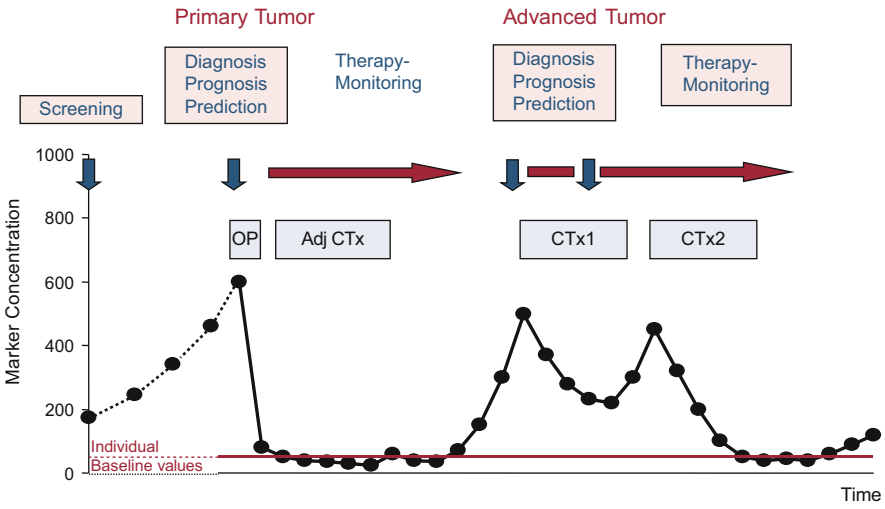

Fig. 1 During the course of cancer disease there are multiple indications for the use of circulating biomarkers: They are applied for (i) cancer detection and differential diagnosis, (ii) estimation of prognosis, (iii) prediction and monitoring of therapy response, (iv) early detection of therapy resistance 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)

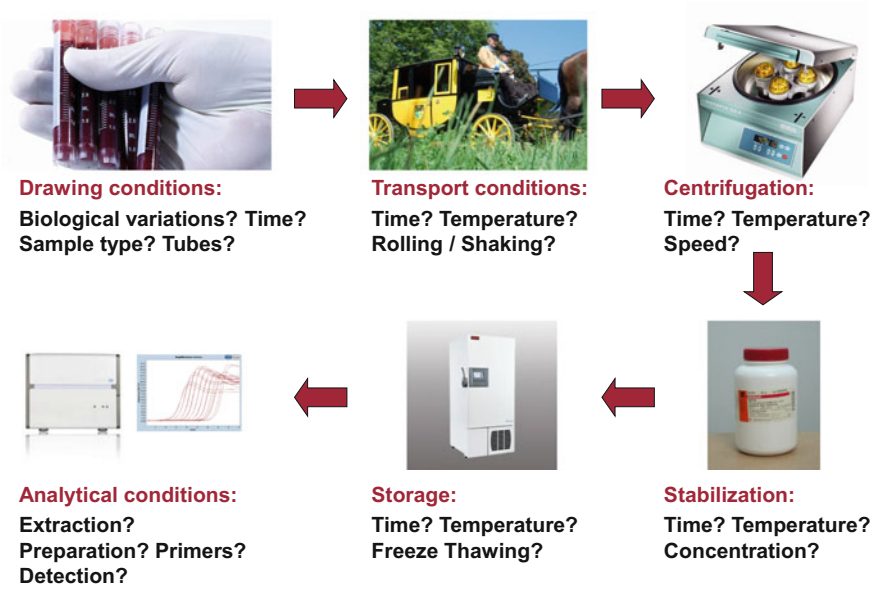

Fig. 2 Preanalytical variations of blood handling may influence the test results. They include the conditions of (i) the individual patient, (ii) the blood drawing procedure, (iii) the material used, (iv) the transport to the laboratory, (v) the handling in the laboratory or biobank facility, (vi) the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

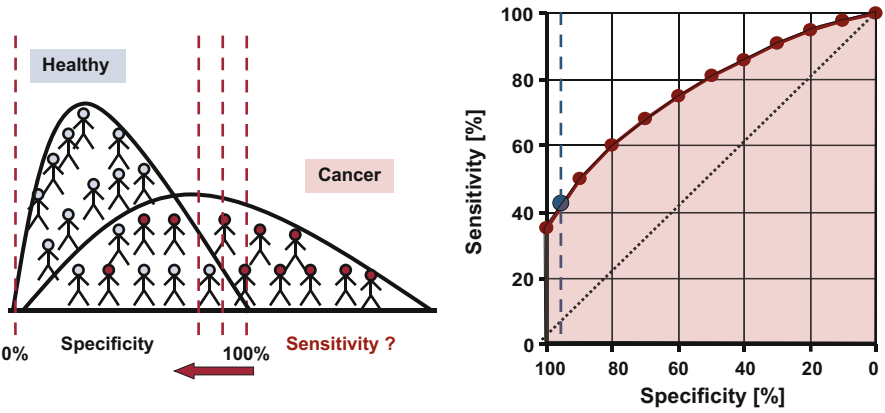

Fig. 3 The value distributions of most cancer biomarkers show an overlap of cancer patients and healthy individuals. The diagnostic performance of a biomarker is illustrated best by ROC curves. To establish this figure, the portion of correctly negative controls (specificity) and correctly positive cancer 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.

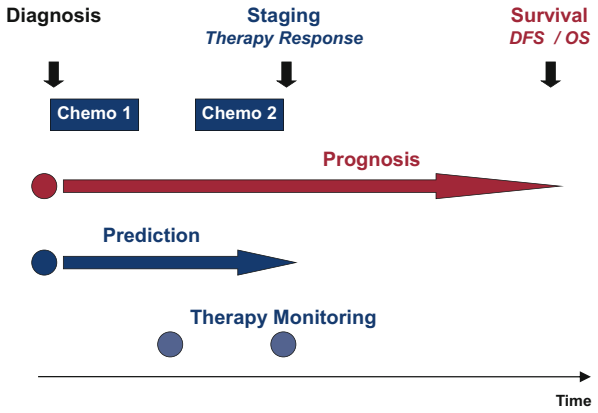

Fig. 4 Biomarkers prior to and during therapy provide prognostic information if they are related to the time of either disease-free (DFS), progression-free (PFS) or overall survival (OS). Predictive biomarkers indicate the response of a specific therapy before the start and are used for therapy stratification,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

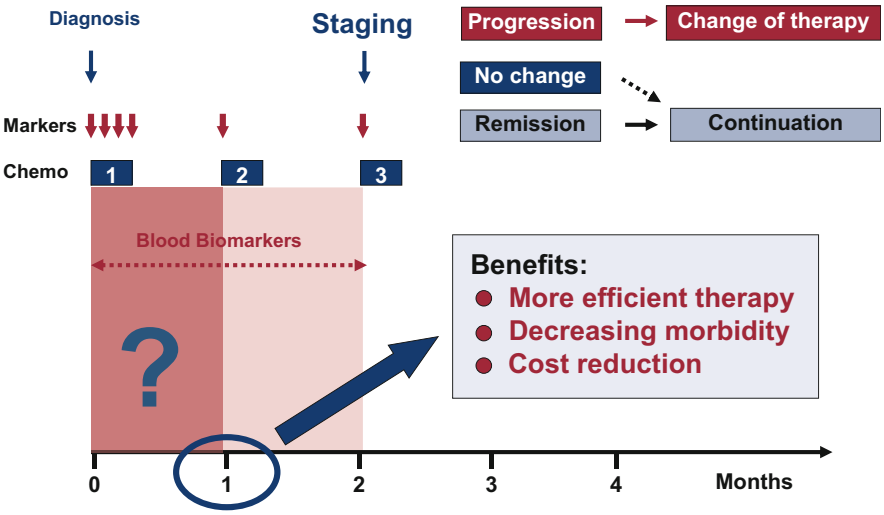

Fig. 5 Early estimation of response to cytotoxic therapies is particularly useful in patients with tumor burden undergoing systemic treatments. As macroscopic changes of tumor volume often are detected by imaging techniques only after weeks or months, the biochemical changes are frequently detected through 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].

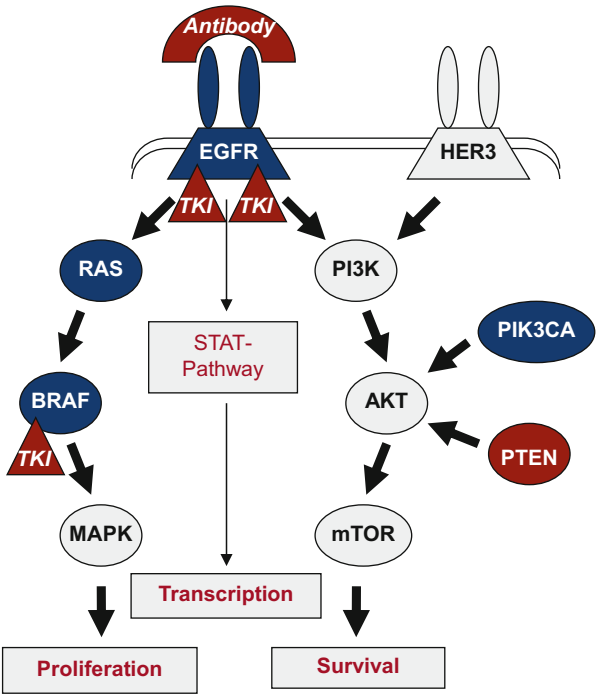

Fig. 6 The *epidermal growth factor receptor (EGFR)* pathway includes the EGFR receptor and diverse components that intracellularly transmit a growth signal to promote processes like transcription, proliferation and cell survival. Mutated parts of this pathway lead to dysregulation and may be treated 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.

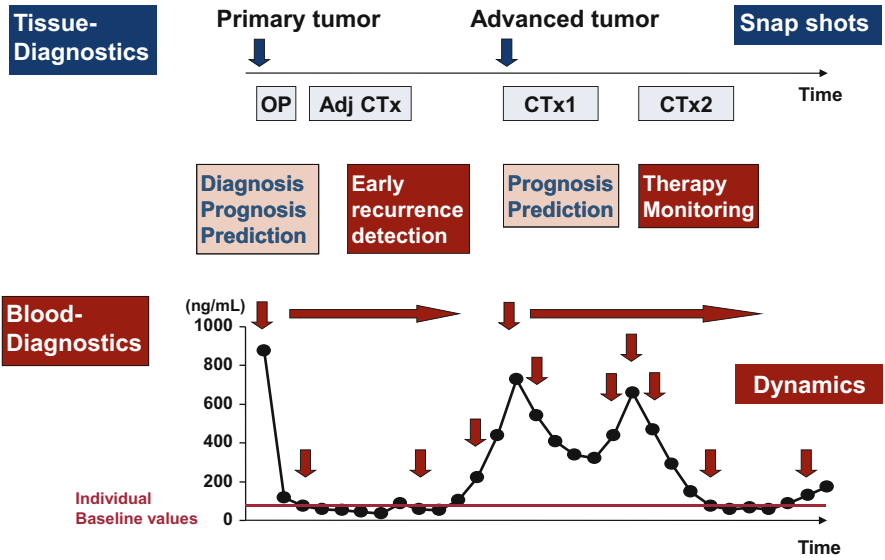

Fig. 7 The combination of pre-therapeutic tissue biopsy and serial liquid biopsies during and after therapy may improve the guidance of cancer patients considerably. The mutation status in tissue is currently required to stratify patients for certain targeted therapies although it allows only a spatially 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").

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Part IV
Other Aspects

Quality Assurance

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Abstract The analysis of cell free DNA is increasingly being used for the early detection of biomarkers relevant to obstetric and oncology practice. Guaranteeing the quality of these analyses is critical to retaining the confidence of the public in these tests. This chapter outlines internal and external quality control and assessment procedures open to laboratories to ensure the production of valid test results. As an example clinical test, the chapter considers non-invasive prenatal testing to determine fetal sex using cell free DNA isolated from maternal plasma. External Quality Assessment as a method through which laboratories can compare their results with a set of peers is also discussed.

Keywords Quality management • External quality assessment • Internal quality control • Test design/validation • Laboratory medicine • Good practice

1 Introduction

The analysis of circulating cfNAs as the analyte for key biomarkers is a developing field in biomedical diagnostics. Much of the interest in this area arises from the improving utility of minimally invasive sampling methods of biological fluids to allow early phase and regular monitoring of both tumor and fetal markers. However many of the potential clinical applications of these techniques critically inform the choice of treatment of a patient with a life threatening condition or a decision

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concerning a pregnancy at risk of a genetic condition. With this in mind there is a duty, on the part of the Directors of all public sector and commercial laboratories involved, to quality assure these tests both during the design and validation phase of laboratory developed assays and following their inception into routine clinical service. Quality assurance can be considered as the sum of two components namely (a) internal control of materials, information, processes and (b) human factors, plus independent scrutiny through inter-laboratory comparisons by External Quality Assessment (EQA) agencies and audit by national accreditation bodies. This chapter will consider the role of these facets of quality assurance as applied to the analysis of cfNAs. It will use prenatal diagnosis of fetal sex as the principle example application and focus on EQA as a powerful tool to facilitate peer review.

2 Quality Management in the Clinical Laboratory

A systematic approach to quality assurance begins with the adoption of a Quality Management System (QMS). These systems have spread from a common set of principles developed first in manufacturing and then in service industry. In the clinical laboratory context, the core values within a QMS aim to engage the service recipient (patient and clinical user) to help define and shape the service product (the clinical test) and how it is delivered. The QMS relies on a set of documented, accessible policies and procedures plus a regular, open and honest review of laboratory performance. All of these components contribute to developing and sustaining a culture of continuous improvement that becomes second nature to the staff working in the laboratory. These principles have become embedded in a set of standards against which certification and accreditation agencies may judge the competence of a commercial company or public service unit. Over time these requirements have become more specific and prescribed. For example the International Organization for Standardization (ISO) EN BS 9001:2010 standards are applicable to certify any QMS in any setting (http://www.iso.org/iso/home.html). More specifically EN BS ISO17025:2005 addresses quality management in general measurement and testing laboratories and finally EN BS ISO15189:2012 describes a set of standards applicable only to medical laboratories. Each country has a recognized authority established to accredit a service provider against the appropriate standards. In the UK, medical laboratories are assessed against a set of standards aligned to EN BS ISO15189:2012 by Clinical Pathology Accreditation which is part of the UK Accreditation Service. In the US similar standards were developed from the Clinical Laboratories Improvement Act (CLIA) and applied by the Centers for Medicare and Medicaid Services and the College of American Pathologists (CAP) (http://www.cms.gov; http://www.cap.org). Other relevant references in considering quality management in clinical laboratories are the Organisation for Economic Co-operation and Development (OECD) Good Laboratory Practice standards plus the OECD recommendations for Quality Assurance in Clinical Molecular Genetic Testing (2007) which are specific to the context of

nucleic acid analysis (http://www.oecd.org/env/ehs/testing/; http://www.oecd.org/science/biotech/38839788.pdf). Although a single world-wide accreditation standard has not been achieved, international comparability remains an aspiration. Nonetheless, accreditation to internationally recognized standards allows patients and clinicians to choose a laboratory with a public 'badge' of competence and have confidence that the analyses are valid and comparable. This is increasingly important since it is common-place for clinical samples to cross national borders for analysis.

2.1 Design and Validation of Laboratory Developed Tests and Verification of Kits

Tests developed in-house and for the use of a single clinical laboratory are a frequent feature of this field and reflect the pace of translation from the research laboratory to clinical service. Although this allows the benefits from new technologies to be more rapidly realized, patients are entitled to expect that laboratory developed tests will be subject to an appropriately rigorous design and validation process. For example, the design of tests using primers for PCR should always include a search for common SNPs that may affect primer annealing and risk allelic drop-out (https://secure.ngrl.org.uk/SNPCheck/).

A series of experiments establishing the robustness (test precision under various input challenges), repeatability (within-run precision) and reproducibility (between-run precision) of the assay should be performed to constitute a thorough validation and define the performance characteristics of the analytical system. The correct outcome of a test should be judged against previous test results using an existing well-characterized analytical system and/or a known clinical outcome or phenotype. Publishing key elements of the information established during validation (e.g. sensitivity and specificity of the test) as a service profile allows the end user to be informed of the limitations of the test and helps give realistic expectations of and increased confidence in the service. Accreditation standards require laboratories to have a formal policy and procedure for validating tests and to document their findings. Publishing a validation study in a peer reviewed journal is one way to ensure these data are robust and to make it public. A series published to validate a prenatal test for fetal sex based on cffDNA in the maternal circulation is an example [1]. Recommendations specific to the validation and verification of clinical molecular genetic tests have been published and form a detailed guide to the development and introduction of clinical tests [2].

As a field matures, commercial products (kits and services) may be marketed with Federal Drug Authority approval and/or CE marking under the European Union In Vitro Diagnostic Device Directive. Accreditation authorities require, and good practice dictates, that the laboratory verifies and documents that the test

'in it's own hands' meets the performance specification indicated by the kit manufacturer before it is introduced into clinical service.

2.2 Aspects of Internal Quality Control

Standard Operating Procedures

The file describing the series of experiments to establish the validation or verification of a test allows a standard operating procedure (SOP) to be written. This forms the key reference for training staff to perform the test in a reliable and reproducible manner, to reduce variability and to consistently produce valid results. The SOP documents the minimum requirements (clinical information, identity, demographics and the condition of the biological material) for samples to be accepted into the laboratory for testing. Examples of acceptance criteria from NIPT include a minimum gestational period (typically 8 weeks), the period from removal of blood to analysis to be less than 72 h and samples to be protected from freezing. The SOP defines individual handling steps and checks to assure the integrity and traceability of the sample through the process. The test examination process should be detailed as a simple step-by-step guide to ensure ease of use. In addition, a risk analysis should be included which covers both safety considerations for the operatives and to manage potential handling errors that might prejudice the secure journey of the patient sample through the analytical process. Finally, the SOP may describe the reporting process and standard wording to be used. Fetal sexing results may be reported variously as: 'This result indicates a male fetus.' 'This result is consistent with a female fetus although the result could be due to insufficient fetal DNA.' and 'The laboratory was unable to obtain a result.' The SOP may indicate reporting caveats e.g. 'The test is not valid for multiple pregnancies and may be affected by a vanishing twin conception.' Finally the SOP will also mandate the process of reviewing and the final authorization of the report. SOPs should always be held within a document control system designed to ensure that procedures are subject to regular review and that only a valid and current version of the document is available for use in the laboratory.

Use of Internal and External Controls

1. The choice, availability and appropriate use of every-day run controls is a key element in the production of a valid result. This is particularly true in the challenging assays represented by the analysis of circulating cfNA biomarkers. Internal controls that may be sourced from surplus archived patient samples may be characterized as an adjunct to the test validation process. Run controls may also be sourced from cell lines or processed DNA from a commercial supplier or cell bank. One of the critical steps in assays using circulating cfDNA is

pre-analytical extraction. This process also requires positive and negative controls to allow the analytical steps to proceed. In the specific context of cffDNA analysis assaying the methylation status of the analyte using a methylation sensitive restriction enzyme for a universal marker such as the RASSF1A promoter is a useful control to ensure that sufficient fetal material is present in the sample for analysis [3]. Periodic use of a higher order reference material is recommended to revalidate an assay against an external standard. Reference materials are formally certified by an accredited institution or standardization body. Examples are the reference materials produced by the US National Institute for Standards in Technology, the UK National Institute for Biological Standards and Controls on behalf of the World Health Organization and the European Union Institute for Reference Materials and Measurements. Commercial sources of external control materials may also be relevant (http://www.nist.gov/; http://www.nibsc.ac.uk/; http://irmm.jrc.ec.europa.eu/html/homepage.htm).

In practice, few reference materials are applicable to this field although the UK National Institute for Biological Standards and Controls has derived a reference material for the standardization of RHD and SRY fetal genotypes in the cffDNA fraction of the maternal circulation [4].

Human Factors – Operator Training and Competence

Clearly operator competence is a critical factor in the reliable production of valid test results. The clinical laboratory must have in place a process, separated from live test situations, for training staff in the pre-analytical, analytical and reporting procedures, giving them confidence and signing off their initial competence. Periodically checking that individual staff competence is maintained by observation (a witness audit) may also be part of the Quality Management routine.

Test Acceptance Criteria

The validation process will establish and define a set of criteria for accepting a set of assays as a valid test result. Assays that fall short of the validation criteria must not be accepted as reportable and must be repeated on the original analyte material or reported as inconclusive.

In the context of NIPT examples of test acceptance criteria that have been used include a minimum number of concordant replicate assays, concordant assays on two different genetic marker loci and/or using two different analytical methods and/or consistent test results from two separate maternal plasma samples collected at different times.

2.3 External Quality Assessment

Outline of External Quality Assessment

External Quality Assessment (**EQA**) is also termed Laboratory Proficiency Testing. It has a long history in Clinical Laboratory practice as a way of systematizing inter-laboratory comparisons amongst a group of peer laboratories sharing a particular test service. In its simplest form it may consist of the informal sharing of a sample to be tested by a small group of laboratories with a subsequent comparison of the results, often called a Ring Trial. At a more formal level a competent agency (accredited to the ISO/IEC17043:2010 standard for proficiency testing) regularly ships a sample to subscribing laboratories. The sample is accompanied by mock identifiers, demographic information and a medical scenario designed to resemble as closely as possible a normal clinical request for a specific test. Participating laboratories are asked to perform the test according to routine testing protocols and to return their results to the EQA agency by a deadline for a formal assessment by a panel of experts. In Clinical Molecular Genetics levels of assessment consist principally of scoring a qualitative genotype or assessing a quantitative result against an accepted range of values around an assigned or consensus value. In addition, the EQA return from the participating laboratory may consist of the test result in its normal report format with the clinical interpretation and comments added. This allows a more detailed evaluation of the report for accuracy of the genotype and use of standard mutation nomenclature. Additional assessment criteria include accuracy of transcription of demographic and identifier information, clarity of presentation of information, accurate description of the methodology used including its limitations, a commentary on the result including any caveats or recommendations for further tests or clinical actions and an indication that the report has been formally authorized for release by a suitably qualified person.

Sourcing Manufacture and Validation of EQA Materials

The ideal EQA challenge is the biological source material normally received by the testing laboratory. For non invasive prenatal-testing or tumor detection this is most frequently a plasma sample. Although processed DNA is accepted as the distributed material for most Clinical Molecular Genetics EQA schemes it is not appropriate as the challenge material for the EQA of circulating cf(f) DNA tests as measuring laboratory performance in the pre-analytical part of the process is a key issue. The challenge for an EQA scheme organizer is to source material that is homogenous, stable in storage and transit and available in sufficient quantity to meet the testing requirements of a relatively large number of laboratories participating in the scheme. Sufficient quantities of material are also required to allow for validation of the genotype or establishment of the assigned quantitative analytical value by one or more reference laboratories in advance of the distribution. If possible,

additional material should also be stored to allow for a limited number of repeat shipments to laboratories that make this request. Some material must be retained to form a reference in the event of the need for an investigation if there is a significant level of discrepancy in the scheme results that cannot be explained by variation in laboratory performance that subsequently calls into question the validity of the scheme. Finally, in advance of distribution, the EQA materials require testing to meet appropriate bio-safety requirements and to be shown to be free of a range of detectable blood borne infectious agents.

For NIPT, a number of approaches to EQA sample manufacture have been explored. These include the production of artificial material, which is a recognized approach in Clinical Laboratory EQA. To assess the feasibility of this approach a batch of material was prepared from fetal (male) DNA isolated from chorionic villus biopsy (CVB) and sheared by sonication to an average fragment length that replicated the size of cffDNA found naturally in maternal circulation. CVB DNA was diluted in quantities designed to match the concentration of cffDNA found in maternal circulation in a sufficient volume of cell free plasma from a non-pregnant female. In a series of tests this artificial product did not meet the minimum requirements for an EQA material; failing to produce a male specific signal to mimic the qPCR signal curve observed in natural plasma samples from women carrying a male fetus (Deans Z, Karamainen O, Patton P, unpublished result).

Since it is not possible to source sufficient material from a single volunteer, an alternative method involves pooling natural cell free plasma samples from a number of pregnant women. The challenges of this method include sourcing sufficient material to satisfy the requirements for an international EQA scheme that may involve many laboratories plus the back-up validation and reference requirements as previously described. This method has been trialed in a small number of pilot schemes involving a limited number of centers in the UK that established that an EQA scheme using this approach is feasible. Research biobanks are a potential source for this program and the recruitment of testing centers is underway in order to prospectively collect suitable samples for pooling for future EQA runs. However the full scale-ability of this method of EQA material manufacture for NIPT remains to be established.

Experience from Pilot EQA Schemes

Selection of Cases

The EQA scheme organizer is responsible for selecting materials to be distributed and matching a mock clinical scenario appropriate to the genotype of the material. It must be remembered that EQA is primarily educational and not an opportunity for scheme organizers to ‘trip-up’ participants with a particularly difficult challenge. Over time the EQA organizers should choose material/clinical scenario combinations that reflect the general range of cases referred to a clinical center. For an EQA of NIPT for fetal sexing these may include cases that reflect samples

from male pregnancies validated as representing a male specific signal well within the detection range expected of a test validated for clinical use. In addition, samples from female pregnancies validated by at least two reference centers as being free i.e. below the limit of detection of contaminating male material by more than one sensitive methodology may be included. In addition, samples can be included from a range of gestational age (within the known detectable limits) to reflect the samples routinely tested by the laboratory. More challenging cases designed to be close to the limit of detection expected of a clinical assay should be presented infrequently and laboratories should not be penalized for failing to detect a signal that falls outside of their stated assay limit of detection.

The information accompanying the EQA referral should be adequate and for an NIPD EQA must include the estimated gestational age of the referred pregnancy. The cases should be sufficiently documented to allow the laboratory to select its approach and analytical methods and answer the clinical question associated with the challenge and issue appropriate recommendations, for example, for a repeat sampling. It is imperative that laboratories understand that their testing protocols should not be amended in order to test the EQA samples and participate in the EQA scheme. EQA is an assessment of routine testing to give a measure of the standard of service provided to their service users.

Assessment of EQA Performance; Genotype, Interpretation and Reporting

The primary measure of performance resulting from molecular genetics EQA is the ability of the participating laboratory to detect and correctly call the qualitative genotype (in this case, either the presence or absence of a male specific signal within the limit of detection).

Molecular genetics EQA schemes frequently include an interpretative element. This may be split into the clinical interpretation of the genotype and the accuracy and clarity of the clinical report. Therefore, EQA assessors make a judgement on the utility of the report as a document conveying information to the recipient, advising on clinical actions and forming a permanent part of the medical record. To do this, they use a number of specific performance requirements informed by Good Practice for the participant to achieve a full score. For both the genotype and interpretative elements the assessment team will assemble a proforma for assigning a quantitative score. A system used by some EQA schemes is a presumptive score of 2.0 with fractions of marks deducted for failure to indicate key interpretive points.

EQA assessors may consider that some elements of a report are essential and deduct marks if they are not present. Assessors may expect that choices for the patient are mentioned; examples include further tests. Although clear advice may be offered to the clinician to help avoid failure to act on critical implications of the test, it is vital that reports should not be in any way be interpreted as being directive to the patient. In assessing the EQA returns, assessors do not penalise the same error

twice. If an incorrect genotype is indicated the interpretation of that genotype is not marked. If a systematic error appears across more than one EQA challenge or case (for example a sample exchange problem) this is penalized once.

EQA schemes comment on the style of report layout and clarity referring to guidelines on clinical reporting (http://www.oecd.org/env/ehs/testing/).

However assessment of report style is usually restricted to comments. The presence of sufficient unique patient and sample identifiers are required on each report and errors in the transcription of essential data for example in such patient identifiers will lead to a deduction of marks.

EQA and Poor Performance

EQA providers have a duty to protect the public from sub-standard and potentially dangerous clinical laboratory practice. Many EQA providers therefore set peer reviewed minimum performance criteria. Laboratories may make errors in EQA schemes indicative of 'poor performance.' The EQA scheme organizer will contact the laboratory director to advise them to take measures to correct the process error detected. Laboratories may be offered either assistance or reference samples or technical advice from a peer center and be offered or required to participate in a supplementary round of EQA.

In some jurisdictions, EQA providers are obliged to report poor laboratory performance to a regulatory body that may monitor the laboratory's performance. Where EQA performance does not improve, the official body may take additional measures to protect the public, for example stopping the lab offering that test. Poor performance in EQA is a powerful indicator of competence in a specific area and EQA providers have reported that some laboratory directors have decided to withdraw from providing individual services on the basis of errors revealed through an EQA scheme.

Learning Lessons from EQA

EQA participation is a valuable opportunity for clinical laboratories to compare their performance against their peers and against agreed standards of practice. It also serves to complement and validate the internal quality control measures they have in place [5]. To be compliant with accreditation standards laboratories should review their performance immediately after they receive their EQA report with all levels of staff and put into action measures to correct serious deficiencies. Less critical comments should also be considered from EQA assessors relevant to accepted good practice (ISO standard 15189). Overall EQA performance especially where laboratories are involved in a number of test specific schemes must be a part of the annual management review of the Quality Management System. A review of EQA records is also a key part of an external accreditation audit.

EQA and Post Market Surveillance

EQA is a valuable method of assessing the performance of *in vitro* diagnostic devices (IVDD). Manufacturers ought to be alerted by the EQA provider when an IVDD performs poorly in a number of laboratories in an EQA challenge. The EQA provider should issue an alert to relevant national and multinational authorities responsible for the surveillance of IVDDs for example the US Federal Drug Administration (FDA) and UK Medicines and Healthcare Products Regulatory Agency (MHRA).

EQA and Good Practice

There is a strong relationship between EQA and the formulation of best practice guidelines. Data gathered from EQA showing an unacceptable variation in performance and practices can help set the agenda of a best practice meeting. In turn, guidelines inform both EQA assessors in marking EQA returns and help laboratories develop their internal quality control systems.

3 Conclusion

Medical tests based on the analysis of cfDNA are often critical to the care of patients. It is vital that laboratories embarking on these tests thoroughly validate their analyses, offer them in the context of an operating Quality Management System and submit their procedures to external audit through EQA as it is developed and accreditation by a recognized authority.

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Societal Aspects: Ethics

Celine Lewis and Lyn S. Chitty

Abstract Non-invasive techniques to diagnose genetic disease have advanced rapidly in recent years and include prenatal diagnosis for fetal aneuploidy and single gene disorders, and early screening and diagnosis for people affected by cancer and diabetes. In this chapter we consider some of the key ethical issues arising as a result of this new technology. We highlight how non-invasive testing offers a number of significant benefits to patients including safe and early testing. Issues that have been raised as concerns include the erosion of informed decision-making, pressure to test, testing for non-medical reasons or for information only and the broader societal impact that the widespread introduction of non-invasive testing may have. These are issues that should be considered when developing practice guidelines.

Keywords Circulating DNA • NIPD • Prenatal diagnosis • Regulation • Consent guidelines • Direct-to-consumer testing • Ethics • Predictive medicine • Non-invasive tests • Social impacts

1 Introduction

The recent advances that have taken place in the molecular analysis of circulating nucleic acids have opened up a whole host of new opportunities for the diagnosis of genetic disease. These include the development of new non-invasive techniques for

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prenatal diagnosis (NIPD) for fetal aneuploidy and single gene disorders, and the possibility for early screening, diagnosis and monitoring of treatment for people affected by conditions such as cancer and diabetes. Whilst these advances have the potential to offer significant benefit to patients, as with any new technology, it is important to pause and consider the wider ethical implications before they are implemented into clinical practice. In this chapter we will reflect on some of the key ethical issues that have arisen in light of these new diagnostic capabilities, the first part relating to current, clinically applied, fetal analyses and the second part considering the more general applications of CNAPS in disease both actual e.g. cancers and diabetes and genetically possible-but-not-probable.

2 cffDNA in Maternal Plasma

The discovery of cell free fetal DNA (cffDNA) in maternal plasma in the late 1990s was a seminal step towards the goal to develop a non-invasive test to diagnose genetic conditions during pregnancy [1]. Traditionally, prenatal diagnosis has involved the use of invasive tests – either amniocentesis from 15 weeks or chorionic villus sampling (CVS) from 11 weeks – for definitive diagnosis. However, these tests carry a small but significant risk of miscarriage, between 0.5 and 1 % [2]. An approach to prenatal diagnosis based on the analysis of cffDNA offers the possibility for a safer, non-invasive approach based on a maternal blood sample rather than current invasive diagnostic techniques which require the insertion of a needle into the uterus. Furthermore, as cffDNA has been detected in the maternal circulation from around 5 weeks and increases with gestation before being rapidly cleared from the circulation after delivery [3], NIPD can potentially be offered earlier than traditional invasive prenatal tests and is pregnancy specific.

The first clinical applications of NIPD have included fetal sex determination [4], Rhesus D genotyping in mothers who are Rhesus D (RhD) negative [5] and paternally inherited single gene disorders or conditions arising de novo, such as achondroplasia [6]. NIPD for recessively inherited single gene disorders where both parents carry the same mutation or for X-linked conditions is more complex as there are high circulating levels of mutant DNA emanating from the mother. However, research taking advantage of new technologies indicates that NIPD may soon be possible for these conditions [7, 8]. Similarly, NIPD for aneuploidies has advanced rapidly and it is now possible to identify pregnancies affected by Down's syndrome from 10 weeks gestation with high accuracy (>99 %) and a low false-positive rate (around 0.3–0.5 %) [9]. Detection rates are also high for trisomy 18 (>99 %) and trisomy 13 (up to 90 %) [10–12]. Indeed, following the publication of a number of large validation studies, several companies now offer NIPD for aneuploidy, and these tests are widely available in the private sector in the US, parts of Asia and parts of Europe [13]. NIPD for aneuploidy is currently not considered as accurate as invasive testing with false negative results being reported, probably due to low levels of cffDNA either because of early gestation or because the fetal fraction

tends to be lower in obese women [14]. False positive or discordant results are also regularly reported and are a result of a variety of factors but reflect the fact that NIPD analyses both maternal and fetal cell free DNA, and that the cffDNA emanates from the placenta. Thus, the reported aetiology of discordant results includes confined placental mosaicism [15], maternal chromosome abnormalities and mosaicism [16] and, very rarely, maternal malignancy [17]. In view of these observations, invasive testing is recommended to confirm a positive NIPD for aneuploidy result (for that reason the test is often referred to as non-invasive prenatal testing or NIPT, however for consistency we will refer to NIPD throughout this chapter). In addition, in some cases (~4 %) the test may have to be repeated, particularly when the fetal fraction is low.

Whilst the clinical benefits of a non-invasive test are clear, it is also important that we consider the ethical and psychosocial issues that arise as a result of this technology, and in recent years a number of studies have been conducted in this area. These include studies examining the attitudes of pregnant women [18–20], couples who have used NIPD for fetal sex determination or for the diagnosis of a monogenic disorder [19, 21, 22], the general public [23], health professionals [24–28] and ethical commentators [29–36]. As a result, the practical and ethical implications of NIPD have been explored widely. Whilst many of the issues raised are not necessarily new to the ethical and social debates associated with existing prenatal diagnostic practices, the ease with which NIPD can be conducted and the risk-free nature of the test may exacerbate existing issues. For that reason, re-examination of the key ethical issues in light of this new technology is important. Here we present a summary.

3 Benefits of NIPD

Research, with key stakeholders, highlights that NIPD is seen as a positive advancement in prenatal care with a number of notable advantages over invasive testing and screening in the case of aneuploidy. The practical benefits are that the test is safe (i.e. there is no miscarriage risk); it reduces the need for invasive testing; and can be conducted early in pregnancy (from as early as 7 weeks for fetal sex determination), allowing more time for decisions around invasive testing and termination of pregnancy. Termination is considered to be safer if conducted earlier in pregnancy as the risk of complications associated with a surgical procedure, which can be performed in most units up until around 12 weeks gestation, is considered to be less than that associated with a medical procedure. For those continuing with the pregnancy, the information can be useful as it allows parents time to prepare practically for the birth of an affected child. In some cases the information may inform pregnancy management, such as the provision of antenatal dexamethasone that is used to reduce external genital virilisation in female fetuses affected by congenital adrenal hyperplasia, or obstetric management around delivery for male pregnancies at risk of haemophilia. Other advantages cited by stakeholders include

that the test is procedurally easy to conduct as it is a blood test and has a high level of accuracy [20–24].

As well as the practical benefits of NIPD such as venepuncture being easier to perform requiring less specialised healthcare professionals, earlier diagnosis has been found to provide a number of psychological benefits. For prospective parents who choose to continue with the pregnancy where the fetus is affected, there is more time to come to terms with the diagnosis and adjust to the information. Knowing the status of the fetus may also provide peace of mind early on during the pregnancy: if the fetus is unaffected the prospective parents can relax and enjoy the remainder of their pregnancy, if the fetus is affected there is peace of mind knowing that appropriate pregnancy management is in place. Women have also spoken about NIPD empowering them to regain, at an early stage, a sense of control over their pregnancy [19, 21, 22]. Finding out that the fetus is unaffected within the first trimester can also help to 'normalise' the pregnancy for some women as all pregnancies are at increased risk of spontaneous miscarriage during that time [22]. For some women, particularly those that have purposely disengaged with the pregnancy due to the risk of the fetus being affected, NIPD enables them to reengage and bond with the fetus much earlier than they would have otherwise [21, 22]. For those who do not want to continue with the pregnancy, the possibility of an earlier termination may also be less emotionally traumatic and procedurally easier as surgical rather than medical termination can be performed. An early termination is also perceived by some as being less contentious ethically [22, 25]. This view accords with the predominant view in most Western countries that the moral status of the fetus increases with gestational age [37]. Thus, NIPD offers a range of practical and psychological benefits to women.

4 Concerns About NIPD

Whilst the practical and psychological advantages of a safe and early test are clear, a number of concerns have been expressed. These include to how to ensure the test is offered appropriately in a way which safeguards informed consent, to whom we offer testing and for what conditions, and the broader societal impact that widespread introduction of such testing may have.

4.1 Informed Consent and Routinisation of Testing

It is widely accepted that a key goal of genetic counselling for prenatal testing is to support women to make an informed choice, which is defined as one that is based on relevant knowledge, consistent with the decision-maker's values, and behaviourally implemented [38] and as such, allows women to exercise their reproductive autonomy [39, 40]. One of the major concerns that has been raised is the potential for

NIPD to undermine informed consent [30, 41], an ethical principle that is seen as having been assured when clients are 'enabl[ed] . . . to make informed independent decisions, free from coercion' [42]. This concern is particularly relevant to implementation of NIPD into routine antenatal practice as part of the Down's syndrome screening pathway. Currently, all pregnant women in the UK are offered prenatal screening for Down's syndrome. These tests are increasingly delivered by a combination of fetal ultrasound and maternal serum biomarkers in the first trimester and provide an individual risk estimate [43]. Women who are 'high risk' ($\geq$150 in the UK) are then offered invasive testing for definitive diagnosis. Thus, the process currently includes two steps; first, a decision whether or not to accept screening followed, for women predicted to be at high risk by the first stage, by a further decision of whether to undergo invasive testing. The nature of having a '2-step' process as well as the risk of miscarriage associated with invasive testing are thought to be psychological barriers that prompt women to think carefully about their options. By reducing the testing process to just 1-step as well as removing the risk of miscarriage, the concern is that women may not fully consider their decision to take the test, particularly if pre-test counselling is not delivered appropriately. This concern is particularly acute given that research has already shown some women do not making fully informed decisions about current screening tests [44]. Furthermore, evidence indicates that health professionals may view the consent procedure for NIPD less stringently than they would for invasive testing [28] and that health professionals may view aspects of testing very differently from the women undergoing testing [45]. These concerns highlight the need for thorough counselling and consent procedures when offering NIPD. Practices that may serve to maintain good levels of informed consent include the availability of written patient information, a delay between the discussion of the test and test delivery, and the use of a formal signed consent form, as is currently the practice when offering invasive diagnostic testing.

The following practices are considered important in enabling informed consent to take place:

- Professionals who provide genetic counselling should have appropriate education and training to enable them to facilitate decision-making and understand the individual needs of the counsellee.
- Provision and understanding of information related to:
  - The condition being test for
  - The risk that the fetus will be affected
  - The test itself (including test procedure, when the test can be conducted, accuracy of test results, risks associated with the test, limitations of the test, when the results will be delivered, how and by whom, and the options available after the results are known)
  - Confidentiality of test results

(continued)

- Whether the test results could have implications for future insurance of individuals involved.

- Assurance of the counsellee's understanding.
- Psychological support which includes ensuring counsellee's:
  - Take time to think through the decision, including what they would do if the fetus is affected
  - Think about the impact of the condition on the child and his or her quality of life
  - Think about the impact of having a child with the condition in the context of their own lives and those of their family members.
- Signposting to support groups and good quality information sources, including the opportunity to access information about quality of life and living with a condition from people with first-hand knowledge (e.g. disease specific lay support organisations).
- Availability of written information.
- Allowing sufficient time between the discussion of the test and test delivery.
- Encouraging independence in the decision-making process.
- Ensuring consent is given freely without undue coercion after having received appropriate information.
- The use of a formal signed consent form.

These guidelines have been adapted from the following sources: [26, 46]

A related concern is that offering NIPD to women on a wide scale through a blood test with no risk of miscarriage may lead to 'routinisation' of testing. This implies that the test will come to be seen as part of routine prenatal care rather than a choice which a person actively chooses (i.e. a test which you opt-in to rather than opt-out of). Commentators have generally aired this concern in relation to using NIPD for aneuploidy as this is the most common genetic condition tested for during pregnancy, although this should not detract from the possibility of NIPD becoming routinised in other circumstances, for example, following sickle cell or thalassaemia carrier screening during pregnancy [33].

4.2 Pressure to Test

As highlighted previously, when a person acts autonomously, they exercise an informed choice free from undue influence. Autonomy is seen to be compromised (and thus informed consent invalidated) when an individual's decision is subject to coercion or illegitimate pressure. It is possible that, by removing the risk of miscarriage, the ease of testing will contribute to women feeling pressured to take

the test as they can no longer argue that the risk of loosing a normal pregnancy precludes them from undergoing testing. This pressure may stem from a number of sources; women's fear of being judged irrational or irresponsible if they don't take the test, due to pressure from a partner or family member, as a result of how the test is conveyed in pre-test counselling, or because it is freely available and offered by a 'trusted' health professional. Subtle pressure may also result from the way we as a society view prenatal testing, for example, evidence indicates that the availability of prenatal tests can create a situation whereby women feel they are being 'bad mothers' if they do not use all the technology available to them [19, 23, 29]. As a result, women who do not want to have the test, for whatever reason (such as they would not terminate an affected pregnancy) may not feel justified in declining NIPD. Again, such concerns highlight the need for appropriate pre-test counselling to ensure that women make informed decisions in line with their own beliefs and values.

4.3 Scope of NIPD

One of the most debated issues raised in relation to NIPD is how the ease and risk-free nature of the test will impact the scope of prenatal testing. Will people test for a wider range of conditions? How will we draw the line between medical and non-medical testing and what we should and should not test for? And how will these technologies be regulated? Whilst the spectrum of conditions tested for will partly depend on the capabilities of the test itself, it is worth reflecting on the potential implications of testing for a broader spectrum of conditions including complex conditions, adult onset conditions and testing for increasingly minor abnormalities, something referred to as 'specification creep'. Whilst these concerns are not necessarily new to prenatal testing, the ease and risk-free nature of NIPD could be an incentive to test for conditions that we would not have tested for previously through invasive testing given the risk of miscarriage.

As stated by Hall et al. [35], it seems likely that existing standards of clinical practice in prenatal testing will be used as a benchmark for deciding whether NIPD should be used for a particular clinical application. At present, prenatal testing (either invasive testing or pre-implantation genetic diagnosis) is allowed in the UK for adult onset conditions which are serious or life-limiting or where there is likely to be exceptional psychosocial burden experienced by parents, such as Huntington's disease or BRCA1 testing, where the information is used to guide pregnancy management. As such, NIPD does not raise any new ethical concerns as long as professionals continue to exert the same standards of care when offering these tests as they do currently. One ongoing concern, however, is where parents want prenatal testing for adult-onset conditions but are not considering terminating an affected pregnancy, and whether this scenario will occur more frequently as a result of the availability of NIPD. In such a scenario, the rights of the future child may be violated as they have not given their consent. Testing of children is

generally regarded as unacceptable because it deprives the child of his or her right to self-determination including the decision of whether to be tested once mature enough to do so [31]. As such, using NIPD for this reason would also be considered morally unsound unless there were extenuating circumstances. Furthermore, in some circumstances, knowledge of carrier status for a gene with high penetrance such as Huntington's disease or BRAC1 may affect the individuals access to health insurance and other benefits. Whilst this is not currently an issue in the UK, the issue is debated frequently and attitudes may change over time and with increasing use of NIPD.

Concerns about the use of NIPD for social sexing are also frequently cited in the ethics literature [41, 47]. Currently, the application of NIPD for fetal sex determination is allowed if the fetus is at risk of a condition that affects a particular sex, such as Duchenne muscular dystrophy or congenital adrenal hyperplasia. The use of NIPD to determine the sex of the fetus for these conditions is not considered to be ethically contentious as the information can be used to guide the need for invasive testing and/or inform treatment [4]. However, the ability to determine the sex of the fetus using NIPD has raised concerns that people might use this technology for non-medical reasons. Parents may want their child to be a particular sex for personal, cultural or economic reasons such as family balancing or because a particular sex is more highly valued within a society, and there is general agreement amongst health professionals that this should not be permitted. There have also been calls for international guidelines to regulate the non-invasive detection of fetal sex [48]. As highlighted by Newson [41], the burden of proof for consent to testing should be made the responsibility of the test provider to ensure that sex selection is conducted for legitimate medical reasons.

A further concern is what impact NIPD will have on the disabled community [23, 29, 49]. Given that the risk of miscarriage related to invasive testing is a key psychological barrier to diagnostic testing [50], it seems inevitable that removing this barrier will invariably lead to an increase in the number of fetuses diagnosed prenatally which may in turn lead to an increase in the number of terminations, particular for Down syndrome, the most common chromosome abnormality. Prenatal testing has been widely criticised in the disability rights literature because it is perceived that the very endeavour of seeking to prevent genetic disability and disease discriminates against and devalues the disabled community [51]. Assuming that NIPD will reduce the number of people being born with genetic conditions, the concern is that this discrimination will be exacerbated or that attitudes about the acceptability of continuing with a pregnancy where the fetus is affected will be subtly altered. For that reason, disability rights activists have highlighted the importance of providing prospective parents with information, based on the experiences of families who have children with disabilities, about what that particular disability is like for the child and their family.

4.4 Genome-Wide Sequencing

Studies have shown that it is now possible to conduct a genome-wide analysis of fetal DNA to diagnose genetic disorders prenatally in a non-invasive way [52–54]. This would, in theory, make it possible to conduct multiple genetic tests concurrently with a single non-invasive test. This new approach raises a number of ethical, legal and social issues that require consideration. One argument for this type of testing is that, assuming parents have made an informed choice to take the test, they are empowered with more information and control over their pregnancy than they would otherwise have had. Thus, the information derived through whole genome sequencing would enable them to make informed decisions which would lead to the best possible outcome for them, i.e. the prevention of the birth of a child with a genetic condition or time to prepare for the birth of a child with a genetic condition. It could also be argued that if it is possible to conduct such a test and if parents make an informed decision to take the test, not allowing them to do so would be to deprive them of their right to autonomous reproductive choice [33]. Despite these potential advantages, a number of concerns have been identified which we will briefly summarise.

One major concern is whether it would be truly possible to give informed consent given the spectrum of genetic conditions that might be identified through whole genome testing. Even if we only tested for the most common chromosomal conditions and single gene disorders, these will all differ significantly in terms of prognosis, treatment and outcome. Complex testing will also inevitably uncover findings of unclear significance which are difficult for health professionals to interpret and patients to understand. Instead of empowering parents, we may end up 'overloading' them with information, creating additional anxiety and confusion, and ultimately diminishing their capability to make informed decisions about testing. Counselling sessions would also require more in-depth discussion about the different conditions being tested for and the possible outcomes of test. This would be likely to require more intensive and lengthy counselling sessions which would be costly.

4.5 Need for Regulation and Ongoing Research

To address these concerns, the development of regulations and best practice guidelines will be essential to ensure that NIPD is offered within agreed clinical pathways. These will need to take into account the views of all stakeholders (including patients, clinicians, scientists, policy makers and ethicists) to ensure that the range of conditions for which NIPD is available lies firmly within the boundaries of what is considered ethically as well as clinically acceptable. Ongoing monitoring and research is therefore vital. An important issue will be how NIPD is regulated, particularly if it is available outside the realm of clinical genetics where high standards of ethical practice are firmly entrenched through bodies such as the Association of Genetic Nurses and Counsellors and the European Society of Human

Genetics. Establishing regulations of testing and guidance from professional bodies outside of clinical genetics is therefore critical.

4.6 Equity of Access to Testing

NIPD for aneuploidy is currently only available in the private sector with costs ranging from £400–£750 in the UK. Implementation of this test has been wholly commercially driven, however, there is currently research being conducted in the UK looking at whether and how NIPD for aneuploidy should be implemented into a public sector health care system [55]. The cost of these tests, which are based on next generation sequencing technology, are high and economic constraints are likely to restrict the way they are offered in the NHS [56]. This may result in the test being offered as a sequential test to those women identified as high risk through current screening (i.e. as an alternative to invasive testing), rather than to all women. However NIPD for aneuploidy is offered, screening policy and practice will need to ensure that access to testing is offered equitably to avoid a 'postcode lottery' system where only women in selected antenatal clinics have access to it, an issue that has caused much furore in the past in the case of the combined test or access to IVF.

4.7 Testing for Information Only

A further consideration is whether, particularly in times of economic hardship, public sector health services should be offering tests if women would not use the information to guide decisions about termination or treatment during pregnancy. Whilst this issue is not necessarily new to prenatal testing, given the non-invasive nature of NIPD, it seems likely that a larger number of women will use the test for information only (i.e. to plan and prepare for the birth) rather than to guide pregnancy management, and raises the question of whether the advantages of a diagnosis that has no immediate clinical benefit are outweighed by the cost of offering that test. Further concerns also relate to the use of NIPD for an early result when the same information can be achieved safely later in pregnancy at the time of other routine tests, for example fetal sex determination in pregnancies at high risk of haemophilia to inform the management of labour. This information is only required at the end of pregnancy and can be easily obtained at no extra cost at the time of the routine fetal anomaly scan [24].

The practical and psychological benefits of NIPD for information only are difficult to quantify from a cost perspective, however we do know that for many women reassurance, control, peace of mind and relief from uncertainty during their pregnancy are important, particularly for those women who may be at increased risk either as the result of a family history of a single gene disorder, as the result of a previously affected pregnancy, or because they have been identified as high risk through screening [19, 21, 22]. Furthermore, women may not know how they will

react to a prenatal result prior to receiving it, and their views on what constitutes the best course of action for them and their family may change after receiving an adverse result. Service providers and health care commissioners will therefore have to carefully consider whether they can justify only offering NIPD to those women who are using the test to make decisions about whether to continue their pregnancy given that the benefits of information for preparation and planning are well established.

4.8 Prenatal Diagnosis of Single Gene Disorders

Autosomal recessive conditions such as sickle cell or cystic fibrosis can only be passed on to the child if both parents are carriers. To be certain that the pregnancy is at risk, the carrier status of both parents must be known. Nevertheless, one can identify certain situations where this might be complicated, for example when the father of the child is unknown or absent, or where the father does not want to be tested. In such cases, the decision to undergo invasive testing is likely to be difficult given the uncertainty around whether the fetus is even at risk of inheriting the condition. An advantage of NIPD is that it enables confirmation of the status of the fetus without the risk of miscarriage, without the need to know the carrier status of the father.

One issue that has raised concern, however, is if the father does not want to know his carrier status. Testing the fetus would violate his right not to know as if the fetus was found to be affected, this would then confirm the father's carrier status. Again, this is not an issue which is unique to NIPD as it also applies to invasive testing, but given the risk-free nature of NIPD it is a situation that may arise more frequently. In the UK, the ultimate decision-maker concerning prenatal testing is the mother, as it is her body, her pregnancy and therefore her right to accept or decline tests. As such, even if her partner did not want to be tested, she would legally have the right to overrule the father's right not to know. One can also apply the ethical argument that finding out the status of the fetus is justified on the grounds that the information is primarily about the fetus; finding out the father's carrier status is a foreseen but secondary consequence. Nevertheless, even though the mother would be ethically and legally justified in testing the fetus without the consent of the father, such situations would still need to be handled sensitively.

4.9 Circulating cell free DNA for Use in Early Diagnosis and Prognosis

Much of the social and ethical discussion around the uses of circulating cell free DNA (cfDNA) has focused on its use prenatally, mainly because the clinical application are most developed in this area. Nevertheless, other possible applications of this technology including early screening and monitoring of patients for

conditions such as cancer and diabetes are being researched [57]. Non-invasive testing for screening purposes has a number of notable advantages over traditional tests. First, the test may be more sensitive and easier to deliver and eliminate the need for more invasive approaches. This may make such screening available to a wider proportion of the population. Moreover, this approach may enable diagnosis at a much earlier stage in the disease process resulting in earlier intervention or treatment and thus a potentially better outcome for the patient. In considering the ethics, some of the concerns raised in relation to NIPD are also relevant here. For example, the relative ease and risk-free nature of the test may create a situation whereby patients feel pressured or coerced into testing. Nevertheless, we have considered some further ethical issues that might apply to using non-invasive testing for screening, diagnosis, prediction of prognosis and monitoring treatment of patients.

4.10 Testing for Conditions of Variable Severity or for Which There Is No Cure

Some conditions have variable severity. This means that one patient who is suffering from the disease may have more severe symptoms than another. For patients affected by conditions of varying severity, receiving an early diagnosis can be a mixed blessing. A good example of this is prostate cancer. Some patients will find an early diagnosis of prostate cancer helpful. They may choose to have surgery or more regular check-ups to monitor the progress of the condition. They may feel that the information enables them to take pro-active steps to look after their health. For others, early diagnosis can cause a great deal of anxiety, particularly if the cancer is slow-growing, does not reduce life expectancy and does not need treatment. Thus, one concern is that because tests based on circulating nucleic acids can be done early and easily, we may end up 'over treating' patients and ultimately cause more harm than good. A good example is the use of mammography in current breast cancer screening programmes, where it now appears that many lesions detected are benign but yet have resulted in significant stress, and in some cases unnecessary surgery. Accurate prediction of prognosis through knowledge of the natural history of disease is an essential prerequisite of any screening programme. It is to be hoped that the increasing understanding of the genetic markers associated with many cancers, will help predict outcome on a personalised basis and thereby facilitate tailored counselling and treatment [58].

A further concern relates to whether the benefits of early diagnosis for conditions for which there is no cure or treatment outweighs the potential harms that may arise. For example, some people may find an early diagnosis useful as it provides an opportunity to plan one's future. For others, the information might cause worry and feelings of helplessness. Any decision to test for conditions with variable severity or for which there is no cure or treatment will require appropriate support and counselling prior to decision-making so that patients do not make decisions they

later regret. Furthermore, the knowledge of carrier status these tests may provide may, as discussed for NIPD, impact on the individual's ability to access life or health insurance, or restrict ability to get a mortgage or impact on the practical aspects of life in many other ways.

4.11 Accuracy and Reliability

In considering the application of this technology to diagnose and treat patients, we must take into account the accuracy and reliability of the tests. Current research indicates, for example in some cancers, that sequencing techniques can be targeted to detect very low levels of known mutations in tumour DNA. However, screening populations to detect any one of many potential DNA changes will require whole genome scanning with the associated generation of vast quantities of data [59]. Given that much of the work in this area is being conducted on a research basis, thorough validation of this approach will be required prior to its use as a screening, diagnostic or monitoring tool [57]. Additionally, there will be issues around data storage, who has access to the data, how long it should be stored for and in what format. Analytical techniques based on sequencing are generating a completely new set of challenges, both technical and ethical which need to be addressed before widespread implementation. Furthermore, some patients may have greater confidence in traditional tests which target the part of the body affected by the illness, for example cervical smears or mammography, and may prefer to go down traditional testing routes. As such, pre-test counselling will require reassurance about the accuracy and scientific background underpinning the test which some may find difficult to understand (this is, of course, also an issue for NIPD and much work developing patient information has been conducted to address this issue).

5 Interpretation and Delivery of Test Results

Pre-test counselling and interpretation of genetic tests has traditionally been delivered by a genetic healthcare specialist. If the promise of non-invasive testing for complex conditions is realised, these may increasingly be delivered by healthcare professionals outside the genetic specialty, such as oncologists or endocrinologists. Whilst these practitioners are clearly best placed to discuss the diseases in question, one concern is how to ensure the practitioners offering these test are sufficiently trained in order to interpret the test results, particularly if they rely on a significant level of knowledge and understanding of genetics and genetic technology. Moreover, if those practitioners offering the test are unable to adequately interpret the test results, genetic specialists will increasingly be relied upon to conduct this work. This raises questions around whether genetic departments will have the capabilities to take on this additional workload and if not how this can be managed. It will be

important to consider these issues now so that we do not reach a situation whereby technology outpaces clinical capability.

6 Direct-to-Consumer Testing

Finally, one area that has received much attention in the past few years is the issue of direct-to-consumer (DTC) testing. Genetic testing (prenatally and postnatally), traditionally has been delivered by specialist health professionals who provide pre-test counselling as well as convey and interpret test results to patients and families. Given the relative ease of conducting non-invasive tests, it is possible that these tests could become easily accessible for a range of conditions outside of the genetic clinic (or other health specialist for that matter) and delivered direct-to-consumer without adequate regulation. DTC tests are becoming increasingly popular given their accessibility over the internet along with the public's growing 'genetic curiosity' in the post genomics era [60]. These tests are fairly controversial, with many ethical arguments for and against them. One of the arguments in support of DTC testing is that such tests enable individuals to make autonomous choices about their health, or in the case of prenatal testing, the health of their future children. Thus, DTC testing might be seen to empower individuals to take control of their health. Moreover, it could be argued that my genetic make-up is something which belongs to me personally and I therefore have a right to access it without the need to involve a health professional.

Nevertheless, whilst these might be persuasive arguments, they must be weighed up against the potential for DTC testing to threaten the wellbeing of the individual. One key criticism of DTC tests is that in order to make an informed choice about whether or not to take a test, the individual needs to be appropriately informed about the value, risks and benefits of that test [61]. Although some DTC companies offer genetic counselling following receipt of test results (usually online or via telephone), it may not stand up in quality to the pre and post test counselling provided through genetic clinics. Genetic counselling, usually conducted face-to-face with a qualified health professional, is viewed as an important aspect of ensuring that individuals make informed decisions in line with their personal beliefs and values based on a clear understanding of the clinical and psychosocial value of the test. Where this is not provided, individuals may misunderstand the nature of the test, misinterpret the test results or receive information that is harmful to them. For these reasons, the professional community have tended to err on the side of caution when it comes to DTC testing (with some actively discouraging the tests) [61]. Further concerns exist with regards to the privacy and confidentiality of personal genomic information stored by private companies [60]. How will such companies use this information and who will have access to it? Will personal information be transferred to third parties for profit? Effective legislation is therefore essential to protect consumers. Wherever you may stand on the issue of DTC tests, patients and the public require clear unbiased information as to the benefits and potential

disadvantages of DTC testing and the legislations in force to protect their privacy so that they can make informed decisions when considering testing.

7 Concluding Remarks

We have tried to present a brief overview of the key ethical issues associated with the use of circulating nucleic acids to diagnose and monitor disease. The application of this new and powerful technology is advancing rapidly. Yet, reflection on how it is likely to impact us as service users and more broadly as a society is vital if we want to ensure the ethical use of the technology and protect against its misuse. It is only by taking part in such dialogue that we can ensure policy and practice is in line with our values and beliefs.

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