Review

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The rising tide of cell-free DNA profiling: from snapshot to temporal genome analysis

https://doi.org/10.1515/labmed-2022-0030
Received March 8, 2022; accepted May 24, 2022;
published online June 21, 2022

Abstract: Genomes of diverse origins are continuously shed into human body fluids in the form of fragmented cell-free DNA (cfDNA). These molecules maintain the genetic and epigenetic codes of their originating source, and often carry additional layers of unique information in newly discovered physico-chemical features. Characterization of cfDNA thus presents the opportunity to non-invasively reconstruct major parts of the host- and metagenome *in silico*. Data from a single specimen can be leveraged to detect a broad range of disease-specific signatures and has already enabled the development of many pioneering diagnostic tests. Moreover, data from serial sampling may allow unparalleled mapping of the scantily explored landscape of temporal genomic changes as it relates to various changes in different physiological and pathological states of individuals. In this review, we explore how this vast dimension of biological information accessible through cfDNA analysis is being tapped towards the development of increasingly powerful molecular assays and how it is shaping emerging technologies. We also discuss how this departure from traditional paradigms of snapshot genetic testing may pave the way for an onrush of new and exciting discoveries in human biology.

Keywords: cell-free DNA; circulating tumor DNA; liquid biopsy.

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Introduction

It is now widely understood that whole or partial genomes of diverse origins, including host cells, fetal cells, microbes, and viruses, are continuously shed into various human body fluids in the form of cell-free DNA (cfDNA) [1–4]. While much remains to be discovered about the fundamentals of the molecular, biological and physiological factors that affect the properties of these molecules, it is clear that DNA can be released into body fluids through various pathways and subroutines related to the mechanisms of accidental cell degradation, programmed cell death, as well as regulated extrusion [1–4]. CfDNA also appears to be released from all types of cells, tissues, and organs, while maintaining the unique genetic and epigenetic features of their originating source. Moreover, emerging research shows that unique bits of genetic, biological and pathological information (e.g., genomic regions, cell identity, tissue type, and mutation clusters) are often partitioned into differently structured cfDNA subtypes via the action of numerous nuclear, cytoplasmic, and extracellular mechanisms and factors that alter the physico-chemical features of specific genomic regions and different cfDNA subtypes in distinct ways.

Capture, profiling and bioinformatic integration of the information encoded in the sequence composition and various physico-chemical features of cfDNA therefore presents the unique opportunity for the minimally-invasive reconstruction of major parts of the host- and metagenome *in silico*. This information can be harnessed in two major ways: First, data collected from a single specimen can be used to detect a broad range of disease-specific signatures, and has so far enabled the development of several breakthrough medical applications, e.g., diagnostic tests for solid tumors [5–7], fetal abnormalities [8–10], allograft rejection [11–16], and sepsis [17, 18]. Second, dynamic analysis of data obtained from longitudinal sampling may open an unprecedented window of access for mapping the scantily explored and still mostly invisible landscape of temporal genomic changes caused by a variety of factors (e.g., aging, pathology, dietary changes, and medical therapies) (Figure 1).

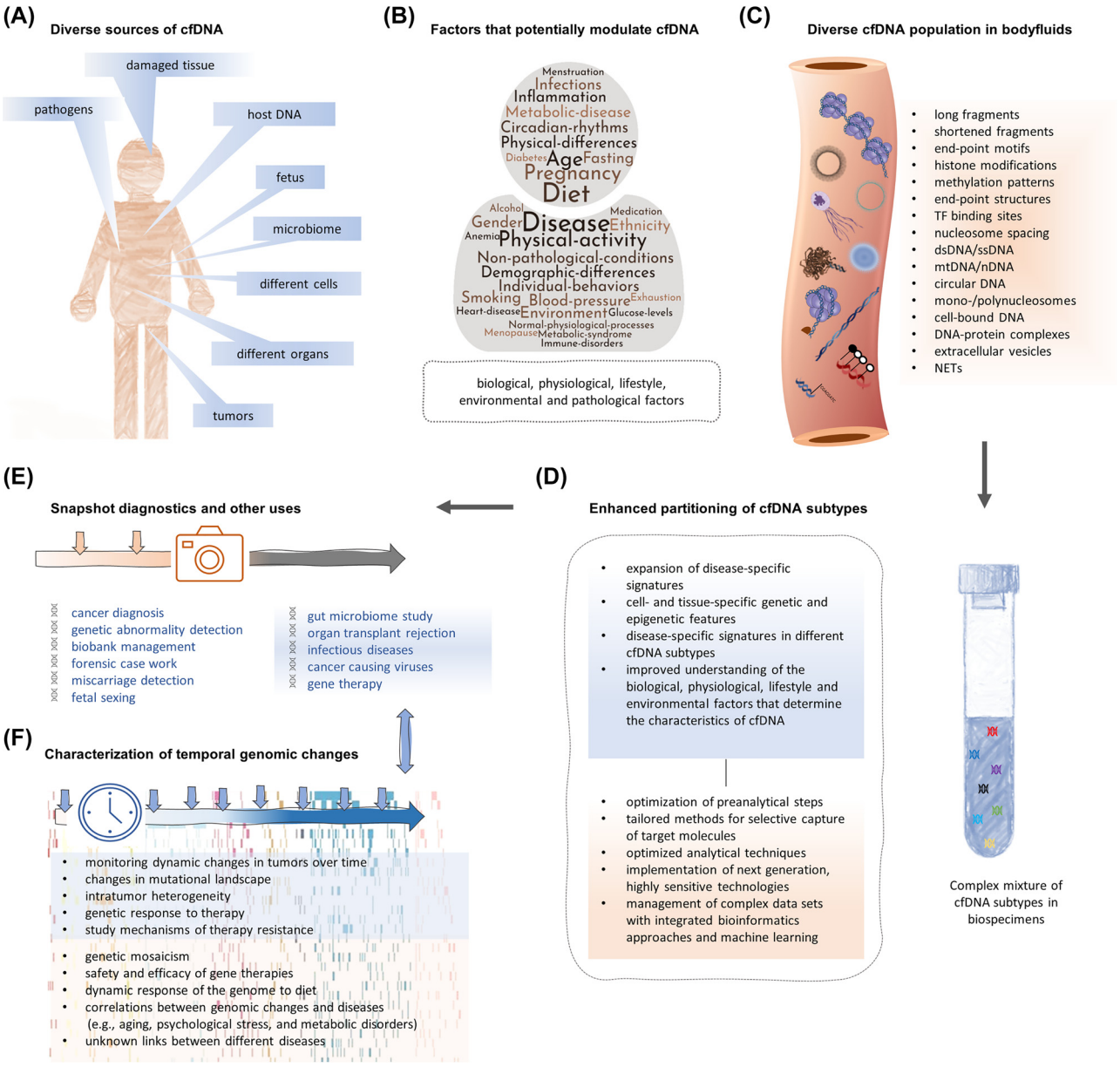

Figure 1: The potential clinical scope of cfDNA profiling.
(A) Genetic material from diverse sources is continuously shed into body fluids. (B) Moreover, the characteristics of cfDNA and its release into body fluids and its subsequent stability and fluctuation are modulated by a wide range of biological, physiological, lifestyle, environmental and pathological factors. (C) Therefore, the composition of the cfDNA population in a biospecimen is highly complex, characterized by the co-occurrence of genomes from various origins and immense genetic, epigenetic, and structural diversity among different cfDNA subtypes. (D) While it is very difficult to analyze a highly heterogeneous cfDNA population in a clinical biospecimen, it is becoming increasingly easy to do so through the coalescence of many ground-breaking advances in the cfDNA research field that allow increasingly effective partitioning and high-fidelity analysis of cfDNA subtypes. These include systematic improvements in preanalytical procedures, analytical techniques, technologies, and bioinformatics, in conjunction with an improved understanding of all the factors that determine the characteristics of cfDNA *in vivo* and in clinical biospecimens, as well as an ever-expanding repertoire of disease-specific markers and tissue-of-origin classifiers. This may enable the development of cfDNA tests that are fit for testing in large-scale cohorts and potential clinical roll-out in the future. (E) On one hand, tests may be developed for the minimally-invasive detection and diagnosis of a wide range of diseases. (F) On the other hand, tests may be developed to map and study temporal genomic changes in individuals or populations in a variety of contexts.

In this review we explore how this new dimension of biological information accessible through cfDNA analysis has already been tapped for the development of new and more powerful cfDNA analysis modalities, how it is shaping emerging technologies, and how it represents a departure from the traditional paradigm of snapshot genetic testing, which may very likely catalyze a surge of new discoveries in human biology.

The immense heterogeneity of the genetic and epigenetic features of the total cfDNA population in a given biospecimen, in combination with the complex network of interacting biological, physiological, and environmental factors that modulate its fluctuation, makes cfDNA a very powerful biomarker and interesting biological phenomenon, but at the same time significantly complicates the selection of appropriate preanalytical steps and analytical differentiation of different cfDNA subtypes. Therefore, in this review we also draw attention to the importance of high-fidelity reverse engineering of a cfDNA sample and explore the technical and analytical challenges and solutions involved in overcoming the heterogeneity of cfDNA samples.

Measurement of total cfDNA levels

Total cfDNA levels have been correlated with a plethora of diseases commonly associated with cell or tissue injury, such as solid tumors [6, 7, 19–21], autoimmune diseases [22] (e.g., systemic lupus erythematosus [23, 24], rheumatoid arthritis [25], and systemic sclerosis [26]), trauma patients [27] (e.g., brain injuries [28, 29] and burn patients [30]), cardiovascular diseases (e.g., acute myocardial infarction [31] and acute coronary syndrome [32]), viral infections (e.g., acute Puumala Hantavirus Infection [33] and Crimean–Congo hemorrhagic fever [34]), benign gastrointestinal tract disorders [35–37], kidney disease [38, 39], lung disease (e.g., chronic obstructive pulmonary disease [40] and pulmonary embolism [41]), thyroid disease [42], pregnancy disorders (e.g., preeclampsia [43] and intrahepatic cholestasis [44]), skin conditions (e.g., psoriasis [45, 46]), stroke [47, 48], and workers exposed to occupational hazards such as pesticides [49], nuclear radiation [50], and toxic paints [51].

CfDNA levels have also been correlated with, for lack of better phrasing, more obscure medical conditions or clinical scenarios such as schizophrenia [52], extra temporal lobe epilepsy [53], sperm quality in men [54, 55], and the quality of embryos [56], pregnancy rates [57], and performance of various stress reduction exercises [58] among women undergoing *in vitro* fertilization. Medical treatments have also been correlated with changes in cfDNA levels, such as surgery [59], radiotherapy [59] corticosteroids [60], hemodialysis [39, 61, 62], and mechanical ventilation [63, 64].

Moreover, many studies have now shown that cfDNA levels are often significantly modulated by a variety of non-pathological conditions, and physiological and lifestyle factors (reviewed in Refs. [1, 3, 6, 65, 66]), such as age [67, 68], body mass index [69, 70], the time of day at which samples are collected [71–74], food intake and creatinine levels [73–75], gender [66, 75], walking [76] and acute exercise [71, 77–82]. Conversely, no correlation has yet been demonstrated between changes in total cfDNA levels and alcohol intake [83], smoking (reviewed in Ref. [66]), a history of betel nut chewing (seeds with stimulatory effects akin to amphetamines and cocaine) [83], frequency of blood donation [84], hematocrit or cannula placement pain [85], height [78, 86], and the menstrual cycle [87, 88].

Taken together, these studies which have focused on the relationship between total cfDNA levels and disease activity, therapy, and various physiological and lifestyle factors seem to suggest that total cfDNA levels could potentially serve as a versatile biomarker for the diagnosis and monitoring of a wide range of diseases and other clinical scenarios. However, it is also clear from these reports that aberrant levels of total cfDNA is not a phenomenon unique to specific pathological states but is instead a common consequence of pathology and many other factors. Therefore, while the findings reported by all the above-mentioned studies are interesting, it should be interpreted with caution. First, measuring the correlation between total cfDNA levels and any specific disease or physiological or lifestyle factor is complicated by the potential influence of many other co-occurring factors in the same category. Second, total cfDNA levels depend significantly on many biological factors (reviewed in Refs. [1–4, 19, 65]). While most early studies have focused on the analysis of cfDNA isolated from the circulatory system, cfDNA molecules have now been detected and are investigated in most body fluids. As such, the composition of the cfDNA population in any specific body fluid is modulated by a wide range of biological factors, many of which are unique to the body fluid in question. While many details remain to be discovered, it is becoming clear that the characteristics of the cfDNA population in any body fluid depends on: (i) the physical location of the fluid (e.g., release of cfDNA can be influenced by the unique vascularization, histology, perfusion, turnover activity, and cell death and proliferation rate in different tissues, while its movement may be affected by biological barriers like the blood-brain barrier); (ii) the relative contribution of

different organs and cell types to the total cfDNA pool [89–91], (iii) tissue or cell-specific genetic and epigenetic features; (iv) the mechanisms by which cfDNA is generated and released (e.g., active extrusion, association with extracellular vesicles, apoptosis, necrosis, and other cell-death subroutines which are well described in the literature, but not necessarily adequately experimentally correlated with cfDNA release, such as autophagic cell death, mitotic catastrophe, regulated necrosis, and other cell death modalities such as anoikis, entosis, parthanatos, pyroptosis, and NETosis); (v) the changes that cfDNA molecules undergo before exiting cells (e.g., fragmentation, or complexing with proteins and vesicles); (vi) the conditions surrounding the movement of cfDNA from immediate extracellular space into the body fluid; (vii) the changes that cfDNA molecules undergo after exiting cells (e.g., enzymatic degradation and interaction with other extracellular components); (viii) the stability/half-life of cfDNA in the fluid (e.g., rate of degradation, clearance, or binding and reuptake by cells).

Accurate measurement of total cfDNA levels is also significantly affected by numerous factors relating to its physico-chemical properties [1–4, 92], the nature of many preanalytical steps [65, 93–96], and analytical decisions [6, 7, 19, 20]. Unsurprisingly, the convergence of so many variables cause greatly overlapping values between different disease types and healthy individuals both in individual studies and in interstudy comparisons. Such overlapping data for total cfDNA has to-date precluded the establishment of a cut-off value or normal reference range for any specific disease or other clinical scenario. Indeed, given the vast number of factors that affect total cfDNA levels, it is likely that certain factors have been wrongly correlated or attributed, while others have been obscured. As such, it does not seem likely that total cfDNA levels alone could serve as a biomarker in a diagnostic setting.

Crucial advancements in genetic and epigenetic profiling of cfDNA over the last two decades have, however, enabled the accelerated discovery of new connections between cfDNA and disease, and enabled the development of clinically meaningful cfDNA assays, some of which are FDA-approved and applied in routine diagnostics, particularly in the fields of oncology and prenatal testing. In the following sections we explore these advancements and show how it may reignite interest in the previously discussed diseases/other clinical scenarios, how quantitative measurements of cfDNA may become an auxiliary marker to qualitative characterization of cfDNA, and how this may inspire researchers to investigate cfDNA in some unexplored domains of biology and medicine.

Qualitative characterization of cfDNA

CfDNA in various biospecimen types have been scrutinized for the detection of sequences or mutations that are unique to specific individuals, diseases, and organisms, and has so far led to many exciting discoveries and the development of diagnostic assays in various clinical fields.

Ample research highlights cfDNA as a prime candidate surrogate marker for various indications during cancer progression, and may become a powerful clinical tool for the management of various stages of the disease (reviewed in Refs. [6, 7, 20]), including pan-cancer screening of healthy populations or at-risk individuals, indication of disease stage and prediction of clinical outcome, guiding the selection of novel targeted therapies, identification of existing and acquired resistance-conferring mutations, tracking clonal evolution, and detection and prediction of minimal residual disease or recurrence. Taken together, this information will likely inform the selection and development of more efficient therapeutic regimes.

Beyond oncology, sequence characterization of cfDNA has immense potential as a diagnostic, prognostic or theranostic tool for (i) investigating miscarriage [97], fetal sexing and the detection of various fetal genetic abnormalities [8, 98–101], (ii) monitoring post-transplant organ rejection, dysfunction and injury [11–16], (iii) detecting pathogenic DNA such as bacteria (*Mycobacterium tuberculosis*-derived DNA [102] and pneumonia pathogens [103]), parasites [104, 105], fungi [106], viruses (e.g., Crimean–Congo hemorrhagic fever [34], and cancer-causing viruses such as Epstein-Barr virus, which is associated with nasopharyngeal carcinoma [107, 108], or Human papillomavirus (HPV), which is associated with oropharyngeal squamous cell carcinoma [109, 110] and cervical cancer [111], and (iv) studying the gut microbiome as it relates to microbial diversity and its role in human health and disease [112–115]. CfDNA may also be interrogated to study environmental DNA in humans, as there is evidence of the presence of meal-derived environmental nucleic acids, such as plant and bacterial DNA, in human body fluids [116, 117]. The relative contribution of such foreign DNA to the total cfDNA population is not known but is likely low. However, as these cfDNA molecules have been shown to be able to be incorporated into the human genome [118–122], they may have underappreciated detrimental effects. A recent study has, for example, suggested that cfDNA may facilitate the horizontal transfer of antibiotic resistance genes [117].

Other potential uses of cfDNA sequence analysis include (i) retrieval of vector-integration sites, which may prove to be a superior approach for assessing the safety and efficacy of various kinds of gene therapy products [123], (ii) biobank management, as analysis of cfDNA in cord blood plasma has shown to be useful for sample identification [124], (iii) non-invasive pre-implantation genetic diagnosis of X-linked disorders [125, 126], (iv) diagnosis of vascular malformations [127], and (v) use as evidence in forensic casework [128–130].

While the majority of cfDNA studies have to-date focused on cell-free nuclear DNA (cf-nDNA), a growing body of evidence indicates the potential clinical utility of cell-free mitochondrial DNA (cf-mtDNA), which is released into body fluids via cellular clearance or repair processes and may be present as free floating mtDNA fragments or be associated with particles such as internal and external mitochondrial membrane fragments [3, 131]. Intact respiratory-competent mitochondria have also been found to circulate in blood plasma, which may also serve as a source of cf-mtDNA [132]. Aberrant cf-mtDNA has been correlated with a wide range of diseases and other clinical scenarios, including cancers [133, 134], such as breast cancer [135], Ewing's sarcoma [136], urological malignancies [137], oral cancer [138], squamous cell carcinoma [139, 140], and lung adenocarcinoma [141]; cardiovascular disease [142–144]; aging [145]; neurodegenerative disease [146], such as multiple sclerosis [147], Parkinson's disease [148] and Friedrich's ataxia [149]; diabetes [150, 151]; trauma, surgery and ICU patients [152, 153]; sepsis [154, 155]; chronic inflammation and cognitive decline in HIV patients [156–158] and type-2 diabetes patients [159]; exposure to carcinogenic pesticides [160]; adverse health effects of spaceflight on astronauts [161, 162]; and poor outcome of patients with adult community-acquired bacterial meningitis [163]. In addition, cf-mtDNA has been shown to be influenced by exercise [164, 165], can be used for non-invasive haplogroup matching [166], and may be useful in studying various psychological issues, as cf-mtDNA levels have been correlated with psychosocial and physical stress [167], acute psychological stress [168], as well as the pathophysiology underlying suicidal behavior [169] and major depressive disorder [170].

Taken together, sequence analysis of cfDNA isolated from a single biospecimen can be used to detect an ever-expanding repertoire of disease-specific signatures, which represents a breakthrough in the application of non-invasive molecular genetic tests for personal, precision diagnostics and clinical assessments. This clinical potential of cfDNA analysis is underscored by several exciting developments, including, but not limited to: (i) four cfDNA sequence-based tests have to-date been approved by the FDA for implementation in routine diagnostics [171], including the detection of PIK3CA mutations in breast cancer, EGFR mutations (exon 19 deletions and exon 21 L858R substitution mutations) in non-small cell lung cancer (NSCLC), KRAS G12C mutations in NSCLC, and BRCA1 and BRCA2 mutations in metastatic castration-resistant prostate cancer; (ii) several Clinical Laboratory Improvement Amendments (CLIA) labs worldwide offer services for the characterization of cfDNA mutational profiles in cancer patients; (iii) numerous non-invasive prenatal testing (NIPT) facilities worldwide have incorporated cfDNA--based tests into their portfolio, including tests for fetal sexing and diagnosis of various fetal genomic aberrations; and (iv) a 24-marker qPCR assay has recently been developed for the detection of sepsis well before the development of clinical signs [18].

Apart from the handful of FDA-approved assays, and despite unprecedented research efforts in the last two decades, the development and implementation of routine tests based on mutational profiling of circulating tumor DNA (ctDNA) has been advancing at a suboptimal rate. The developmental timeline of ctDNA assays (and by extension cfDNA assays that target specific sequences in other diseases) that bear the required diagnostic sensitivity and specificity for implementation in routine clinical practice is extended by various factors that challenge the analytical detection of ctDNA. The main factors include: (i) a low proportion of ctDNA molecules (or any cfDNA molecules targeted in other diseases) vs. highly abundant background DNA originating from diverse sources, especially in early disease stages where tumors shed miniscule amounts of DNA into extracellular space, (ii) dilution of ctDNA through preanalytical steps that contribute to the release of germline DNA from peripheral blood cells, (iii) an ever expanding list of selectable products, analytical techniques and technologies, many of which show varying degrees of efficiency and bias towards specific applications, sample processing procedures, and physicochemical features of cfDNA, and lastly (iv) ctDNA profiling is complicated by the presence of cancer-specific mutations in clonal hematopoiesis (CH)-derived cfDNA in both cancer patients [172] and healthy subjects that do not have cancer at the time of measurement and may never develop cancer [173–178]. It is currently not clear how the biological noise created by CH-derived cfDNA may be overcome to prevent the misdiagnosis of CH-derived mutations in cfDNA as malignancy. One possibility is that CH-derived cfDNA may be distinguished from ctDNA on the basis of fragment size differences, as ctDNA fragments have been shown to be shorter than CH-derived cfDNA

[179]. It is worth noting here that CH mutations should not be considered merely as false-positive results in ctDNA assays, but should be evaluated for potential pathological and clinical significance as CH mutations have been correlated with increased risk of developing severe Covid-19 [180], hematological malignancies, and cardiovascular disease [181]. Moreover, cancer patients with CH are more likely to develop myeloid neoplasms after chemotherapy vs. non-carriers [181].

Concerning the low proportion of ctDNA molecules to background cfDNA, several strategies may be considered to maximize the chances of capturing ctDNA molecules, thereby increasing the sensitivity and specificity of assays (reviewed in Refs. [6, 7]). These include: (i) collecting and processing larger volumes of body fluid samples (e.g., drawing more blood); (ii) collecting body fluids that are closest to the region of interest, which usually have higher proportions of the target molecule compared to other body fluids (e.g., CSF for brain tumors, urine for bladder cancer, and stool for colorectal cancer); (iii) using optimized analytical techniques, such as performing independent assays on aliquoted replicates or interrogating multiple mutations; (iv) the use of preanalytical strategies that minimize the release of germline DNA from peripheral cells [65, 182]; (v) the use of extraction procedures that provide the highest yield of cfDNA or that are biased toward capturing target molecules; (vi) the development and use of increasingly sensitive assays (e.g., targeted sequencing); and (vii) use of molecular barcoding and integrated bioinformatics approaches that allow the construction of consensus sequences and elimination of random PCR and sequencing errors.

In addition to the implementation of these steps, a growing body of evidence indicates that the probability of capturing ctDNA molecules can be significantly increased by extending the analysis of hotspot mutations to interrogation of other disease-specific alterations in genetic and epigenetic features of cfDNA, thereby markedly increasing the sensitivity and specificity of mutation-based assays.

Beyond hotspot mutation analysis

Every year, cfDNA is characterized with higher resolution, and just in the last few years more information on the physico-chemical features of cfDNA has been mined than in the previous three decades [92, 183–185]. Through this continual unfolding of new knowledge on the composition of cfDNA has it become clear that cfDNA molecules do not merely maintain the primary genetic and epigenetic information stored in the DNA of their originating cells, but often carry extra layers of information in various secondary physico-chemical features that arise upon changes to its primary form. Here, primary genetic information refers to (i) the DNA code itself, which indicates origin, e.g., nDNA, mtDNA, metagenomic DNA, including features like gene sequences, the repetitive element landscape, GC-content, sequence motifs (e.g., transcription factor binding sites), mutations, and copy number variations, (ii) structural and numerical chromosomal abnormalities, and (iii) topological forms like extrachromosomal circular DNA [186–188]. Primary epigenetic information refers to classic DNA methylation (e.g., hypermethylation of promoter CpG islands, hypermethylation of tumor suppressor genes, hypomethylation of oncogenes, and global hypomethylation across the genome), a wide range of histone modifications, as well as nucleosome spacing patterns. Secondary physico-chemical features refer to the various changes that can occur to DNA molecules outside the context of its normal function in the nucleus, such as (i) the binding of cfDNA to DNA binding proteins. There are many DNA-binding proteins that exist in the human body and in the extracellular space (e.g., HMGB-1, fibrinogen, HDL, albumin, CRP, SAA). However, there is a lack of studies concerning the binding of cfDNA to these proteins, and its effects on cfDNA purification, its effects on downstream analysis, or its potential clinical utility; (ii) the binding of DNA to macromolecules (e.g., heparin [189–191]); (iii) packaging of DNA into or association with vesicles (e.g., micronuclei [2, 192]), extracellular vesicles [193–205], apoptotic bodies [206, 207], and outer membrane vesicles [208–211], (iv) association with complex macromolecular structures [212–214], (v) binding to cell membranes on the outer surface [215, 216], (vi) unique fragmentation patterns [184, 217–224], and (vii) unique fragment end-points and motifs [225–227]. These changes may occur during the movement of cfDNA from the nucleus to the cytoplasm, in the cytoplasm, during movement from cytoplasm to the extracellular space, and finally in the extracellular space. There are many ways by which the characterization of these various genetic and epigenetic features may be beneficial towards the development of increasingly powerful cfDNA-based clinical assays.

First, in contrast to the limited number of recurrent hotspot DNA mutations, which typically affect only small regions of the genome, many of these modifications already occur early during tumorigenesis and across a much larger portion of the genome, in turn corresponding to higher proportionality in the total cfDNA population, significantly increasing the probability of detection. For these reasons, profiling of non-classical genetic and primary and secondary epigenetic features of cfDNA is now

widely considered as a potential auxiliary marker to the profiling of hotspot mutations in cfDNA. Moreover, in some cases, the characterization of epigenetic features may even outperform mutational profiling and serve as a standalone biomarker. One breakthrough in this regard includes the FDA-approved Epi proColon 2.0 CE test, which is a qualitative *in vitro* diagnostic test for the measurement of aberrant methylation of SEPT9, which has been associated with colorectal cancer, in patients over the age of 50. Patients with positive results may then be referred for diagnostic colonoscopy. Also of significance, a landmark study on breast cancer patients was recently conducted. Based on the unique methylation patterns observed in breast cancer tissue vs. healthy tissue, researchers used cfMeDIP-Seq to identify breast cancer methylation signatures in the cfDNA of asymptomatic individuals, which enabled minimally-invasive stratification between breast cancer cases and cancer-free subjects before clinical presentation and up to five years before clinical diagnosis using conventional approaches [228].

While not yet ready for implementation in routine clinical practice, numerous studies have demonstrated very strong correlations between epigenetic characteristics of cfDNA and various indications in different types of cancer [229–247]. It is also interesting to note that, beyond the field of oncology, epigenetic characterization of cfDNA has shown potential for the diagnosis and monitoring of cardiovascular disease [248], diabetes [249, 250], liver fibrosis [251, 252], psychosocial stress [167], aging [68], multiple sclerosis [253], psychotic episodes [254], and paracetamol overdose [255].

Second, whereas tissue biopsies and other conventional screening methods are limited to the detection of cancer in specific tissues, characterization of genome-wide methylation patterns may enable parallel interrogation of multiple cancer types. In the context of cancer screening, it is essential to identify the tissue of origin of underlying cancers. While this remains a complex issue and challenging task, much progress has been made in mapping the landscape of tissue specific methylation patterns [232, 256–258], as well as various other tissue-of-origin classifiers. For example, the tissue-of-origin of cfDNA molecules have been determined through the profiling of nucleosome-depleted regions, transcription-factor binding sites [259, 260], fragmentation profiles [226], unique fragment endpoints and end-motifs [225–227], and post-translational histone modifications [261].

Third, while the existence of secondary epigenetic changes to cfDNA has been known for some time, there is a major ongoing breakthrough in the discovery that these secondary changes to DNA that are encoded into cfDNA are not merely random changes that indiscriminately affect the entire genome. Instead, these epigenetic modifications of DNA are often unique to specific scenarios, reflecting the unique mechanistic underpinnings of processes that act on specific genomic regions, in specific cell types, in specific tissues, or in specific disease states. In other words, this means that unique bits of biological and pathological information are partitioned into different cfDNA subtypes. Different cfDNA subtypes can then be analyzed to gain insight not only into the tissue-of-origin, but also be scrutinized to study other biological phenomena and pathological events. Therefore, in addition to enhancing mutation-based cfDNA assays and aiding in determining the tissue-of-origin of cfDNA molecules, it is likely that systematic mapping of these additional epigenetic cfDNA features will lead to the identification of unique, currently unknown features that exhibit more disease-specific qualities, which will increase the pool of molecules that can be grouped under disease-defining variants, thereby increasing the differentiating power of the method.

As with hotspot mutational profiling, epigenetic profiling of cfDNA has many biological, preanalytical, technical, as well as analytical issues and limitations that need to be overcome on route to the development of clinically meaningful assays. The major limitations include: (i) overlapping modifications in pathological and ordinary biological processes, (ii) biological noise induced by stochastic fluctuations in epigenetic marks or markers, and (iii) methodological biases, such as fragment-length biases of different sequencing chemistries and DNA library preparation methods (reviewed in Ref. [92]).

In addition to interrogating a wide range of genetic and epigenetic features of cfDNA, increasing evidence indicates that the sensitivity and specificity of cfDNA tests may be increased substantially by the parallel assessment of cfDNA and other non-DNA liquid biopsy markers. While the most promising approach thus far appears to be the combinatorial analysis of cfDNA and various proteins [262, 263], there may be great synergistic potential in combining cfDNA analysis with the profiling of other biomarkers [264], such as circulating tumor cells (CTCs) [265–268], extracellular vesicles [269, 270]), miRNAs [271], mRNA transcripts [272], or metabolites [273].

Conclusion – new perspectives on the importance of cfDNA biology

The composition of the total cfDNA population in any body fluid, especially blood plasma, is highly complex and

consists of DNA fragments from diverse origins, including host DNA originating from multiple organs and cell types as well as heterogeneous metagenomic DNA. In addition to differences in sequence information, cfDNA molecules can vary significantly in their physico-chemical features, which not only reflect the characteristics of their originating cell but include a wide range of possible alterations effected through various processes and factors encountered in the different biological compartments through which DNA molecules move following the disruption of its primary structure in the nucleus. Moreover, there is often an overlap of prominent genetic and physico-chemical features in cfDNA fragments that originate from disparate sources and processes. In line with this, the proportion of different cfDNA subtypes is determined by numerous biological, physiological, lifestyle, and environmental factors, many of which are inextricably linked. Additionally, the measured quantitative and qualitative characteristics of cfDNA depends significantly on the nature of preanalytical steps, the efficiency and biases of purification methods, and analytical decisions.

The vast biological and structural diversity of cfDNA, along with the complex network of factors that modulate its fluctuation is a double-edged sword. On one side, the richness of information accessible through cfDNA analysis represents an unparalleled treasure trove that can be mined to infer the changing physiological and pathological state of an individual.

First, data captured from a single biospecimen can be used to detect a broad range of disease-specific signatures, which represents a breakthrough in the application of non-invasive molecular tests for precision clinical assessments and diagnostics. This clinical potential of cfDNA is demonstrated by FDA-approved ctDNA assays, mutational profiling of ctDNA in CLIA labs, and the widespread clinical implementation of NIPT assays. It is conceivable that such routine clinical assays may in the near-future be developed for the detection of pathogens, monitoring of organ transplant procedures, and many other pathologies and clinical scenarios, likely including many that have not yet been considered in cfDNA research.

Second, data captured from the profiling of longitudinally collected cfDNA samples can significantly expand the window through which the genome can be studied, allowing a much deeper understanding of how the genome functions, reacts to the environment, and changes over time in response to changes in physiology and pathology. This may prime the ground for a significant leap in genomic sciences, as our current understanding of human genome biology is achieved mainly through snapshot analyses.

What is meant by snapshot analyses is that, through various means we procure a fraction of a whole organism (e.g., tissue biopsies and cell culture models) and in this bounded setting isolate and analyze various molecules (DNA, RNA, proteins, lipids, etc.) that serve as mere proxies for a process taking place. In other words, we isolate a specific process or sub-set of processes from a greater overarching process in order to infer or extrapolate its character and function in a whole organism. On one hand, such a reductive approach has been invaluable for studying numerous integral phenomena in molecular biology, e.g., elucidating most structural features of the genome. On the other hand it should be recognized that contextual logic is substantially traded off through the application of scientific reductionism, wherein sub-processes are inexorably sequestered from the context of the larger process(es) within which it is embedded. This obfuscates the relationships between sub-processes and its relation to the whole, which inherently precludes a proper study of the true dynamic nature of the genome. However, analysis of cfDNA may have an underappreciated role in overcoming this "static" view or interpretation of the genome. A good example of the latter, and why a dynamic view of the genome is highly desirable, is the recent successes achieved in assessing and monitoring dynamic genomic changes in tumors over time, such as changes in the mutational landscape, intratumor heterogeneity, genetic response of a tumor to therapy, and mechanisms that lead to the emergence of resistance against therapy. This approach has already significantly improved the outcome of cancer patients (reviewed in Refs. [6, 7, 20]). Conversely, accurate monitoring of dynamic tumor changes is virtually impossible with tissue biopsies in most cases.

The benefits of temporal genome analysis will not be limited to the domain of oncology, as all cells in the body seem to share the capacity to shed their genome into body fluids, while conserving the unique genetic and epigenetic traits of their originator cells. Moreover, as mentioned previously, a growing body of evidence indicates that the tissue or cellular origin of cfDNA can be identified by virtue of cell and tissue-specific epigenetic signatures, such as nucleosome spacing, fragmentation profiles, unique sequence motifs such as fragment end-points and transcription factor binding sites, histone modifications, and differentially methylated regions. While much remains to be discovered on this front, there is currently an unprecedented effort underway to map cell-specific genetic and epigenetic signatures. It therefore seems plausible that serial collection and characterization of cfDNA may in the future allow the investigation of time-dependent genomic changes over the whole body or specific regions of interest

under a wide variety of conditions, whether relating to normal genomic functioning, malfunctioning processes, or the positive or negative impacts of drug and environmental effects.

Some intriguing possibilities include the study and monitoring of (i) the nature of genetic mosaicism, (ii) the safety and efficacy of gene therapies, (iii) the dynamic response of the genome to diet, (iv) correlations between genomic changes and a variety of diseases, such as aging, psychological stress, and metabolic disorders, and (v) unknown links between different diseases.

Beyond probing dynamic changes to the genetic and epigenetic code, recent studies have shown that gene expression programs could be inferred from nucleosome occupancy patterns [259, 274] and post-translational histone modifications [261], representing a new modality in the perusal of cfDNA. Further refinement of such approaches may in the future create the possibility of non-invasively identifying and studying activities/factors that cause positive vs. negative gene expression responses. Additionally, cfDNA could potentially be leveraged to monitor gene expression patterns in various poorly understood biological processes, such as the developmental transitions in growing embryos, which is currently studied through extremely costly and tedious longitudinal collection and dissection of thousands of rat organs.

Taken together, cfDNA analysis clearly shows immense potential not only as an important and versatile clinical biomarker, but also as a powerful research tool in basic molecular biology and genetics. However, on the other side of the double-edged sword mentioned earlier, there are some serious drawbacks and complications posed by the biological and structural diversity of cfDNA and the concurrent array of related preanalytical and analytical challenges. For example, it significantly limits the clinical utility of total cfDNA levels, it makes it very challenging to differentiate analytically between cfDNA subtypes, and it complicates the detection of scarce biomarkers (e.g., ctDNA hotspot mutations) in specific assays, whether for clinical or basic research interests.

Therefore, unlocking the full potential of cfDNA requires not only high-fidelity reconstruction of the quantitative, genetic and epigenetic features of cfDNA contained in biospecimens, but is dependent on the accurate elucidation and systemic mapping of all known and unknown features as it relates to various biological, physiological, lifestyle, environmental, preanalytical, and analytical variables in the widest sense possible. In other words, the importance of a deep and structured enquiry into the structure and biology of cfDNA cannot be overstated. While there is still a significant lack of knowledge, studies on the complete genetic and epigenetic features of cfDNA molecules as it relates to a variety of contexts has come rapidly to the front and will in the next couple of years likely become the principal center of research interest and be one of the major drivers of progress in the field.

As discussed above, an improved understanding of cfDNA biology will on the one hand expand the repertoire of disease-specific markers, facilitate the discovery of new links between the properties of cfDNA and various diseases and clinical scenarios, and enable the characterization of temporal genome changes and gene expression programs.

On the other hand, deeper knowledge of cfDNA biology may facilitate improvements in the sensitivity and specificity of various cfDNA assays, allowing rapid advancements in each of the former avenues of cfDNA research. First, a better understanding of the biological and physiological factors that determine the release and extracellular stability of different types of cfDNA molecules will likely inform the development of various strategies that maximize the likelihood of capturing or detecting target molecules. For example, (i) research indicates that ctDNA molecules are enriched in body fluids that are in closest proximity to the tumor in question, (ii) prior to sample collection, there may be optimal patient conditions that either favor the release of target molecules or limit the release of background molecules into the body fluids in question (the large number of factors that can affect the characteristics of cfDNA demonstrates the importance of collecting and documenting the right meta-data from patients, and documenting patient conditions prior to and during biospecimen collection). Second, when the mechanisms of release of specific cfDNA molecules are understood, it may become feasible to leverage existing or newly developed drugs or mechanical methods (e.g., temporarily opening the blood-brain barrier by focused ultrasound and microbubbles) to either limit non-specific release of cfDNA or promote the release of cfDNA molecules into the body fluids in question. Third, knowledge of the exact physical and chemical properties of specific cfDNA molecules or the structures with which they become associated will enable the selection, tailoring or development of new extraction procedures that are either biased towards the capture of specific cfDNA molecules, or eliminate non-specific DNA molecules, such as contaminating DNA originating from the lysis of peripheral blood cells. The utility of such biology-informed methods is exemplified by studies that have shown that selective capture of short cfDNA fragments, which have recently been shown to harbor increased amounts of cancer-associated mutations, substantially increases the analytical and diagnostic sensitivity and specificity for the detection of various cancer-specific mutations

[220]. Similarly, cancer-specific aberrations in DNA methylation have recently been demonstrated to correlate with physico-chemical changes that are characterized by increased affinity for gold nanoparticles, which indicates the intriguing possibility for the development of extraction methods that selectively purify tumor-derived cfDNA [275]. Similarly, some studies suggest that specific extracellular vesicles (exosomes) carry specific DNA cargo, which represents another possible approach for the selective capture of specific cfDNA molecules [198–200]. Lastly, many of the different forms in which cfDNA subtypes exist are differently affected by many of the steps that precede extraction and analysis. Although these differences have not yet been thoroughly explored, knowledge of this will enable the selection of preanalytical methods that suit specific study objectives [65].

Acknowledgments: Abel Jacobus Bronkhorst was supported by the Georg Forster Research Fellowship Programme of the Alexander von Humboldt Foundation. The financial assistance of the Alexander von Humboldt Foundation is hereby acknowledged.
Research funding: This work was funded by Alexander von Humboldt Foundation.
Author contributions: All authors have accepted responsibility for the entire content of this manuscript and approved its submission.
Competing interests: Authors state no conflict of interest.
Informed consent: Not applicable.
Ethical approval: Not applicable.

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