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CURRENT OPINION

Cell-Free DNA: Features and Attributes Shaping the Next Frontier in Liquid Biopsy

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Abstract

Cell-free DNA (cfDNA) is changing the face of liquid biopsy as a minimally invasive tool for disease detection and monitoring, with its main applications in oncology and prenatal testing, and rising roles in transplant patient monitoring. However, the processes of cfDNA biogenesis, fragmentation, and clearance are complex and require further investigation. Evidence suggests that cfDNA production relates to mechanisms of cell death and DNA repair, both of which further influence fragment size and its applicability as a biomarker. An emerging domain, cfDNA fragmentomics is being explored for advancing the field of diagnostics using non-mutational signatures such as fragment size ratios and methylation patterns. Thus, this review examines structural diversity in cfDNA with various fragment sizes. In examining these cfDNA subsets, we discuss their distinct biological origins and potential clinical utility. Development of sequencing methodologies has broadened the application of cfDNA in diagnosing cancers and organ-specific pathologies, as well as directing personalized therapies. This has been achieved by identifying and uncovering different subsets of cfDNA in biofluids using different methodologies and biofluids. Different cfDNA subsets provide important insights regarding genomic and epigenetic features, enhancing the understanding of gene regulation, tissue-specific functions, and disease progression. Advancement of these key areas further asserts increasing clinical relevance for the use of cfDNA as a biomarker. Continued exploration of cfDNA subsets is expected to drive further innovation in liquid biopsy and its integration into routine clinical practice.

1 Introduction

Over the past few decades, our understanding of cell-free DNA (cfDNA) has evolved significantly from its initial discovery in the 1940s to the current state of technological advancements. cfDNA refers to fragmented DNA molecules present in bodily fluids, including but not limited to blood, urine, and saliva. These DNA fragments are shed by cells through various biological processes and can originate from various cell types, such as tumor cells, immune cells, and placental cells [1].

The study of cfDNA reflects indirect reporting of a complex landscape of biological processes. Previous

Key Points

Ongoing research analyzing cfDNA subfractions (ultrashort cfDNA, mitochondrial cfDNA, nucleosomal cfDNA) separately, rather than in bulk, may significantly enhance biomarker resolution, improve tissue-of-origin identification, and refine disease classification for precision diagnostics.

Beyond mutations, non-mutational cfDNA analysis through fragmentomics, end motifs, and methylation profiling provides deeper biological insights, offering powerful diagnostic and prognostic tools beyond traditional mutation-based approaches.

Advancements in cfDNA technology, such as biofluid-specific analysis, long-read sequencing, single-stranded library prep, and AI/ML-driven cfDNA analysis, are revolutionizing early cancer detection, treatment monitoring, and personalized medicine.

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studies have indicated that during apoptosis, the presence or absence of cfDNA reflects protection due to association with nucleosomal proteins or transcription factors, which are altered by the gene expression state [2]. These biological processes shape the features and attributes of cfDNA, including fragment size, end-motifs, methylation patterns, mutational profiles, and concentration.

Since crucial information of biological processes is embedded in these molecules, the analysis of cfDNA has the potential to revolutionize personalized medicine. Some applications include early detection and diagnosis, treatment monitoring and response prediction, or personalized therapy. These applications can all be acquired in a minimally invasive manner, reducing the need for biopsies and other invasive tests [3, 4].

Although cancer-related cfDNA work originally revolved around cfDNA mutation detection, the landscape has recently expanded to include non-mutational features, like fragmentomics and end-motif analyses that rely on the morphological features of cfDNA. Recent analyses and technical advancements are contributing to bettering our understanding of cfDNA morphological variation. In this current opinion article, we will explore the various subsets of cfDNA and its unique features. We aim to provide an overview of the impact of different sequencing methodologies, biological processes, and biofluids on cfDNA. Lastly, we will identify the latest advancements and challenges in its use as a liquid biopsy tool.

2 Morphology of cfDNA

The identification of various cfDNA conformations is now possible due to advancements in DNA preparation techniques, diverse sequencing methodologies—including both short-read and long-read sequencers—and sophisticated bioinformatic tools [5, 6].

Morphologically, studies have shown that cfDNA appears as linear fragments [7, 8] or circular structures [9]. Linear fragments may be nucleosome-associated DNA [10] or result from DNA fragmentation. Circular cfDNA may indicate the presence of extrachromosomal DNA of varying lengths, like microDNA (100–400 bp), small polydispersed circular DNA (100–10,000 bp), episomes (up to 1000 kb), and telomeric circles [11]. These fragments can be derived from errors in DNA repair mechanisms [12], homologous recombination, or microhomology-mediated end joining [11]. Current opinion discussions involve cfDNA with linear morphological features. In addition to linear and circular structures, cfDNA may exist in other conformations, such as stem-loop or tetrad, which can be attributed to the occurrence of intercalated motifs, like cytosine-rich or G-quadruplex structures, in cfDNA, mostly seen in very short lengths of cfDNA [13–17].

As has been observed, the post-sequencing fragment profile indicates that the peaks (i.e., maximum bulk) of cfDNA occurs at certain nucleosomal lengths: mononucleosomal ~167 bps (mncfDNA), dinucleosomal ~320 bps (dncfDNA), and trinucleosomal ~480 bps (tncfDNA). The nucleosomal size of cfDNA results from histone-bound DNA ~147 bps with the addition of a linker DNA 10 bps (Fig. 1B) [18]. More recently, cfDNA of lengths longer than 3 kbps have been identified using long-read sequencing [6]. Similarly, in addition to peaks at the mono- and dinucleosomal length, an additional peak has been observed between 40 and 70 bps, named ultrashort cfDNA (uscfDNA) (Fig. 1A) [14, 15].

In pathological conditions, notably cancer, it has been observed that the average length of nucleosomal lengths of cfDNA fragments are shorter, typically by 10–20 bps, when compared to those of a healthy patient [19–21]. Therefore, the relative abundance of the short and long fragments is being used as an effective cfDNA biomarker [19]. It has been demonstrated in early-stage pancreatic cancer that the shorter fragments of cfDNA are enriched by mutations [21]. It may be of interest to see if uscfDNA is also enriched with tumor-derived cfDNA using targeted sequencing.

Different conformations of cfDNA can also be distinguished based on their strandedness and length. For instance, uscfDNA is predominantly observed in a single- stranded [15] form, whereas emerging evidence suggests that mncfDNA typically exists in a double-stranded conformation. Further subtypes include jagged cfDNA and nicked cfDNA, which potentially result from DNA breakage or repair [18]. Similarly, single-stranded cfDNA may be a result of DNA replication, repair, or transcription, while double-stranded cfDNA is typical of genomic DNA [8].

3 Processes Contributing to cfDNA Formation and its Features

The biomarker potential of cfDNA leverages on their original biogenesis which affects their processing, and functional associations (Fig. 2). Current evidence indicates that cfDNA is generated from nucleic acid cleavage during processes such as cell death [22] or from DNA repair mechanisms [23]. The factors influencing cfDNA production can be understood at the cellular level by examining processes like inflammation and cell death, or the molecular activity of nucleases involved in its release. This section explores the biological and molecular processes that contribute to

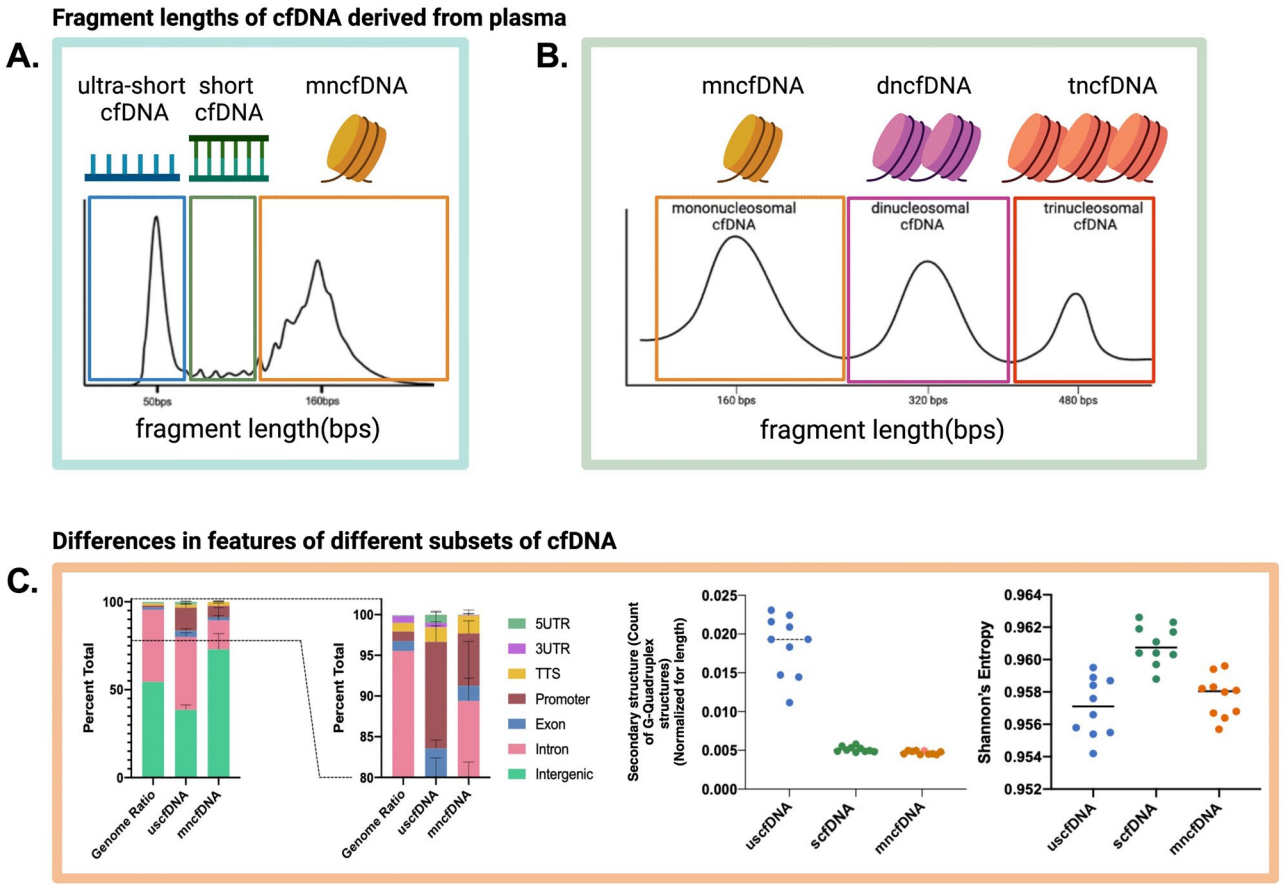

Fig. 1 Plasma derived cfDNA possess different fragment lengths and characteristics. **A** Low molecular weight DNA extraction with single-stranded DNA library preparation of plasma cfDNA reveals a mononucleosomal cfDNA peak (~166 bps) along with an ultrashort cfDNA peak (40–70 bps) and short cfDNA valley (70–120 bps) (blue box). **B** Multiples of the nucleosomal peaks (~166 bps, ~320 bps, ~480 bps) are revealed with routine double-stranded library DNA protocols (green box). **C** Contrasting characteristics of different subsets of cfDNA: Genomic region mapping of called peaks differ amongst subsets of cfDNA; differences in abundance of potential secondary structures seen in cfDNA; differences in end motif cleavage for different subsets of cfDNA. Data derived from [15, 16]. *Created in BioRender.com*

the origins of cfDNA while providing insight into its structural features and interactions, particularly in the context of different size-based subsets of cfDNA.

3.1 Biological Processes Contributing to cfDNA Formation via Bio-Catabolism of the Genome

3.1.1 Cell Death (Apoptosis, Necrosis)

Apoptosis is one of the major biological processes contributing to the generation of cfDNA. DNA cleavage forms an important step during apoptosis: cellular DNA is cleaved into smaller fragments by endonucleases, such as caspase-activated DNase (CAD, DFFB) and endonuclease G (EndoG) [24]. The generation of cfDNA from apoptosis is a two-step process. First, nucleosomal-length cfDNA is generated through chromatin cleavage into nucleosomes, mediated by DNase1, DNase1L3, and DFFB. Each nucleosome consists of approximately ~147 bp of DNA wrapped around a core of histone proteins. Finally, the nucleosomal-length cfDNA is further degraded into smaller fragments with a 10 bp periodicity, resulting in the characteristic fragmentation pattern of apoptotic cfDNA [25]. The observation of the uscfDNA peak may be indicative of other different mechanisms of cfDNA release, in addition to cell death.

Understanding and assessing apoptosis-associated genome-wide cfDNA generated by healthy tissues can help map and establish the physiological cfDNA signatures for different subsets. As disruption of cell death processes (i.e., necrosis or apoptosis) occurs, especially during oncogenesis and tumor progression, variation in physiological cfDNA signatures can aid in clinical detection or monitoring.

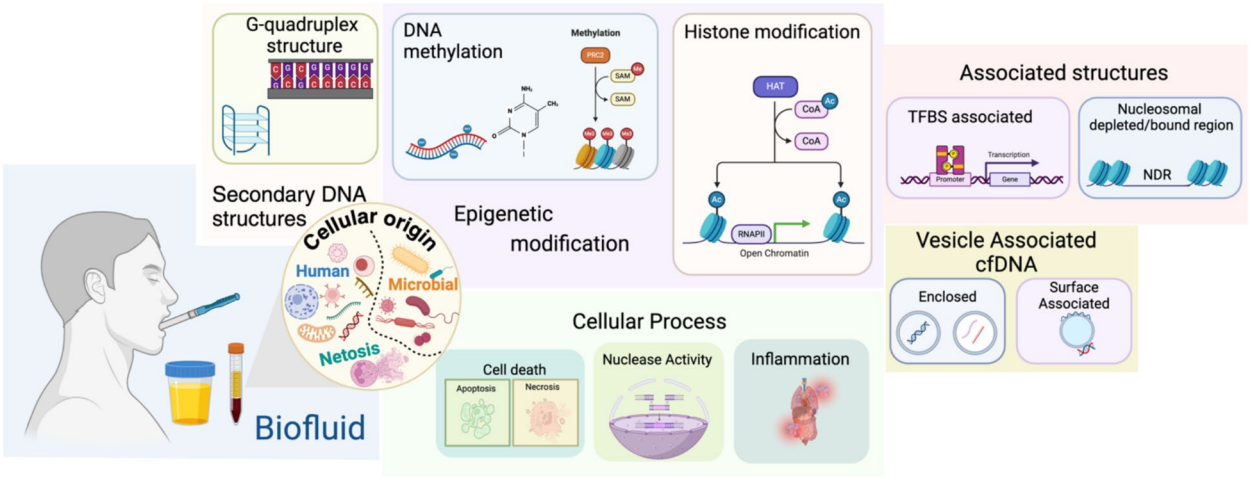

Fig. 2 Factors affecting cfDNA characteristics: Processes and structural features that lead to cfDNA formation: biological and cellular processes, genomic structures, nucleosomal associated structures or epigenomic modifications. Source of cfDNA, biofluids, and cells of origin. *Created in BioRender.com*

3.1.2 Inflammation

The relationship between cfDNA and inflammation is bidirectional. cfDNA can both trigger and be influenced by an inflammatory response or ongoing inflammation. Occurrence of cfDNA is regularly attributed to extracellular traps by neutrophils [26] and eosinophils [27], in response to various noxious stimuli. Increased inflammation and the presence of autoimmune disorders and cancers have been shown to affect fragmentation patterns and the bulk of cfDNA [28]. During systemic inflammation, it has been observed that cfDNA is released in a biphasic manner: first from hematopoietic cells and then followed by non-hematopoietic cells like endothelial cells [29]. Additionally, interleukin-8 has been found to affect cfDNA levels [30]. Current findings suggest that inflammation plays a major role in cfDNA origins.

Although many findings have been reported regarding cfDNA in general, the impact of these processes on different subsets of cfDNA may lead to new and interesting avenues in understanding the clinical relevance of cfDNA.

3.2 Molecular Processes Contributing to cfDNA Formation via Bio-Catabolism of the Genome

3.2.1 Nuclease-Dependent DNA Cleavage and its Impact on Fragmentomics

DNA nuclease activity plays a crucial role in shaping the fragmentomic landscape of cfDNA. Endonucleases, such as DNase1, DNase1L3, or DFFB, cleave DNA at specific sites, generating fragments with distinct ends and sizes of cfDNA [22]. These enzymes can be activated during cellular processes, like apoptosis, necrosis, DNA replication, and DNA repair, and can release cfDNA with characteristic fragment end patterns [22, 31, 32].

Nuclease enzymes are expressed by all cell types. Some nucleases are expressed ubiquitously by major cell types and organs like DNase1 [33], while others like TREX have been demonstrated to be only expressed in certain tissue types. For example, it is known that Trex2 is predominantly expressed in keratinocytes, which cooperate with DNase1L2 to promote DNA degradation in the oral epithelium [34]. This process may contribute to the emergence of different end-motif profiles from intracellular degradation and can help identify cfDNA emerging from oral keratinocytes. Appreciating tissue-specific nuclease activity could open a new landscape of information where the deconvolution of cfDNA can be assessed with end motif sequences.

3.3 Structural Features and Associations of cfDNA

3.3.1 Nucleosomal Protein-Associated cfDNA

The analysis of nucleosomal cfDNA can provide insights into chromatin structure, gene regulation, and disease states. Nucleosomal proteins, such as histones, play a crucial role in packaging DNA into nucleosomes, the basic units of chromatin. Nucleosomal cfDNA typically consists of 147 bp + 10 bp linker DNA, reflecting the nucleosomal structure [18]. After the alignment of sequenced cfDNA, certain regions in the genome have different coverage patterns due to the open chromatin behavior. Nucleosomal-depleted regions (NDRs) are regions characterized by a lack of nucleosomes and correspond to regulatory elements like promoters, enhancers,

and silencers [35, 36]. Thus, the surviving cfDNA reflects footprints of protein-DNA interaction. For example, studies in stage IV hepatocellular carcinoma have shown a strong correlation in the NDRs of active gene regions with the RNA expression data from the HepG2 cancer cell line [35].

Moreover, specific histone modifications or nucleosomal positioning can indicate gene activation or repression [35, 37]. In addition, the majority of cfDNA is associated with nucleosomal structures containing histone modifications like H3.1, H3K27me3, and H4K16ac. This suggests that cfDNA is not "naked" but linked to nucleosomal proteins [38].

Interestingly, shorter sequences, like uscfDNA, were observed to be enriched in promoter sequences when compared to mncfDNA fragments, possibly indicating a different genomic source [16]. The distinct genomic origins of uscfDNA and mncfDNA may indicate varying relationships between these cfDNA subsets and their association with nucleosomes.

3.3.2 Transcription Factor Binding Site-Associated cfDNA

With cfDNA occurring from NDR portions of the genome, it has been observed that cfDNA contains specific sequence motifs which are associated with transcription factor binding sites (TFBS) [39]. Activating histone modifications in NDRs, in particular, can expose the DNA to various transcription factors and, thereby, contribute to transcription factor-bound cfDNA.

Specific TFBS may indicate the activity of specific transcription factors or signalling pathways. The analysis of TFBS-associated cfDNA can provide a new perspective on the complex interactions between DNA, proteins, and other regulatory elements and indicate molecular functions associated with cfDNA fragments [39, 40].

Current exploration of TFBS in cfDNA has identified binding sites for TFs, like HOXA and FOX, to be clinically useful cfDNA fragments [39]. Interestingly, if cfDNA could be categorized based on the superclass of TFBS they possess, it may demonstrate structural and biological differences that may help to reveal the evolutionary conservation of regulatory mechanisms. For instance, zinc-coordinating DNA-binding domains, like the Cys4 zinc finger of nuclear receptor type, may be enriched within uscfDNA, which have increased occurrences of sequences with potential secondary structures. This enrichment feature would be a potential biomarker in deducing the origin of cfDNA [41].

3.3.3 Methylated cfDNA

DNA methylation can also impact DNA cleavage, an essential step in the generation of cfDNA [42]. Methylated DNA is more resistant to cleavage by certain endonucleases, leading to the generation of longer fragments. This can result in a shift towards larger fragment sizes in methylated cfDNA, which can then be detected using long-read sequencing.

The analysis of methylated cfDNA can provide valuable insights into disease mechanisms and progression. For example, methylated cfDNA can be used to detect cancer at an early stage, monitor treatment response, and identify potential therapeutic targets [43]. Researchers have used methylated cfDNA, especially mononucleosomal lengths, to identify disease-associated DMRs in cfDNA and have used this information for cellular deconvolution [43]. When comparing methylation status between different subsets of cfDNA, uscfDNA was shown to be hypomethylated when compared to mncfDNA, which was hypermethylated [44].

3.3.4 Secondary Structures and Repeat Elements

Repeat and transposable elements, such as LINE-1 and Alu, are abundant in cfDNA and play a significant role in shaping its landscape [45]. These elements can influence cfDNA fragmentation patterns, methylation profiles, and overall structure. For instance, LINE-1 elements have been shown to be enriched in cfDNA from cancer patients, suggesting their potential as biomarkers for disease diagnosis [45, 46].

Transposable elements can also give rise to secondary structures in cfDNA, such as circular [47], stem-loops, and cruciform, which can impact its stability and functionality [48]. These secondary structures can also affect the binding of proteins and other molecules to DNA, potentially influencing its role in intercellular communication [49]. Such interactions should be explored within different subsets of cfDNA. For example, it has been observed that secondary structures, like G-quadruplex structures, were more enriched in uscfDNA when compared with mncfDNA [16].

Furthermore, the presence of repeat and transposable elements in cfDNA can also impact its detection and analysis, requiring specialized bioinformatic tools and techniques. The study of repeat and transposable elements in cfDNA is an active area of research, with new technologies and techniques being developed to accurately detect and quantify these elements. For example, machine-learning algorithms can be used to identify patterns of repeat element enrichment in cfDNA from different disease states [45]. Additionally, the development of new sequencing technologies, such as single-molecule real-time sequencing, can provide high-resolution views of cfDNA structure and repeat element composition.

3.3.5 Vesicle-Associated and/or Enclosed cfDNA

Bioactive cfDNA has been studied in association with extracellular vesicles and exosomes, either enclosed or on the surface. Although there are a lot of studies on synthesis of the vesicles, the mechanisms for release of DNA associated

with these vesicles have been very sparsely studied [50]. It has been observed that vesicle-associated cfDNA is derived from nuclear DNA, mitochondrial DNA, or cytosolic DNA (DNA released into cytosol from the nucleus and micronuclei due to genomic instability and DNA damage; or mitochondrial DNA) [50]. The topology of cfDNA has been observed to be different with different vesicles [51].

Collectively, different subsets of cfDNA display unique characteristics, influenced by their biological origins, associations, and epigenetic modifications. These distinctions are important to consider in order to gain a more complete understanding of the potential of cfDNA in disease detection and therapeutics. A detailed, subset-specific analysis can uncover distinct biological and pathological insights that might otherwise be hidden in bulk cfDNA analysis, ultimately enhancing clinical relevance [16]. Therefore, careful examination of each subset could improve the accuracy and effectiveness of cfDNA applications in personalized medicine (Fig. 1C) [15, 16]. Application of these unique cfDNA features, in addition to single nucleotide polymorphism (SNP) detection, variant calling, and copy number alterations (CNAs), have been used in various clinical contexts as discussed in the clinical relevance section, with special guidelines for application of cfDNA for cancer patients [52].

4 Other Sources of cfDNA

4.1 Mitochondrial cfDNA

Mitochondrial cfDNA (mtcfDNA) is a lesser-known form of cfDNA that is derived from the mitochondrial genome [53]. Mitochondrial DNA (mtDNA) is a separate entity to nuclear DNA and is responsible for regulating mitochondrial function. The mitochondrial genome is comprised of H and L strands and house genes for cellular respiration. Disruptions in mtDNA have been observed in cancer [54]. Detection of cfDNA originating from the mitochondrial genome has received a lot of traction as an effective biomarker for LB assays [53]. mtcfDNA can be released into biofluids through various mechanisms, including mitochondrial damage, apoptosis, or active release.

The analysis of mtcfDNA can provide valuable insights into mitochondrial biology and disease mechanisms. It has been observed that mtcfDNA ranges between 40 and 70 bps, which overlaps with the uscfDNA peak originating from the nuclear genome. The detection and analysis of mtcfDNA can be challenging due to the presence of contaminating nuclear DNA. However, advances in sequencing technologies and analytical techniques have made it possible to detect and quantify mtcfDNA with high sensitivity and specificity. More recently, the analysis of methylated mtcfDNA has shed some light on its association with certain disease phenotypes [55, 56].

4.2 Microbial cfDNA

Microbial cfDNA is a type of cfDNA that is derived from microorganisms such as bacteria, viruses, and fungi [57, 58]. Microbial cfDNA can be present in biofluids, particularly in the context of infections or dysbiosis, where the balance of the microbial community is disrupted. Moreover, commensal microflora can be a contributor to the microbial cfDNA for the localized biofluids like saliva, which has cfDNA from various oral commensals [17]. This type of cfDNA can provide valuable information about the microbial community and its impact on human health, including the presence of pathogens, the diversity of the microbiome, and the response to antimicrobial therapy [59].

The detection and analysis of microbial cfDNA can be challenging due to the presence of human DNA and the variability of microbial genomes. Sequencing and analytical advancements have enhanced microbial cell-free DNA detection and quantification, providing improved sensitivity and accuracy [58]. Technically, the assessment of microbial cfDNA poses a significant challenge due to the diversity of microflora and the varying assemblies of the microflora reference genome. The development of microbial cfDNA-based biomarkers has the potential to revolutionize the diagnosis and monitoring of infections, as well as the development of personalized therapies targeting the microbiome. Further research is needed to fully understand the potential of microbial cfDNA analysis and its applications in human health and disease.

5 cfDNA in Different Biofluids

cfDNA in different biofluids, such as blood, urine, and saliva, can exhibit distinct characteristics and concentrations. cfDNA in blood is typically present at higher concentrations than in other biofluids and is often used for liquid biopsy tests. In contrast, cfDNA in urine and cerebrospinal fluid is typically present at lower concentrations [60] but may be more informative for diseases affecting the urinary tract, etc.

Local environments with proximity to biofluids may have a significant impact on different features of cfDNA. The fragment profile observed from plasma demonstrates differences when compared to fragment profiles within urine or saliva (Fig. 3) [17, 61]. The fragmentation patterns and sizes of cfDNA can also vary between biofluids. The cfDNA in urine and saliva is typically more fragmented than in blood, as both urine [62] and saliva [63] have a high content of nucleases such as DNASE1, resulting in higher

fragmentation of DNA, leading to more fragmented urinary and salivary cfDNA [61]. This may indicate different clearance mechanisms and half-lives of cfDNA in these biofluids. Moreover, it has been observed that oral microbial species make a significant contribution to salivary cfDNA [17]. This is an important observation as it highlights the heterogeneity for origins of cfDNA in a given biofluid. Additionally, the relative proportions of nucleosomal and non-nucleosomal cfDNA may differ between biofluids [64], potentially reflecting different mechanisms of cfDNA release and clearance.

The analysis of cfDNA in different biofluids can provide complementary information about disease mechanisms and progression. For example, the analysis of cfDNA in blood may provide insights into systemic diseases, while the analysis of cfDNA in urine and saliva may provide insights into urogenital and oral health issues, respectively. Understanding the differences in cfDNA between biofluids can aid in developing more accurate and informative liquid biopsy tests. Currently, urinary cfDNA has been used for the early detection of bladder cancer through the use of fragmentation hotspots, as well as detection of minimal residual disease (MRD)and survival prediction of bladder cancer patients using variant allele frequency, inferred tumor mutational burden, and copy number-derived tumor fraction levels [65, 66]. Similarly, non-mutational cfDNA features found in saliva have shown promise in the early detection of gastric cancer [17].

6 Other Biological Considerations

6.1 Role of cfDNA in Signalling, Immune Modulation, and Biofilm Maintenance

Apart from biomarker potential, other physiological functions of cfDNA have been very sparsely studied. For instance, the immunogenic or immunomodulatory effect of cfDNA [67, 68]. Furthermore, cfDNA can induce toll-like receptors and mediate inflammatory responses in mesenchymal stem cells (Fig. 4) [69].

Additionally, cfDNA derived from cancer cells was observed to develop metastatic phenotype in non-cancer cell lines [70–72]. Cell-free DNA can alter gene expression of MMP9, CD44, and miRNA like hsa-miR-99b-5p in the prostate cell line to induce a malignant phenotype [71].

Interestingly, microbial communities harness cfDNA for promoting and maintaining biofilms [73, 74]. Extracellular DNA or cfDNA in biofilms can be derived from bacterial apoptosis, or in response to stress, often controlled by quorum sensing. It can also be derived from host cells via bacterial toxins or neutrophil extracellular traps, and can also occur via lysis-independent mechanisms.

These processes highlight the importance of cfDNA as a downstream molecule. The downstream impact of cfDNA has been assessed using an entire cfDNA population; it would be interesting to evaluate if size-based populations have a similar downstream effect.

6.2 Clearance/Half-Life of cfDNA

The presence of cfDNA in biofluids is transient and has a half-life ranging from several minutes to a few hours. This dynamic nature of cfDNA, especially the different subsets, makes it a fascinating area of study with important implications in enhancing our understanding of human health and disease.

The clearance of cfDNA from circulation involves a complex interplay of biological processes. Local tissue uptake, circulatory dynamics, hepatic metabolism, renal excretion, and splenic filtration all contribute to removing cfDNA from the bloodstream [75]. This multifaceted process ensures that cfDNA levels remain within a narrow range, maintaining homeostasis and preventing potential harm.

In pathological conditions, such as cancer, the dynamics of cfDNA clearance are altered. The tumor tissue and microenvironment release circulating tumor DNA (ctDNA), which

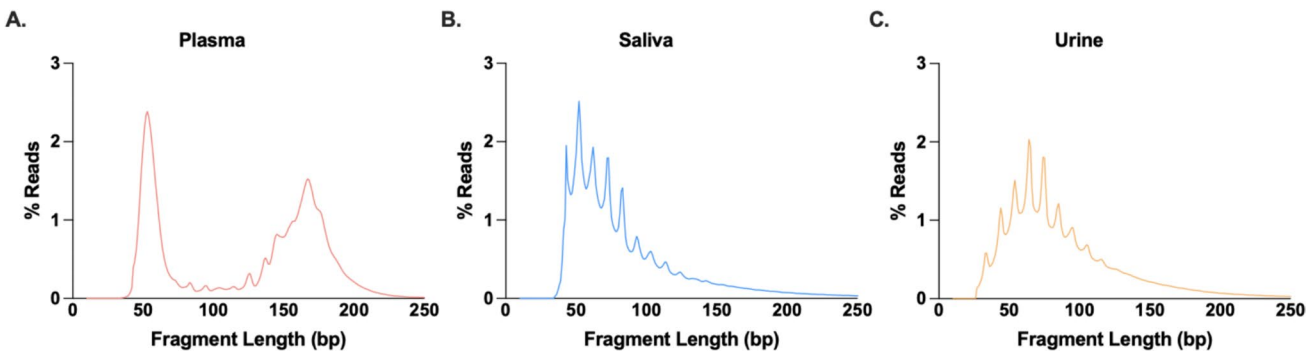

Fig. 3 cfDNA profile in different biofluids using a single-stranded library preparation: **A** plasma, **B** saliva and **C** urine. Data derived from [15, 17, 76]

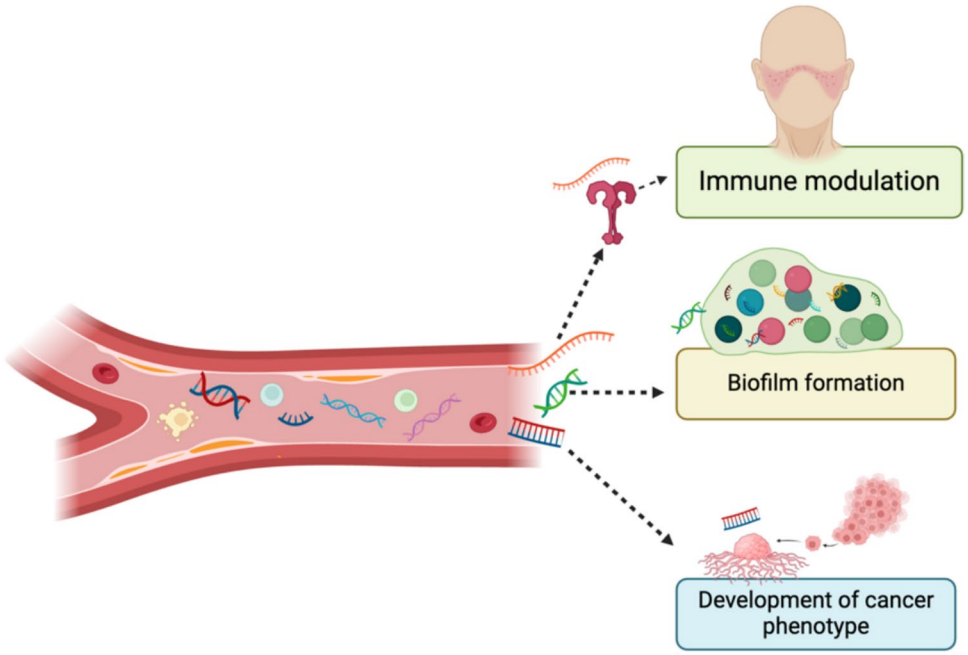

Fig. 4 Downstream functions of cfDNA: Immune modulatory effects, horizontal transfer of information and inducing transformation to cancer phenotype, biofilm formation. *Created in BioRender.com*

carries distinct mutations and variations. However, distinguishing ctDNA from cfDNA poses a significant challenge due to its low abundance in circulation. Predicting ctDNA within the cfDNA bulk, referred to as the tumor fraction (TF), has become one of the key features in the analysis of liquid biopsy. TF can be predicted by employing various features of cfDNA, which have been observed to be altered when derived from tumor cells like shorter fragment lengths [76], occurrence of variant alleles [77], or differences in the end motif.

Recent advances have enabled the selective priming of ctDNA in mouse models to enhance its recovery [78]. This breakthrough highlights the importance of understanding the differential clearance mechanisms of cfDNA and ctDNA. By elucidating these processes, researchers can develop more effective strategies for detecting and analyzing ctDNA, ultimately improving disease diagnosis and management.

7 Technical Considerations for cfDNA Analysis

Technical considerations play a crucial role in the analysis of cfDNA. Preparation of cfDNA greatly impacts downstream analysis, DNA extraction [15], or library preparation (Fig. 5) [5]. Extraction methodologies have an impact on the incorporation of different populations of cfDNA. The use of silica columns or magnetic beads affects incorporation of shorter pieces of cfDNA. Interestingly, a probe-based approach of extraction of cfDNA has been applied for direct extraction of cfDNA from the biofluid [79]. For an NGS-based approach for cfDNA detection, DNA preparation is a crucial step. Fragmented DNA poses a significant challenge in DNA preparation. Library preparation methodologies, such as single-stranded library preparation or double-stranded library preparation (with or without repair methodologies), affect the cfDNA populations that are sequenced.

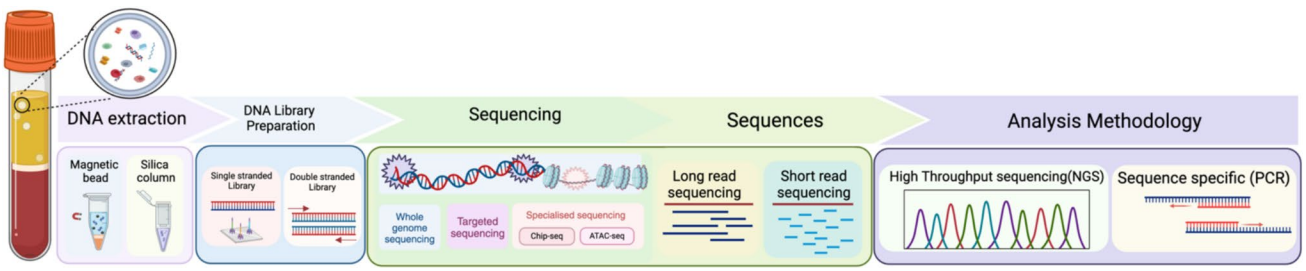

Fig. 5 Different technical considerations that affect cfDNA attributes. *Created in BioRender.com*

Sequencing methodologies such as whole genome sequencing (WGS) [19], Chromatin Immunoprecipitation Sequencing (ChIP-Seq) [80], and targeted sequencing approaches [81] can detect and quantify cfDNA. WGS provides a comprehensive view of the genome which can be used for biomarker discovery [16], while ChIP-Seq enables the study of protein-DNA interactions and can be employed to study the epigenetic landscape of cfDNA. Targeted sequencing allows for focused analysis of specific genes or regions [81, 82]. The choice of sequencing methodology depends on the research question and experimental design.

Different sequencers, such as Illumina, PacBio, and Oxford Nanopore, have distinct characteristics that affect cfDNA data quality and interpretation. The ability of sequencers to sequence short or long DNA fragments affects the analyzed DNA fragments. Long-read sequencers, like PacBio or Oxford Nanopore, can be used to evaluate the fragments longer than 600 bps [83]. Long-read sequencing has uncovered cfDNA fragments measuring around 39.8 kbps [6]. Meanwhile, Illumina is a short-read sequencer and generates high-quality, high-throughput data.

Bioinformatic considerations are equally important in cfDNA analysis. Proper bioinformatic quality control is essential for ensuring data quality and preventing errors in downstream analysis. This includes assessing sequencing quality, aligning reads to the reference genome, and detecting potential contaminants. Effective bioinformatic analysis of cfDNA data requires careful consideration of factors such as sequencing depth, coverage, and bias [36, 84, 85]. Additionally, specialized bioinformatic tools and pipelines are required for cfDNA data processing and analysis, such as tools for fragment size analysis and methylation profiling.

Specific cfDNA target assessment methodologies include PCR or EFIRM-based detection [86, 87]. PCR techniques, such as quantitative PCR and digital PCR, are essential for amplifying and detecting cfDNA. However, PCR bias and amplification artifacts can impact cfDNA analysis. Recently, methodologies have been developed to evaluate shorter and longer pieces of cfDNA using PCR [88]. Moreover, different PCR techniques have different sensitivities and specificities which need to be considered when designing experiments.

By addressing these technical and bioinformatic considerations, researchers can ensure accurate and reliable analysis of cfDNA and its different populations.

8 Clinical Relevance

Different characteristics of cfDNA—including mutations, SNP detection, CNA, TFs, variant detection, methylation, and emerging topological features such as fragmentomics and end motif profiling—have been leveraged to tackle a broad spectrum of clinical challenges, and be included within international guidelines for application like cancer monitoring [52, 89]. Here, we primarily focus on its applications in oncology; however, cfDNA has also been widely utilized in non-invasive prenatal testing [90], transplant monitoring [91] and infectious disease detection [92, 93].

8.1 Cancer Detection and Screening

Leveraging cfDNA within liquid biopsy has been shown to be promising in detecting early-stage cancers by analyzing genomic changes and methylation patterns. Studies have reported promising results in cancer screening by using single or multi-cancer early detection (MCED) tests based on cfDNA methylation [94–100] (Galleri test, GRAIL; Cancerguard, Exact Sciences); topological characteristics like transcription factor binding sites and CNA [101–103] (under development, Freenome); fragmentomics [104, 105] (DELFIDiagnostics); and cfDNA mutation profiling [106–108] (Guardant 360 CDx, Shield, Guardant).

8.2 Cancer Treatment Monitoring, Prognosis, and Prediction

The quantity and mutations of cfDNA have also been widely used as prognostic markers. High levels of cfDNA have been correlated with advanced stages of disease and poor prognosis [109]. Predictive biomarkers, such as EGFR mutations in lung cancer, have advanced from single gene tests to more extensive analyses of plasma ctDNA [89].

The quantitative and qualitative changes in cfDNA provide real-time information on therapy effectiveness. Analyzing the levels of cfDNA, as well as the fragmentomic profile of cfDNA, enables the assessment of cancer progression [110], treatment efficacy [111, 112], and MRD [113]. Studies have shown that a decline in the levels of cfDNA is usually associated with a good therapeutic response, while stable or rising levels could indicate resistance or residual disease. By using ctDNA variant detection, there was a significant improvement in MRD detection (PhasED-Seq, Foresight Diagnostics) [114].

Similarly, tumor-specific mutations in ctDNA have shown promise in detecting MRD after therapy or surgery [115, 116] (Signatera, Natera). TFs have been shown to improve the sensitivity and specificity of the cfDNA based assays [117]. Moreover, TFs can differentiate false-negative from true-negative patients with low levels of ctDNA shedding, especially in ambiguous cases [118, 119].

The clinical relevance of cfDNA currently relies predominantly on bulk cfDNA analysis; however, focusing on distinct cfDNA subsets tailored to specific clinical conditions could significantly enhance its diagnostic and

prognostic potential, especially when employing non-mutational cfDNA features.

9 Future Perspectives

The future of cfDNA research holds great promise for unravelling the complexities of human biology and disease. As the field continues to evolve, we can expect a deeper understanding of cfDNA and its role in intercellular communication, its relationship with the microbiome, and its potential as a biomarker for various disorders. Moreover, cfDNA may reveal new insights into the epigenetic regulation of gene expression and disease development.

The current field of cfDNA analyses focuses on the overall assessment of cfDNA. Recent evidence shows that the features demonstrated by cfDNA in plasma can vary based on lengths of cfDNA fragments. Information harbored by different subsets, either in totality or relative to one another, can be useful biomarkers. Analyzing all subsets together may dilute the information that may be represented exclusively by a single subset, such as differences in occurrence of secondary structures in different size-based subsets of cfDNA. Current understanding of different subsets have emerged from the use of different sequencing methodologies. Although interesting, the present understanding of uscfDNA is fairly limited as it has only been explored using low-pass sequencing methodologies. The current landscape of cfDNA, especially considering nucleosomal lengths, has been explored with different sequencing methodologies, yet such exploration is lacking with uscfDNA. Moreover, our knowledge of cfDNA coming from different biofluids offers another exciting domain for exploration. In addition to the exploration of the physical and biomarker potential of the different subsets of cfDNA, it would be interesting to look into the clearance and biological process associations for the different subsets of cfDNA. As cfDNA research continue to push the boundaries of our understanding of human biology and disease, we can expect to see new therapeutic applications. For example, cfDNA may be used as a vehicle for drug delivery or as a template for gene editing.

From a technical point of view, advancements in sequencing technologies, PCR techniques, and bioinformatics tools will be crucial for unlocking the full potential of cfDNA. Future developments may also include the integration of machine-learning algorithms to improve data analysis and interpretation and the development of novel methods for cfDNA isolation and detection. Furthermore, standardization of protocols and establishment of reference datasets will be essential for ensuring the reproducibility and comparability of cfDNA research.

Declarations

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Conflict of Interest David Wong, Neeti Swarup, Ho Yeung Leung, Irene Choi, Mohammad Arshad Aziz, and Jordan Cheng declare that they have no conflicts of interest that might be relevant to the contents of this article.

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