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Review Article

Fragmentomic cfDNA Patterns in Noninvasive Prenatal Testing and Beyond

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Abstract

The release of fetoplacental cell-free DNA (cfDNA) into the maternal bloodstream opened up new avenues towards noninvasive prenatal testing (NIPT) for aneuploidies, hereditary DNA mutations and other pregnancy-related developmental disorders. Increasingly, cfDNA catches interest for its noninvasive screening value in other areas as well, including oncology. Although there are indications that cfDNA fragmentation is a non-random process, the etiology and different structural aspects of cfDNA are still not well known. The emerging field of cfDNA fragmentomics investigates the existence of tissue and disease specific cfDNA signatures and the chemistry and biology underlying the fragmentation process. This review sheds light on recent developments in cfDNA fragmentomics and illustrates their significance in NIPT improvement and beyond.

We discuss aspects of fragment size distributions, epigenetic correlations and putatively enriched cfDNA fragment-end compositions. Combinatorial fragmentomic efforts have provided more insights into the roles of different enzymes that contribute to the fragmentation process in the tissue of origin and in the bloodstream. Altogether, these studies revealed multiple fragmentomic-related biomarkers that can be used to make noninvasive screening and other types of clinical use of cfDNA more robust, by raising its distinctive capacities. This includes multiple complementary approaches to determine the fetal fraction, a key determinant in NIPT. Furthermore, these developments translate to a better understanding of the encountered cfDNA patterns and will catalyze the expansion of screening possibilities in NIPT and beyond.

Keywords: Fragmentomics; cfDNA; ctDNA; NIPT/NIPS; prenatal testing
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INTRODUCTION

Invasive prenatal testing methods such as amniocentesis and chorionic villus sampling (CVS) pose iatrogenic risks for both mother and fetus.[1] This problem could be overcome by using safer methods based on maternal blood sampling in prenatal screening. The presence of intact nucleated cells of fetal origin in the bloodstream of pregnant women was first reported in 1893[2], and later prompted speculation of their clinical significance and potential applications in noninvasive prenatal testing (NIPT).[3] Although the existence of double-stranded cell-free DNA (cfDNA) in blood was first demonstrated in 1948[4], qualitative evidence for presence of cell-free fetoplacental DNA (cffDNA) traces among the cfDNA pool of the carrying mother was not found until 1997.[5] Parallel advancements in nucleic acid analysis methods consequently promoted development of strategies for quantification of cffDNA from maternal blood samples as an alternative approach to perform prenatal genome screening.[6] The first successful demonstrations of cffDNA-based screening advocated

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the improved patient safety, operational ease, time efficiency and scalability as compared to invasive methods.[7-9]

In the subsequent decade, fast-paced concurrent developments in sophisticated sequencing technology further enabled increasingly accurate restoration of the fetal genome from maternal plasma, even reaching the point of full recovery of the entire fetal genome.[10]

The levels of cffDNA in pregnant women are dynamic during the entire pregnancy, reaching highest levels near delivery.[11] Delivery is succeeded by rapid biphasic clearance from the maternal plasma, with estimated cffDNA half-lives of 1 hour and 13 hours respectively, rendering it undetectable typically within two days postpartum.[12] Increased levels of cffDNA have been correlated to fetal aneuploidy[8,13] but also to pregnancy anomalies including pre-eclampsia[14,15], hyperemesis gravidarum[16] and premature labor.[17] However, increased cfDNA levels alone have limited diagnostic value at the individual patient level. Initial cfDNA-based screening offered opportunities to perform fetal sex determination, identify paternally inherited traits and detect fetal aneuploidies.[7,9,18] The major breakthrough came in 2008 with the first description of exploring cffDNA with next-generation sequencing, allowing for noninvasive detection of fetal trisomies by increased levels of cfDNA originating from chromosomes 13, 18 or 21 in maternal blood.[19,20] Noninvasive restoration of the entire fetal genome[10] was followed by genome-wide noninvasive aneuploidy detection[18] and noninvasive aneuploidy detection in multiple pregnancies.[21-23] The discovered potential of cfDNA as an alternative, reliable and noninvasive diagnostic source led to rapid clinical implementation as a second (after combined testing) or first-tier test in prenatal screening[24-29] (Figure 1).

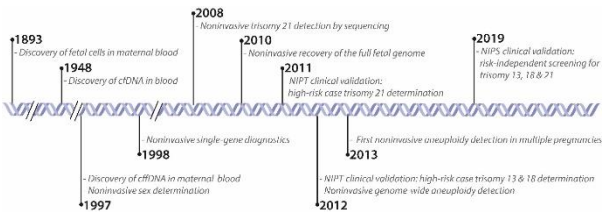

Figure 1. Timeline of the main events leading towards the development of NIPT

Analogously to the successive developments preceding clinical NIPT integration, the detection of tumor-derived cfDNA, referred to as circulating tumor DNA (ctDNA), was pivotal in the development of noninvasive cancer detection methods, also known as liquid biopsies.[30] The fields of oncology and prenatal screening and diagnostics perpetually exchange developments, and sometimes even intersect when ctDNA is detected during NIPT.[29,31] Also in cardiology and neurogenetics, liquid biopsies have been shown useful for clinical monitoring, for instance for heart failure[32] and neuronal atrophy.[33] Somewhat less known, but not less promising, are the pioneering efforts in cfDNA-based organ transplants monitoring[34,35] and cfDNA-based diagnostics of parasitic[36], viral[37] or bacterial[38] infections. Moreover, other medical disciplines have reported presence of cfDNA in body fluids other than blood plasma, including urine and saliva, which in turn provides stimulus and opportunities for further expansion of noninvasive medical research.

Despite its widespread use in clinical practice, most molecular features of cfDNA itself remained poorly studied for a long period and much of the underlying biology remains to be resolved. Recent attempts to elucidate other cfDNA characteristics have consistently demonstrated reproducible cell-type specific patterns in fragment size distributions, genomic origin and other features such as methylation profiles. These consistent observations indicated that cfDNA fragmentation is a non-random process, which sparked impulse in the emerging field of 'cfDNA fragmentomics'. Deciphering the architecture and etiology of cfDNA landscapes may lead to better understanding of the involved biological processes and could provide novel insights for improving diagnostics and screening including, but not limited to, NIPT. This review sheds light on the recent developments in the field of cfDNA fragmentomics and aims to illustrate their significance for NIPT.

FRAGMENT SIZE DISTRIBUTION PATTERNS

Early observations of increased cfDNA levels in cancer patients raised questions regarding the tissue of origin of the excess cfDNA.[39] Tumor-specific altered methylation patterns allowed Jahr and colleagues to discriminate between ctDNA and non-ctDNA pools, and to determine the tumor-derived fraction in patient samples.[40] The same authors demonstrated a correlation between the presence of apoptotic or necrotic tissue and increased overall cfDNA levels, supporting the concept that tumors likely contribute to elevated cfDNA levels. Their results suggested necrotic cells dispose of large DNA fragments (>10,000 bp) whereas apoptotic tissue produced smaller fragment sizes of approximately 180 bp, and multiples thereof, reminiscent of earlier reported nucleosomal fragmentation patterns found in apoptotic cells.[41] cfDNA size features continued to be studied hereafter, with use of electrophoresis techniques, quantitative PCR and more recently massively parallel paired-end sequencing.[42]

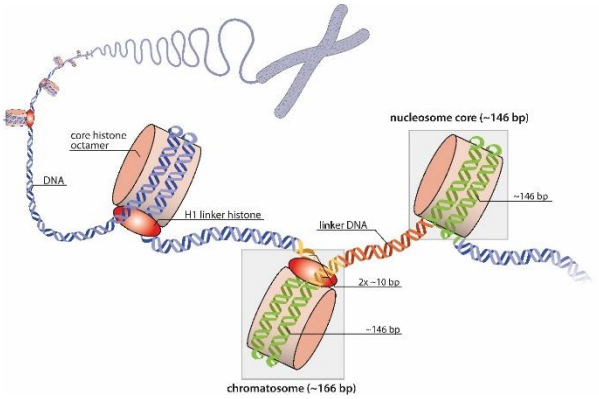

Figure 2. Schematic overview of the 'beads-on-a-string' architecture of nuclear DNA. ~146 bp DNA winds around a core histone octamer to yield a nucleosome core unit. When the linker histone H1 binds to this complex, an additional ~10 bp on both DNA ends are bound, yielding a chromatosomal unit encompassing ~166 bp in total. These units are interspaced by linker DNA of arbitrary length. Intermediate condensed structures are not displayed.

In pregnant women, it was found that the size distribution of cffDNA shifts to be slightly shorter on average than that of cfDNA fragments originating from maternal tissue.[43] A paired-end sequencing study by Lo and colleagues in 2010 aided in the accurate determination of cfDNA size distributions, revealing a major ~166 bp peak, a less pronounced ~143 bp peak and recurrent 10 bp-interspaced declining peaks for cfDNA from pregnant women, which is composed of both maternal- and fetal-derived fractions. In the fetal derived cfDNA, the difference between the 166 bp and 143 bp peak was smaller compared to the difference seen for the maternally derived fragments, confirming that the fetal fraction is enriched for the shorter (~143 bp) fragments.[10] As with DNA from apoptotic tumor tissue, these fragment sizes are reminiscent of the nucleosomal 'beads-on-a-string' organization of nuclear DNA, since they reflect the dimensions and periodicity of histone-wound sequences[44] (Figure 2).

This remarkable shift in size distribution instigated approaches to discriminate fetal from maternal cfDNA from the combined pool present in maternal plasma. Especially in early gestation, a low fetal fraction and high background maternal signal pose a major hurdle for the sensitivity of NIPT.[45] The discovery of distinct size distributions prompted the concept that low-abundance cffDNA could be enriched for by size selection. This led to multiple novel size-based approaches to effectively increase the detected fetal fraction.[46-51] Yet, while increasing the detected fetal fraction, this type of enrichment was also shown to reduce the total count of cffDNA molecules detected, counterproductively impacting the statistical significance of any aberrations of interest detected.[52] Instead, by applying size-based discrimination *in silico*, size distribution shifts were found to be of diagnostic purpose in sex-independent fetal fraction determination[53], identification of aneuploid pregnancies[54] and distinguishing fetal aberrations from maternal ones.[55] Especially in aneuploidy detection, both size distribution shifts and read counts of potentially aneuploid fetal chromosomes can function cooperatively to achieve supplemented and accurate results. Similarly, ctDNA fragment size as fragmentomic marker has shown to be of value for enhanced noninvasive detection of cancers.[56]

EPIGENETIC PATTERNS IN FRAGMENTOMICS

The first concrete evidence linking the size distribution of cfDNA to the nucleosomal organization was found in 2008 by using paired-end sequencing to demonstrate that the ends of cfDNA fragments map to genomic chromatin regions covered by nucleosomes, such as regions surrounding transcribed genes.[20] The authors emphasized consistent periodic alignment of cfDNA fragments just downstream of transcriptional start sites, which is in line with characterized eukaryotic histone occupancy at such genomic loci.[57] This relation between nucleosome positions and distribution and length of cfDNA has been confirmed with different approaches by several independent groups, even allowing the determination of genome wide nucleosome pattern based on starting positions of sequence reads.[53,58,59] Moreover, it was found that cfDNA originating from mitochondria and viruses are abundantly enriched for smaller sized (<50bp) fragments which explicitly do not obey the periodic distribution patterns as found for cfDNA of nuclear origin.[10,58] Given that the mitochondrial and viral genomes use alternative, non-nucleosomal, DNA packaging mechanisms[60], these findings support the theory that nuclear-derived cfDNA fragmentation is not a random process and might be associated with detailed aspects of nucleosomal organization.

Nucleosomal occupancy is strongly associated with the transcriptional activity of specific genomic regions.[61,62] Methylation of cytosine residues at the DNA level has been shown to moderate a nucleosome's positioning and its regulatory effects.[63,64] Studies on tissue-specific differential methylation of genomic regions, and especially dysregulation thereof, contributed to profound understandings in cancer research.[65] The discovery that epigenetic nucleic acid modifications, rather than the actual DNA sequence itself, could be used for discriminating cfDNA originating from different tissues caused interest in the development of tissue-specific diagnostic and therapeutic strategies.

For the NIPT field, this logically translated into attempts to discriminate cffDNA from a maternal cfDNA pool, based on fetoplacental tissue-specific hyper-methylated genomic regions. The *SERPINB5* locus on chromosome 18 was the first of such regions on which this principle was demonstrated to determine fetal trisomy 18.[66] A decade later, this extrapolated to methylotyping over 5,000 regions in parallel, simultaneously distinguishing cfDNA from a range of tissues including the placenta.[67] Major advantages over conventional methods include that the procedure does not require knowledge on parental haplotypes nor on fetal sex. However, distinguishing hypo-methylated fetal DNA typically involves toxic bisulfite treatment which extends the laboratory workflow and notably is known to degrade more than 90% of the DNA present in a reaction.[68] For NIPT in particular, this is a major drawback, as the fetal fractions studied already are very low. Methylation-sensitive enzymatic restriction, as a cheaper alternative method, is mainly limited in terms of which sequences can be targeted. Methylated DNA immunoprecipitation offers a relatively flexible and sensitive approach, but involves more costly tailored antibodies. A combination of these strategies has recently allowed for a methylation-based estimation of the fetal fraction.[69]

Methylation-sensitive digestion of the cfDNA pool, in which methylated nucleotides are protected from degradation, depletes abundant hypo-methylated maternal sequences, allowing to increase the fraction of cffDNA. Hyper-methylated fetoplacental genomic loci, such as *RASSF1A, SOX14 and TBX3*, may serve as useful marker candidates in such enrichment approaches.[70,71] Further exploration of the fetoplacental methylome showed that, overall, it is initially hypo-methylated, but dynamically changes with gestational age.[72] This suggests that the most pronounced maternal-fetal methylome differences occur within the first trimester, when most NIPT analysis are performed, as compared to the third trimester, in which cffDNA was found to be relatively more methylated. In contrast to most

Table 1. Overview of characterized fragmentomic markers suitable for distinguishing between maternal and fetoplacental cfDNA, and their relevant applications in NIPT.

Fragmentomic marker	Maternal cfDNA	Fetoplacental cfDNA	Applications in NIPT	References
Predominant fragment size (mode)	Approximately 166 bp	Approximately 143 bp	• Fetal fraction determination • *In vitro* & *in silico* enrichment for fetal fraction • Distinguishing between fetal and maternal aberrations	46-51,54,55
Alignment of fragment ends related to genomic nucleosome positions	Most fragment ends map to the linker DNA regions between nucleosomes	Most fragment ends map to the boundary regions of nucleosomal DNA	• Fetal fraction determination • *In silico* enrichment for fetal fraction • Distinguishing between fetal and maternal aberrations	53,76,81
Methylation profiles of fetoplacental-specific activated/silenced genes	Baseline methylation profiles	Hypo- or hypermethylated with respect to the maternal cfDNA baseline profiles	• Fetal fraction determination • *In vitro* enrichment for fetal fraction • Aneuploidy detection based on allelic ratio • Distinguishing between fetal and maternal aberrations • Detection of methylation-associated fetal dysmorphologies	66,67,70-73
Tetranucleotide 5' fragment end motifs	Baseline end-motif frequencies	- Increase in CCCA, CCAA, CAAA end motifs - Decrease in ACTT, ACCT, CTGG end motifs	• Fetal fraction determination • *In silico* enrichment for fetal fraction	87
Fragment end staggered end length (mean)	21 nt	19 nt	• Fetal fraction determination • *In silico* enrichment for fetal fraction	86

conventional genetic markers, this highlights that reversibility and fluctuation of epigenetic markers need to be taken into account when setting up assays. Dysregulation of the normally tightly regulated placental epigenome is associated with impaired fetal development and placental dysmorphology, prompting recent efforts to demonstrate methylotyping of cffDNA could also serve an inherent diagnostic purpose, rather than solely providing complementary approach for fetal fraction determination.[73]

Apart from DNA-methylation, a myriad of histone modifications has been characterized that mediate nucleosomal positioning.[74] By maintaining a tailored nucleosomal organization, these factors constitute a tissue-specific epigenetic transcription control. As a consequence, nucleosomal organizations retain genotype-independent information on the corresponding tissue of origin. Deep sequencing of cfDNA pools has been shown to yield alternate coverages around tissue-specific genes compared to housekeeping genes, indeed suggesting that cfDNA mapping can be used to trace back the nucleosomal landscape architecture and thereby link it to the tissue of origin.[75] Whereas in healthy individuals the majority of the cfDNA pool is of hematopoietic origin, cfDNA pools of different types of cancer patients reflect increased contributions coming from corresponding tumor tissues.[59] A differential tissue-specific histone architecture is expected to result in a shift of mapped cfDNA fragment ends, as histone depleted regions are expected to be degraded, and vice versa. Bioinformatic analysis of such cfDNA fragmentation patterns enabled researchers to determine which tissues largely contributed to the cfDNA pool.[76,77]

These results suggest that in cfDNA research, nucleosomal profiling offers alternative approaches for distinguishing genetic material based on the tissue of origin. This was quickly demonstrated to be useful for the detection and monitoring of several types of cancer.[59,76,77]

For NIPT, adequate characterization of fetoplacental-derived sequences can serve as a complementary strategy to determine the fetal fraction with improved accuracy, but it enables more than just that. Knowledge on the tissue of origin offers possibilities, for instance, to distinguish between fetuses in multiple pregnancies or to distinguish between fetal anomalies and maternal mosaic anomalies if for instance a trisomy 8 is found.[78] Similarly, noninvasive detection of epigenetic markers on ctDNA allows for tracing back the tissue where the tumor is located.[79] At the intersection of perinatal medicine and oncology, NIPT results sometimes display maternal cancer signatures[29], which can be linked to a potential tissue of origin for adequate confirmation by further diagnostics.

cfDNA FRAGMENT END PATTERNS

The fact that cffDNA fragments are generally shorter than maternal cfDNA fragments prompted to study differences in genomic preferred end-sequences between maternal and fetoplacental cfDNA. Ultra-deep cfDNA sequencing of these fragment ends led to the characterization of fetal-specific preferred genomic sites.[80] The same study demonstrated the use of the identified preferred fragment ends for determination of the fetal fraction. An even more accurate estimation of fetal fraction could be calculated by combining information on both fragment size and preferred fragment ends.[81] The authors showed that such a combinatorial approach can increase the reliability of noninvasive prenatal trisomy 21 detection by enrichment for fetal fragments.

As briefly discussed above, fetal fragments are somewhat shorter than maternal fragments. The shorter fetal fragments are too short to fully wrap around a histone octamer twice, in contrast to maternal fragments which typically measure ~166bp, the length needed to span a complete nucleosome, including H1 (Figure 2). This difference is reflected by the breakpoints of both fetal and maternal cfDNA. Breakpoints of long (170-250 bp) fragments map to linker DNAs that interspace nucleosomal sequences, whereas ends of short (60-155 bp) fragments map to the border and central regions of nucleosomal DNA. This confirms that fragmentation of fetoplacental DNA frequently occurs at histone-bound sequences, whereas maternal DNA is mostly cleaved at the linker DNA sequences in between histones. Further proof was obtained by determining chromatin-accessibility using a transposase, which makes cuts in non-nucleosomal DNA. Such transposase treatment resulted in cleavage of nucleosomal fetoplacental DNA, but not of nucleosomal maternal DNA.[81,82] This suggests that fetoplacental DNA is less tightly packed around histones, resulting in increased accessibility for putative enzymes involved in fragmentation during apoptosis, which leads to shorter fragments as compared to maternal DNA fragmentation.

Researchers reasoned that if cfDNA fragmentation is indeed non-random, and there are genomic cleavage hotspots depending on the tissue of origin, the fragmentation process would lead to so-called cfDNA 'preferred ends'. In 2015, significantly elevated cytosine-rich fragment ends were found in cfDNA as compared to the random distribution in sonicated genomic DNA.[58] This preference for C-rich nucleotide fragment end motifs has been confirmed by other researchers as well.[83-85] Notably, this pattern was explicitly not observed for mitochondrial-derived cfDNA fragments, again supporting the view that the mitochondrial genome is degraded by a distinct mechanism.[58]

When initially examining the fragment end nucleotide signatures in samples from pregnant women through deep sequencing, insignificant differences were observed between fetal and maternal end patterns, suggesting that the same enzymatic fragmentation mechanism is active in both maternal and fetoplacental tissue.[58] Five years later, the discovery that the vast majority of cfDNA fragment ends are staggered, instead of blunt, suggested that relying on deep sequencing only, may miss out on additional information about fragment end patterns.[86] Fragment end restoration prior to sequencing revealed that different tissue types, amongst which the placenta, bear their own preferred fragment end identity. The study characterized such preferred tetranucleotide sequences and demonstrated their potential value as fragmentomic marker for cancer detecting cancers, monitoring organ transplants and prenatal testing.[87]

DECIPHERING THE FRAGMENTATION MECHANISM

The above-mentioned organized consistencies found in cfDNA size distributions, related epigenetic patterns and fragment end compositions all support the upcoming paradigm that cfDNA fragmentation during apoptosis is a non-random process. The non-random fragmentation patterns have resulted in characterization of multiple fragmentomic markers that can aid or complement the NIPT diagnostic pipeline (**Table 1**). Proper understanding of the underlying fragmentation mechanism may lead to explanations for why maternal and fetal fragmentomic patterns deviate from each other, and could improve the use and evaluation of the derived fragmentomic markers in NIPT, and in other fields that intersect with cfDNA research.

In particular the observed size- and sequence-based preferences for fragmentation suggest that the mechanism involves DNA endonucleases that preferably cleave genomic DNA into nucleosomal fragments with C-rich ends. As DNase1 is one of the most prominent and widespread desoxyribonucleases found in mammals[88], it was a straightforward candidate for further studies regarding its role in cfDNA fragmentation. However, since DNase1 preferentially cleaves 'naked' DNA instead of protein-bound DNA[89], DNase1-deficient murine models did not result in significantly deviating cfDNA patterns, suggesting that other nucleases are more dominantly involved.[90]

The *DNASE1L3* gene codes for DNase γ, another major mammalian endonuclease found in blood plasma. cfDNA analysis of murine models with a deficiency for this enzyme revealed increased levels of large multinucleosomal fragments as well as almost halved frequencies of the normally C-rich end motifs.[83] Similar deviant cfDNA fragment size and end motif profiles have been found in plasma of human subjects with DNase γ-deficiency.[84] The same trend was observed in plasma of hepatic cancer patients, where the *DNASE1L3*-expression was around 10-fold lower than in surrounding healthy liver tissue.[87] Surprisingly, the DNase γ-deficient mouse models also showed an increase in shorter fragments (20-120bp), which was attributed to the autoimmune response phenotype affiliated with this deficiency, resulting in anti-DNA antibodies that presumably lead to increased fragment degradation.[83] This was verified in double-deficient mice for DNase γ and CD40 ligand, which were incapable to mount such an immune response and showed normal levels of short fragments. Functional DNase γ originating from the fetus could partially rescue the aberrant patterns of cfDNA originating from the DNase γ-deficient murine mother, implying that the protein can enter the maternal bloodstream and continue to perform its effector function

systemically. Moreover, viral transfection of a DNase γ-deficient mouse with a functional copy of *Dnase1l3*, the mouse homologue, resulted in an *in vivo* rescue of the aberrant profiles.[84] The same study demonstrated *in vitro* that the DNase γ, unlike DNase1, prefers protein-bound DNA substrates over naked DNA substrates to generate fragment sizes reminiscent of a nucleosome. Furthermore, DNase γ has a strong preference for cleaving at 2 subsequent cytosines, irrespective of the organization of the DNA (i.e. histone-bound or naked). These data strongly support the major role DNase γ plays in cfDNA fragmentation.

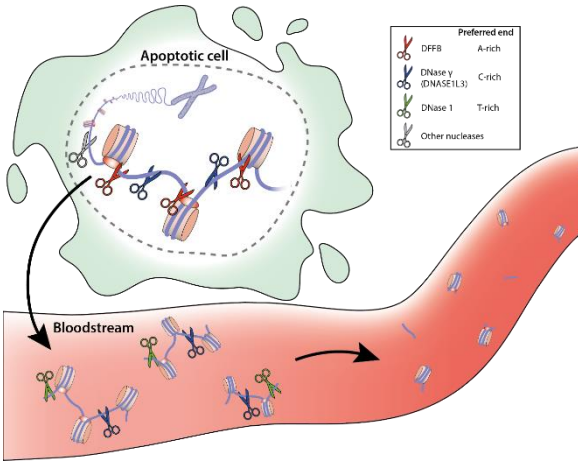

Figure 3. Schematic overview of the cfDNA fragmentation model. During apoptosis, DFFB, DNase γ, and most likely a set of other nucleases, break down the genomic DNA into multinucleosomal fragments. Once released into the bloodstream, they are further degraded systemically by extracellular DNase γ to mononucleosomal units with C-rich ends. DNase 1 can digest these further to smaller sizes with

Since DNase γ is secreted and continues to cleave DNA in the blood stream, researchers set out to investigate properties of 'freshly' released cfDNA, in order to investigate whether there are cellular nucleases involved in DNA fragmentation before cfDNA is released into the bloodstream. By simulating apoptotic conditions in *in vitro* cell models, Han and colleagues succeeded in extracting and analyzing cfDNA directly after excretion.[85] End motifs of newly released fragments turned out to be relatively A-rich, suggesting another non-random cleavage process preceding the DNase γ-mediated C-rich motif end generation. The authors continued to show that cells deficient for the major apoptotic endonuclease, DNase Fragmentation Factor subunit Beta (DFFB), also known as caspase-activated DNase, resulted in virtually no difference between end motifs of newly released cfDNA and cfDNA fragments that already circulated in the bloodstream. Similarly, they showed that the A-rich motifs are accumulated in cfDNA pools from DNase γ-deficient mice, suggesting that the reaction product of DFFB is the substrate for DNase γ.

Even though C-rich ends are the most prevalent among the studied cfDNA ends, it was observed in wild-type murine plasma that smaller (<150 bp) fragments have a slightly increased likelihood of bearing thymine-end motifs.[85] In vitro disruption of the nucleosomal organization by disruptive agents led to even more pronounced T-rich motifs, whereas DNase1-deficient mice did not present this preference. Given that DNase1 prefers naked DNA as substrate, it was hence suggested to act as one of the last in the fragmentation process, preferably releasing cfDNA fragments with T-rich ends. Furthermore, the disruption of nucleosomes also eliminated the 10 bp periodicity normally found for fragments under 150 bp. This periodic fragment degradation is suggested to be resultant of a balance between histon-protected intranucleosomal DNA and degradation of exposed DNA ends, in which both strands are alternately exposed due to the inherent helical 10 bp-per-turn structure. Altogether, these findings led to the first model for enzymatic cfDNA fragmentation as a biphasic process occurring during apoptosis and in the bloodstream[85] (Figure 3).

CONCLUSION

Cell-free nucleic acids increasingly catch interest for their high diagnostic value, in combination with a minimally invasive clinical procedure. Nevertheless, the underlying chemistry and biology of DNA fragmentation in apoptotic cells is still to be understood fully. Numerous patterns demonstrate the non-randomness of the cfDNA fragmentation process and hint for underlying mechanisms to be characterized. These patterns include fragment size distributions, epigenetic correlations and fragment end compositions. The upcoming field of cfDNA fragmentomics examines such patterns to explore new routes towards tracking down the underlying biology of cfDNA fragmentation. Similarly, other cell-free nucleic acids including mitochondrial-derived cfDNA and cfRNA are thoroughly being explored.[91] A close understanding of the responsible fragmentation mechanisms involved enables developments of using cfDNA as a biomarker for more accurate, perhaps more personalized, diagnostics and screening, monitoring and therapeutics.

Rapid technological advancements have enabled scientists involved in cfDNA fragmentomics to study the link between disturbed patterns and clinical features. So far, cancer- and pregnancy-related research has the closest ties with the field of cfDNA, but other disciplines increasingly show efforts to study cell-free nucleic acids as well. Integrating cfDNA fragmentomic information has already been shown to improve testing sensitivity in noninvasive cancer diagnostics and screening.[92,93] One of the major hurdles in prenatal screening is the accurate determination of the fetal fraction. This review has touched upon several identified fragmentomic markers that can be used to approach the fetal fraction, or more generally, to determine the maternal or fetal origin of variants observed using NIPT. Mostly, these are based on patterns that are essentially different between mother and fetus, allowing for distinguishing them *in silico*. The combined outcome of the most recent findings on the involved nuclease biology mark a point in cfDNA research where the fragmentation process is modelled and assigned to several effector nucleases. This model may form the base to further explore the underlying fragmentomic mechanisms that cause maternal and fetal cfDNA patterns to deviate from each other.

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