Review

New Perspectives on the Importance of Cell-Free DNA Biology

Abel J. Bronkhorst *, Vida Ungerer, Angela Oberhofer, Sophie Gabriel, Eleni Polatoglou, Hannah Randeu, Carsten Uhlig, Heiko Pfister, Zsuzsanna Mayer and Stefan Holdenrieder *

Munich Biomarker Research Center, Institute for Laboratory Medicine, German Heart Centre, Technical University Munich, Lazarettstraße 36, D-80636 Munich, Germany
* Correspondence: bronkhorst@dhm.mhn.de (A.J.B.); holdenrieder@dhm.mhn.de (S.H.)

Abstract: Body fluids are constantly replenished with a population of genetically diverse cell-free DNA (cfDNA) fragments, representing a vast reservoir of information reflecting real-time changes in the host and metagenome. As many body fluids can be collected non-invasively in a one-off and serial fashion, this reservoir can be tapped to develop assays for the diagnosis, prognosis, and monitoring of wide-ranging pathologies, such as solid tumors, fetal genetic abnormalities, rejected organ transplants, infections, and potentially many others. The translation of cfDNA research into useful clinical tests is gaining momentum, with recent progress being driven by rapidly evolving preanalytical and analytical procedures, integrated bioinformatics, and machine learning algorithms. Yet, despite these spectacular advances, cfDNA remains a very challenging analyte due to its immense heterogeneity and fluctuation in vivo. It is increasingly recognized that high-fidelity reconstruction of the information stored in cfDNA, and in turn the development of tests that are fit for clinical roll-out, requires a much deeper understanding of both the physico-chemical features of cfDNA and the biological, physiological, lifestyle, and environmental factors that modulate it. This is a daunting task, but with significant upsides. In this review we showed how expanded knowledge on cfDNA biology and faithful reverse-engineering of cfDNA samples promises to (i) augment the sensitivity and specificity of existing cfDNA assays; (ii) expand the repertoire of disease-specific cfDNA markers, thereby leading to the development of increasingly powerful assays; (iii) reshape personal molecular medicine; and (iv) have an unprecedented impact on genetics research.

Keywords: cell-free DNA; circulating tumor DNA; liquid biopsy; cfDNA; ctDNA

1. Introduction

Most human body fluids are, through a complex network of release and clearance mechanisms [1], constantly replenished with a population of genetically diverse cell-free DNA (cfDNA) fragments (Figure 1). Since cfDNA samples can be obtained in a one-off and serial fashion through minimally-invasive procedures, e.g., through a blood draw; cfDNA profiling represents an unprecedented treasure trove of real-time genetic data minable for wide-ranging diagnostic, prognostic, and theranostic purposes. Remarkable progress has already been made on this front with the development of cfDNA assays that trump many of the inherent limitations of traditional methods and are slowly transforming the way in which solid tumors, fetal genetic abnormalities, organ transplant rejections, and infections are diagnosed, monitored, and treated. Furthermore, profiling of cfDNA from serial biospecimen collections holds the potential to revolutionize the characterization of temporal genome dynamics in a variety of contexts. The vast dimension of temporal genomic information accessible through cfDNA analysis has, for example, already been tapped towards the development of methods for the longitudinal assessment of various aspects of tumor biology, including residual disease, metastases, intratumor genetic heterogeneity, shifting mutational landscapes, genetic responses to chemo- and radiotherapy, and mechanisms that underlie the emergence of therapy resistance. All of the abovementioned information, which is virtually impossible to procure through tissue biopsies, has been

invaluable in guiding therapeutic regimes and has already had an overwhelmingly positive effect on the management and survival of cancer patients (reviewed in [2]). These successes indicate the intriguing possibility of developing serial cfDNA tests for monitoring many other pathologies. First, serial cfDNA analyses may provide new insight into many slowly progressive or chronic illnesses which have been correlated with aberrant cfDNA profiles, such as cardiovascular disease, diabetes, autoimmunity, and neurodegenerative disease. Second, serial cfDNA analyses may be especially useful for monitoring progressive diseases or clinical scenarios that are characterized by rather tight temporal thresholds around rapid malignant transformation or the sudden onset of detrimental effects, such as Parkinson's disease, Alzheimer's disease, sepsis, stroke, traumatic injuries, and adverse effects of gene therapy. Moreover, serial analysis of cfDNA may be useful for studying the role of the gut microbiome in human health and disease, the biological footprint and effects of assimilated environmental DNA, and may even have applications in forensic casework and biobank management.
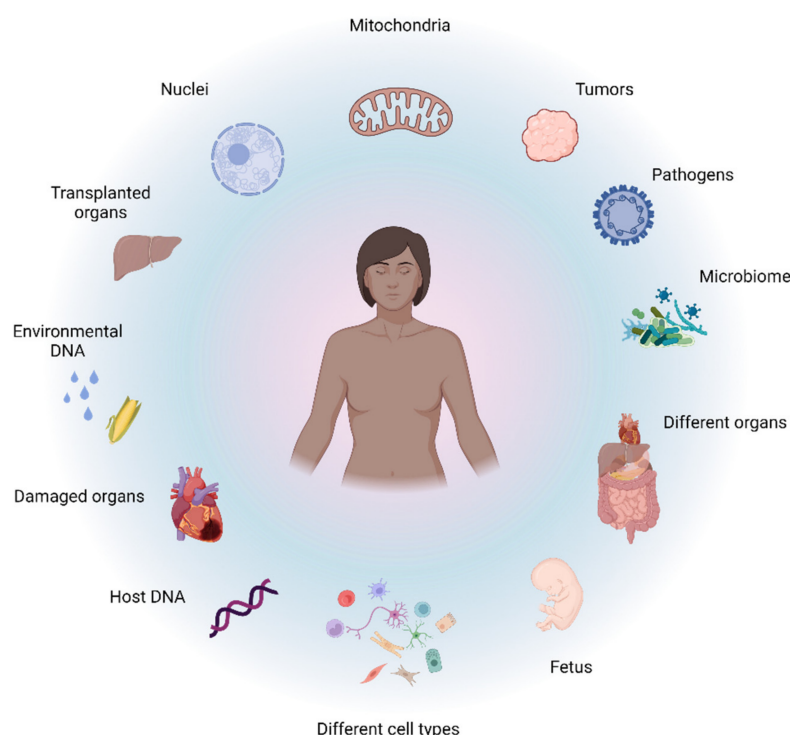
Figure 1. The diverse possible origins of cfDNA in humans. Through various pathways of cell death, clearance, and regulated release, whole or partial genomes of diverse origins are constantly shed into human body fluids in the form of fragmented cfDNA.

Beyond their use as clinical biomarkers, cfDNA molecules demonstrate underappreciated biological functionality. On one hand, numerous studies have implicated cfDNA in the development, progression, and treatment-resistance of various pathologies, such as cancer, autoimmunity, and COVID-19. Therefore, an improved understanding of the molecular mechanisms that underlie the detrimental effects of cfDNA will grant deeper insight into the pathogenesis of specific diseases and likely reveal currently unknown links between cfDNA and other diseases. At the same time, targeted elimination of cfDNA molecules may represent a new therapeutic modality. On the other hand, empirical evidence and elegant theoretical deliberations indicate that cfDNA may be a hugely underestimated factor in several important biological processes, such as immunity [3], intercellular communication [4], and even evolution [5].

Taken together, cfDNA not only shows significant potential as a surrogate marker for numerous disease indications but is also delicately poised as both a pathological factor and important molecule in several biological processes (Figure 2).
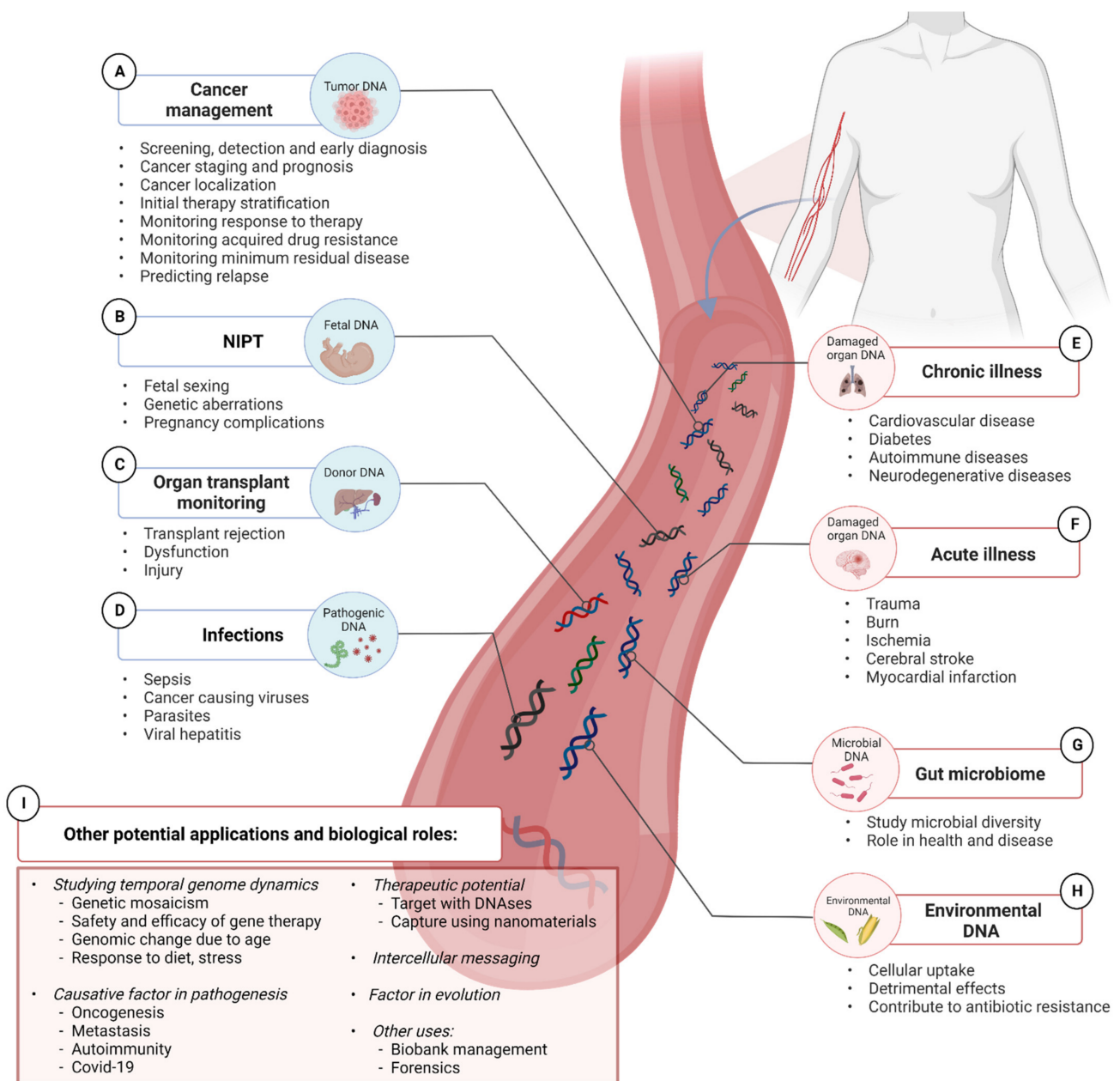
Figure 2. Clinical applications and potential roles of cfDNA in human biology. Human body fluids are constantly replenished by cfDNA fragments of various origins. CfDNA profiling thus offers the unique opportunity to reconstruct major portions of the host- and metagenome, and this information can be harnessed to develop tests for the diagnosis, prognosis, and monitoring of wide-ranging pathologies, such as (**A**) various cancer indications, (**B**) fetal genetic abnormalities and pregnancy complications, (**C**) organ transplant complications, (**D**) infections, (**E**) chronic illnesses, and (**F**) acute illnesses. CfDNA profiling may also be used to characterize (**G**) the gut microbiome and (**H**) assimilated environmental DNA. (**I**) Beyond its use as a clinical biomarker, cfDNA may have many other potential uses and roles in human biology and pathology.

Despite the obvious significance of cfDNA in human biology and pathology, the translation of cfDNA research into useful clinical tests has been advancing at a sub-optimal rate, while the biological functionality of cfDNA is poorly understood and understudied [1,3,6]. As reviewed elsewhere [2,7,8], the development and implementation of clinically meaningful tests is hampered by an array of persistent obstacles that have yet to be overcome, such as a lack of universal preanalytical standards, limited best practice guidelines, analytical lim-

itations, no standard reference materials, insufficient analytical validation, and inadequate clinical trials. Another major factor that negatively impacts both translational and basic cfDNA research relates to the difficulties in achieving high-fidelity reverse engineering of both the quantitative and qualitative characteristics of cfDNA molecules in a biospecimen. Much progress has been made in this regard through rapidly evolving technology, molecular methods, integrated bioinformatics, and machine learning (ML) algorithms, coupled with major efforts in optimizing and standardizing preanalytical procedures. However, accurate measurements of cfDNA are still challenged by numerous obstacles that relate to the biological characteristics of cfDNA. The most prominent challenges in this regard are: (i) the immense heterogeneity in the characteristics of cfDNA in vivo, and the difficulties in differentiating analytically between different cfDNA types (ii) the complex network of biological, physiological, pathological, lifestyle, and environmental factors that modulate the characteristics of cfDNA, (iii) the existence of numerous possible preanalytical steps that are biased toward the preservation, degradation, elimination, or capture of specific cfDNA subtypes, and (iv) a poor understanding of all of the former. Therefore, in this review we explored the biological features of cfDNA and show how a deep and structured enquiry into cfDNA biology may (a) augment the sensitivity and specificity of currently existing cfDNA assays, especially clinical tests based on the detection of hotspot mutations, (b) expand the repertoire of disease-specific cfDNA markers, thereby leading to the development of new and more powerful assays and thus significantly expand the liquid biopsy toolbox and clinical scope of cfDNA assays, (c) open an unprecedented window of access for studying temporal genomic changes as it relates to a wide range of processes, and (d) ultimately shed new light on poorly understood processes as well as reveal hidden biological processes, likely catalyzing a surge of new discoveries about genome function.

2. Measurement of Total cfDNA Levels in Different Contexts

2.1. Serious Medical Conditions

Elevated total cfDNA has been detected in a wide range of disorders, such as cancer [2,9], autoimmune diseases [10] (e.g., systemic lupus erythematosus [11,12], rheumatoid arthritis [13], and systemic sclerosis [14]), trauma patients [15] (e.g., brain injuries [16,17] and burn patients [18]), cardiovascular diseases (e.g., acute myocardial infarction [19] and acute coronary syndrome (ACS) [20]), viral infections (e.g., acute Puumala Hantavirus Infection [21] and Crimean–Congo hemorrhagic fever (CCHF) [22]), benign gastrointestinal tract disorders [23–25], kidney disease [26,27], lung disease (e.g., chronic obstructive pulmonary disease exacerbations [28] and pulmonary embolism [29]), thyroid disease [30], pregnancy disorders (e.g., abnormal placentation, such as preeclampsia [31] and intrahepatic cholestasis [32]), skin conditions (e.g., psoriasis [33,34]), and stroke [35,36].

2.2. Other Clinical Scenarios

In addition to the more common medical conditions listed above, which are clearly associated with cell or tissue injury and damage, total cfDNA levels have also been correlated with a variety of other more obscure clinical scenarios. Elevated total cfDNA levels have been found in psychiatric disorders, e.g., schizophrenia [37]. Conversely, cfDNA levels were found to be lower in patients with extra temporal lobe epilepsy vs. healthy subjects [38]. CfDNA levels were also found to be correlated with indications during in vitro fertilization (IVF) procedures, while also reflecting male and female fertility status. For example, cfDNA levels in the fluid of the blastocoel cavity of embryos correlated positively with embryo morphology, indicating promise as a candidate marker of embryo quality and implantation potential [39]. Similarly, increased cfDNA levels have been demonstrated in low pregnancy rates among women undergoing IVF–embryo transfer [40]. In line with this, the performance of various stress reduction techniques among women undergoing infertility treatment resulted in a decrease in total cfDNA levels [41]. Interestingly, cfDNA levels in the seminal plasma of azoospermic (no sperm in ejaculate) men has been shown to be higher than in normozoospermic men [42,43].

2.3. Medical Treatments and Therapy

Various kinds of medical treatments have been shown to result in elevated cfDNA levels in comparison with untreated subjects, including various surgeries [44], radiotherapy [44], high doses of corticosteroids, particularly in patients with lymphoid hyperplasia or sustained immunologic stress (which may be ascribed to the lympholytic effect of steroids) [45], patients with end-stage renal disease (ESRD) receiving haemodialysis [27,46,47], and intensive treatment unit patients receiving ventilation [48,49].

2.4. Different Physiological States

Studies have demonstrated higher cfDNA levels in older individuals (over 60 years) vs. younger individuals, a phenomenon linked with various age-associated processes, including increased cellular senescence, inflammation-induced cell death, and reduced clearance and phagocytosis capacity [50,51]. Increased mass of adipose tissue in overweight and obese pregnant women has been shown to result in increased cfDNA levels, often complicating non-invasive prenatal screening tests (NIPTs) [52,53].

Some reports suggest that measured cfDNA levels depend on the time of day when samples are collected and show significant intra- and interindividual variation [54,55]. One study, for example, reported that the majority of subjects presented with maximum cfDNA levels at midday [56], while fasting subjects in a different study showed the highest cfDNA levels in the morning, which decreased up to three-fold after breakfast and lunch [57,58]. While it is speculated that circadian rhythms, postprandial effects, or the effects of fluctuating blood lipid content (in response to food intake) on DNA isolation methods may play a role in intra-day fluctuating cfDNA levels, the true cause is not yet clear.

An evaluation of 25 studies that reported on the relationship between cfDNA and biological sex revealed a greater likelihood of increased cfDNA levels in males vs. females. However, these differences are considered to be minor and may reflect lifestyle differences between males and females, and as such requires further investigation [59]. Other variables such as haematocrit or cannula placement pain [60], height [61,62], and the menstrual cycle [63,64] were not found to affect total cfDNA levels.

2.5. Lifestyle Factors and Occupational Exposure Hazards

Studies have shown that high-intensity or acute exercise markedly increases plasma cfDNA levels. While the cause of this phenomenon is not yet known, it is possible that these cfDNA molecules originate from strenuous physical exertion-induced muscle damage, oxidative stress-induced DNA damage, or leukocyte inflammatory responses [54,62,65–67], but this explanation is challenged by observations that cfDNA levels increase dramatically almost immediately after beginning exercise [54,68]. Conversely, cfDNA measurements in subjects engaging in chronic exercise showed less pronounced spikes and inconsistent levels [69]. To-date, only two studies have investigated the effects of alcohol intake on total cfDNA levels, but no correlation was found [70,71]. However, more research is needed, as these studies had incomplete methodological reporting, used a non-specialized method for cfDNA isolation, failed to specify alcohol intake for subjects in the 'moderate-severe' category [71], have not in their cohort categorization accounted for possible differences between social drinkers and non-drinkers [70], and were performed in the context of a study on cancer [59].

Despite the DNA-damaging effects of cigarette smoking, surprisingly few studies have assessed the correlation between smoking and cfDNA levels. Of the ten studies that have been conducted so far, highly conflicting results were obtained, together showing virtually no correlation between smoking and cfDNA levels [59]. It is possible that a correlation may be found in clinical studies that have enrolled appropriately powered, equally balanced, and sex- and age-matched subjects into their cohorts, for example.

Significantly elevated total cfDNA levels have been demonstrated in greenhouse workers exposed to pesticides for 5–15 years vs. controls [72], while increased cfDNA levels coupled with increased DNA damage were demonstrated in workers exposed to toxic

paints in automobile paint shops [73]. Conversely, another study demonstrated a much lower level of cfDNA in nuclear-site workers exposed either to gamma-neutron or chronic tritium β-radiation [74]. To our knowledge, no other studies have evaluated the effect of occupation-related exposure to toxic substances on total cfDNA levels. Yet, it seems likely that cfDNA measurements could potentially be used to monitor worker health in various occupations that are at increased risk of chronic exposure to harmful substances. These include, for example, miners that inhale silica, construction workers that are exposed to asbestos and regularly use products that release toxic fumes (e.g., insulation, glues, paint, polyurethane, blown foam, and solvents), welders that work with metals that become toxic when heated, and textile workers exposed to chemicals such as formaldehyde, arsenic, and cadmium.

As mentioned earlier, total cfDNA levels are influenced by some therapies, and may be affected by food intake. Apart from these studies, and those on alcohol use, cigarette smoking, and one study that found no correlation between cfDNA levels and a history of betel nut chewing (seeds with stimulatory effects akin to amphetamines and cocaine) [70], there are no further data on the correlation between nutritional, medicinal, or drug abuse status and changes in total cfDNA levels. Detecting changes in the baseline values of cfDNA in response to the consumption of certain foods, dietary supplements, medicine, or drugs of abuse may be complicated by the short half-life of cfDNA, which is currently estimated between 16 min and 2.5 h [75–77]. However, it may, for example, be interesting to investigate changes in total cfDNA levels during the chronic use of medications that may cause muscle atrophy, such as cholesterol medication, or in individuals with a long-term diet of inflammatory foods, or in individuals that abuse drugs that cause chronic inflammation, for example inflammation of the immune system [78] or pancreas [79]. Other lifestyle factors such as frequent blood donation were not found to correlate with cfDNA levels [80].

2.6. Limitations of Quantitative cfDNA Measurements

At first glance, the reports discussed in the sections above suggest that total cfDNA levels could potentially serve as a stand-alone biomarker for detecting and monitoring several disorders and other clinical scenarios. However, the use of total cfDNA levels for this purpose is highly unlikely due to the convergence of several factors that result in greatly overlapping data between different disease types and healthy individuals both in individual studies and in interstudy comparisons, thereby precluding the establishment of cut-off values or normal reference ranges for any specific condition (Figure 3). These factors include: (i) elevated cfDNA levels is not a phenomenon unique to specific pathological states but is instead a common consequence of many diseases; (ii) cfDNA levels increase in response to a wide range of ordinary non-pathological conditions and also correlates with numerous lifestyle factors; (iii) as will be described in greater detail in Section 9, accurate measurements of total cfDNA are also significantly affected by a plethora of biological factors as well as factors relating to its physico-chemical properties, the nature of various preanalytical steps, and analytical decisions. It is also likely that the co-presence of so many overlapping variables has resulted in the reporting of erroneous correlations between total cfDNA levels and a specific factor, while interesting correlations between a specific factor and total cfDNA levels have been obscured to date.

Although total cfDNA levels have limited clinical utility, the surge of early studies on the correlation between total cfDNA levels and clinicopathological data sparked widespread interest in the genetic and epigenetic characterization of cfDNA. This primed the ground for rapid advancements in molecular methods and technologies which accelerated the discovery of unknown correlations between qualitative characteristics of cfDNA and various disease indications, particularly in the medical fields of oncology, prenatal testing, and organ transplant monitoring. This, in turn, enabled the development of several cfDNA tests that have been approved for use in routine clinical practice. In the next sections we discuss new advances, approved and potential clinical applications, as well

as challenges related to qualitative cfDNA assays. In outlining these new advances in the research field, it is interesting to note how it may reignite interest in the conditions discussed in Sections 2.1–2.5 or inspire enquiry into unexplored domains of biology and medicine in relation to cfDNA, and how quantitative measurements of total cfDNA may be resurrected as a potential auxiliary marker to qualitative characterization of cfDNA.
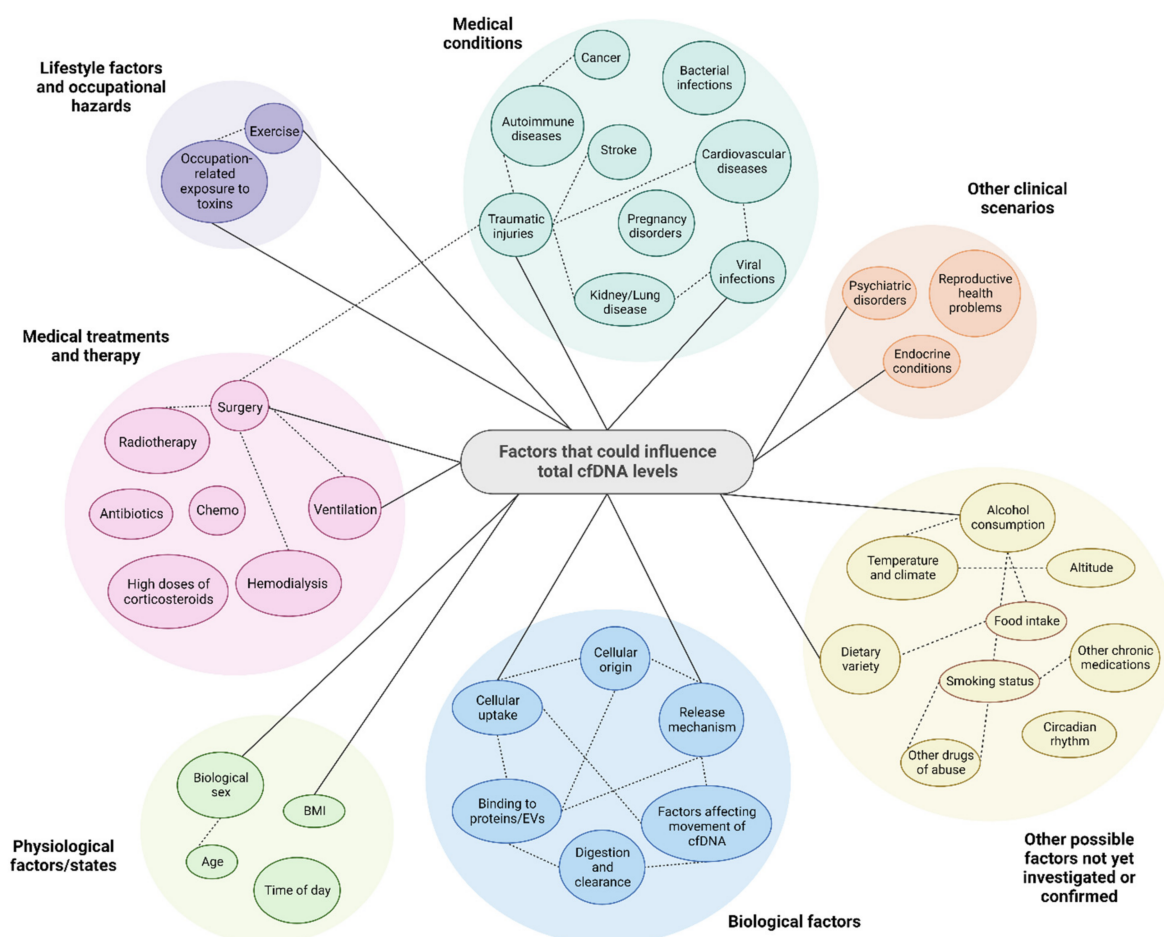
Figure 3. Factors that can potentially affect total cfDNA levels. Here we summarize the wide-ranging factors that have been experimentally shown to modulate total cfDNA levels. We also show several other factors that possibly affect total cfDNA levels, but which have not yet been conclusively demonstrated or have not yet been investigated. This makes it very difficult to correlate total cfDNA levels with a specific factor and limits the clinical use of total cfDNA measurements.

3. Sequence Analysis of cfDNA

Hundreds of studies have investigated cfDNA in various biospecimen types for the detection of mutations or sequences unique to individuals, organisms, and diseases. This led to many exciting discoveries, while the use of increasingly sophisticated molecular analysis methods for cfDNA analysis has enabled the development of diagnostic assays in various clinical fields, some of which are already clinically available. Below we give an overview of the potential applications of cfDNA sequence analysis.

3.1. Cancer

The tremendous value of cfDNA as a surrogate molecular marker throughout cancer management is now well understood (reviewed in refs. [2,81–83]). In-depth profiling of cancer-specific mutations in cfDNA may enable the development of pan-cancer screening tests for at-risk groups or unsuspected healthy populations, thereby allowing early detection and prompt treatment (reviewed in [8,84,85]). As the level of cancer-specific mutations

shows a high correlation with tumor size, quantitative measurements could be used as an indicator of disease stage and may even predict the clinical outcome of patients. As will be discussed in a later section, various cell and tissue-specific information is encoded into both classical and newly discovered epigenetic features of cfDNA, which may not only act as auxiliary markers for tumor detection but may enable clinicians to pinpoint the location of tumors, especially those of unknown primary. Furthermore, cfDNA can be used to guide the selection of new targeted therapies which only work when specific mutations are present. Tissue biopsies collect only a fraction of tumor tissue, and therefore do not capture the spatial genetic heterogeneity of the complete tumor. On the other hand, tumor-derived cfDNA, which is released into body fluids from many parts of the tumor, provides a much more holistic representation of the genetic landscape of the whole tumor. Thus, given the minimally-invasive nature of venipuncture, longitudinal cfDNA sampling enables the assessment of dynamic changes in cfDNA levels, the identification of acquired resistance-conferring mutations, and the tracking of clonal evolution. These temporal data can be harnessed not only to monitor the response of cancer to surgical removal, which makes possible the detection and prediction of minimal residual disease or recurrence, but also monitor the response to other therapies, which makes it possible to detect and study the emergence of acquired resistance. The information provided by cfDNA analysis will facilitate the development of better therapies and inform the selection of more effective therapy regimes.

3.2. Fetal Genetic Abnormalities

Since the discovery of the presence of cell-free fetal DNA in maternal plasma, non-invasive prenatal tests have been developed for fetal sexing [86], screening of various fetal genetic abnormalities [87,88], and monitoring pregnancy complications [31], many of which are now routinely screened for (reviewed in ref. [89]).

3.3. Organ Transplant Monitoring

Characterization of unique single-nucleotide polymorphisms in cfDNA has allowed the differentiation between host- and recipient-derived cfDNA in organ transplant patients and is emerging as a potentially useful clinical tool for monitoring post-transplant organ rejection, dysfunction, and injury [90–96].

3.4. Detecting Pathogenic DNA

Pathogenic DNA or RNA from bacteria (e.g., *Mycobacterium tuberculosis*-derived DNA [97] and Pneumonia pathogens [98]), viruses (e.g., Crimean–Congo hemorrhagic fever (CCHF) [22]), and parasites (e.g., *Leishmania*, *Plasmodium*, *Schistosoma*, *Trypanosoma*, and *Wuchereria* spp.) have been detected in body fluids [99]. Characterization of pathogenic DNA in body fluids may facilitate the early detection of a wide range of infections. For example, a 24-marker quantitative real-time PCR (qPCR) assay has recently been developed for the detection of sepsis well before the onset of clinical symptoms [100]. Similarly, detection of parasitic cfDNA using nucleic acid amplification tests (NAATs) showed improved accuracy over traditional microscopic and serological diagnostic tests, while the possibility of convenient, cost-effective, non-invasive, and painless collection of parasitic DNA from specimens such as urine and saliva is highly practical for implementation in large-scale epidemiological screening programmes [99]. Furthermore, routine screening of cancer-causing viruses such as the Epstein-Barr virus (EBV), which is associated with nasopharyngeal carcinoma [101,102], or Human papillomavirus (HPV), which is associated with oropharyngeal squamous cell carcinoma [103,104] and cervical cancer [105] may enable early detection and prompt treatment.

3.5. Studying the Gut Microbiome

The major role that the gut microbiome plays in human health and disease is becoming increasingly appreciated. The detection of high levels of cfDNA fragments in human plasma

that originates from both known and unknown resident microorganisms [106–108] suggest that the metagenome encoded into the total cfDNA population may not only be harnessed to study and gain further insights into the gut microbiome, such as the mechanisms by which it contributes to human health or disease or the effects of antibiotics, but also gain deeper insights into the microbial diversity within humans, which appear to be much more diverse that initially thought [108].

3.6. Studying Environmental DNA in Humans

Environmental nucleic acids ingested through fluids and meals, such as bacterial or plant DNA, has been found in human body fluids. The fate of these nucleic acids is uncertain, but it is suggested that they may remain in body fluids for extended periods before it is degraded, excreted, or assimilated by cells [109,110]. While the relative contribution of environmental DNA to the total cfDNA population has not yet been determined, it is generally considered to be low. However, foreign cfDNA molecules may have underestimated detrimental effects, as studies have shown that they are able to enter the host cell nuclei and incorporate into the genome [111–114]. For example, a recent study suggested that cfDNA may facilitate the horizontal transfer of antibiotic resistance genes [110]. While no research has been carried out on the topic, we could speculate here that cfDNA may serve as a potential surrogate marker to study the presence and potential effects of genetically modified crops or animals on human health.

3.7. Other Potential Uses

While thorough investigation is lacking, some reports have indicated that cfDNA may have potential use in (i) biobanking; analysis of cfDNA in cord blood plasma has been useful for sample identification [115], (ii) forensic casework; cfDNA has been genetically profiled in samples recovered from externally found body fluids, such as blood, sweat, and feces [116–119], and (iii) quality control assurance of blood donations [120] or transfusions [121].

4. Limitations of cfDNA Hotspot Mutation Analysis

An ever-increasing range of disease-specific signatures can be detected through the characterization of cfDNA sequence information. This constitutes a major breakthrough in the use of molecular tests towards minimally invasive personal medicine. The clinical significance of cfDNA sequence analysis is underscored by several recent breakthrough advancements in the field (Table 1).

Table 1. Developments that demonstrate the potential clinical utility of sequence-specific cfDNA assays.

Development	Use/Implementation
Food and Drug Administration (FDA)-approved cfDNA assays for use in routine diagnostic settings	BRCA1 and BRCA2 mutations in metastatic castration-resistant prostate cancer
	EGFR mutations in non-small cell lung cancer
	KRAS G12C mutations in non-small cell lung cancer
	PIK3CA mutations in breast cancer
CfDNA Assays performed in Clinical Laboratory Improvement Amendments (CLIA)-certified laboratories	CtDNA assays that have been validated in CLIA-approved clinical laboratories are increasingly offered to cancer patients worldwide
CfDNA assays performed in non-invasive prenatal testing (NIPT) facilities	NIPT facilities worldwide offer cfDNA-based tests for the screening and early characterization of various fetal characteristics, such as sex and chromosome conditions, i.e., aneuploidy, trisomies, and microdeletions

Despite these exciting achievements and despite the spectacular progress made in the cfDNA research field in the last two decades, the development and implementation of ctDNA assays into routine diagnostic settings has been slow and challenging. The main

reason for this is that the timeline for the development of ctDNA assays that bear the required diagnostic sensitivity and specificity for testing in large-scale clinical trials, and thus the development of kits that are fit for clinical roll-out, is extended by numerous, often non-mutually exclusive factors that challenge the robust analytical detection of ctDNA. The most challenging factor is low absolute amounts of ctDNA molecules in the blood, especially in early disease when small tumors shed miniscule amounts of DNA into extracellular space. While the analytical sensitivity of ctDNA assays have surged tremendously in recent years, the detection of scarce ctDNA molecules in a biospecimen is still challenged by the vast background of non-target molecules originating from diverse origins. The analytical challenges involved in detecting low amounts of ctDNA is further exacerbated by the selection of counter-productive preanalytical steps, such as (a) blood-drawing techniques, processing procedures and storage conditions that result in the degradation of target molecules or dilution by the release of germline DNA, (b) extraction procedures that fail to capture a large portion of ctDNA molecules, either due to general recovery inefficiency (e.g., automated methods extract much less DNA than some manual spin-column methods) or size bias (e.g., ctDNA mutations occur in both long and short fragments, but most kits are biased toward the extraction of short fragments).

Moreover, in the rapidly advancing cfDNA research field, which is currently experiencing an influx of new ideas, discoveries, potential applications, and companies, there is not only an ever-expanding menu of products for each preanalytical step, but also a growing repertoire of analytical techniques and technologies open to selection. Although many of these products and technologies have different degrees of efficiency and bias towards specific sample processing procedures, applications, and different cfDNA subpopulations, they are somewhat arbitrarily used by both basic and translational researchers. This significantly complicates the harmonization of cfDNA preanalytics and analytics among researchers, institutions, and clinics. These issues are increasingly addressed [7,58,122–129], yet preanalytical optimization and standardization remains a major, evolving issue that requires ongoing surveillance and active problem solving. As reviewed elsewhere [130], ctDNA profiling is also challenged by clonal hematopoiesis (CH)-derived cfDNA that bear cancer-specific mutations. This is especially problematic as this is a common phenomenon in both cancer patients [131] and healthy subjects [132–135]. It is currently not clear how the misdiagnosis of CH-derived mutations in cfDNA as malignancy may be prevented, but some suggest that ctDNA and CH-derived cfDNA may be differentiated on the basis of fragment size, as tumor-derived cfDNA is generally shorter than DNA from other origins [136]. However, as discussed in Section 9, it is also noteworthy that there is a wide range of cfDNA size populations in human body fluids, many of which share overlapping origins.

5. Beyond cfDNA Hotspot Mutation Analysis

Significant research efforts in the last decade uncovered a rich landscape of cfDNA physico-chemical features (reviewed in [130,137–140]). Beyond hotspot mutations, numerous cfDNA features are candidate biomarkers, including (i) various genetic features, such as DNA sequence features (Figure 4A), chromosomal abnormalities (Figure 4B), and topological forms (Figure 4C); (ii) primary epigenetic markers such as DNA methylation (Figure 4D) and histone modifications (Figure 4E); and (iii) various secondary epigenetic features that cfDNA molecules attain intracellularly or extracellularly following disruption of the primary structure of DNA, such as binding to proteins, extracellular vesicles, or cell membranes (Figure 4F), and fragmentomic features (Figure 4G).

One major advantage that most of the abovementioned features have over hotspot mutation profiling is that they occur across a large portion of the genome, which markedly increases their probability of detection. Indeed, the characterization of many of these markers are now considered as auxiliary tests to hotspot mutational profiling, while some epigenetic assays may even outperform mutational profiling and be used as stand-alone tests. Two breakthroughs are noteworthy here. First, the FDA-approved Epi proColon

2.0 CE test is routinely used to screen high-risk groups (patients over the age of fifty) for aberrant methylation of SEPT9, an indicator of colorectal cancer [141]. Second, a recent study used cfMeDIP-Seq to ID breast-cancer specific methylation signatures in the cfDNA of asymptomatic subjects, which allowed the detection of breast cancer several years before clinical presentation and diagnosis [142]. While they have not yet achieved the diagnostic sensitivity and specificity required for clinical implementation, numerous other studies have also demonstrated strong correlations between epigenetic features of cfDNA and wide-ranging disease activities, including various indications in different cancer types [143–161], CVD [162], liver damage [163,164], diabetes [165,166], multiple sclerosis [167], aging [51], and even psychological distress [168,169].

cfDNA features that can be interrogated as biomarkers

Primary genetic features

A) DNA sequence features

i. Gene sequences (indicating origin)
  • Nuclear DNA
  • Mitochondrial DNA
  • Metagenomic DNA
ii. Repetitive elements
  • SINEs (ALUs, MIRs)
  • LINEs (LINE-1, LINE-2, L3/CR1)
  • LTR elements (ERV class I, ERV (K) class II, ERV (L) class III, MALR)
  • DNA elements (hAT-Charlie, TcMar-Trigger)
  • Satellites
  • Minisatellites
iii. GC-content
iv. Sequence motifs
v. Mutations
  • Missense   • Deletions
  • Nonsense   • Duplications
  • Insertions   • Frameshift
vi. Copy-number variations
vii. Telomeres

B) Chromosomal abnormalities

i. Structural:
  • Deletions
  • Duplications
  • Translocations
  • Inversions
  • Insertions
  • Rings
  • Isochromosomes

ii. Numerical:
  • Aneuploidy

C) Topological forms

i. Extrachromosomal circular DNA
ii. Single- vs. double-strandedness

Primary epigenetic features

D) DNA Methylation

i. Hypermethylation of promotor CpG islands
ii. Hypermethylation of tumor suppressor genes
iii. Hypomethylation of oncogenes
iv. Global hypomethylation
v. Hypomethylation of pericentromeric blocks

E) Histone modifications

i. Methylation
ii. Acetylation
iii. Phosphorylation
iv. Ubiquitination
v. Others:
  • GlcNAcylation, citrullination, crotonylation, sumoylation, butyrylation, propionylation, hydroxylation, biotinylation, neddylation, ADP ribosylation, N-formylation
  • Histone variants

Secondary epigenetic and physico-chemical features

F) DNA associated with proteins

i. Extracellular vesicles
  • Exosomes
  • Apoptotic bodies
  • Microvesicles
ii. DNA bound to macromolecules
iii. Binding to cell membranes

G) Fragmentomics

i. Fragmentation profiles – different sizes
ii. Nucleosome spacing/variable linker DNA lengths
iii. Fragment end-point motifs
iv. Preferred ends
v. Jagged-ends
vi. End-orientation

Figure 4. Features of cfDNA that could serve as clinical biomarkers. In addition to DNA hotspot mutations, various disease- and tissue-specific genetic, epigenetic, and structural features are encoded into cfDNA. Much of this information can be leveraged for the detection and monitoring of a wide range of diseases, physiological states, and other clinical scenarios.

Another major advantage of epigenetic cfDNA features is that it may enable the parallel characterization of several cancer types. This is especially important in the context of cancer screening, where the identification of the tissue-of-origin of underlying cancers is crucial. This is a challenging task, but in the next section we explore how this is becoming increasingly possible through rapid progress in mapping various tissue-of-origin classifiers in the genome and in cfDNA [170,171].

5.1. Various Cell and Tissue-Specific Epigenetic Signatures in cfDNA

Cell-specific epigenetic features are conserved by cfDNA molecules and have been used for a variety of tissue-of-origin analyses in recent years. In particular, distinct approaches employing methylation patterns, nucleosome positioning, transcription factor binding site occupancies, and fragmentomic features were successfully developed to determine the tissue-of-origin of individual cfDNA molecules (Figure 5). Tissue-of-origin analysis enables estimation of the contributions of various tissues from the human body to

the plasma DNA pool and thereby identify tissues where increased cell death occurs, potentially yielding sensitive screening tests for early diagnosis of a wide range of pathologies, therapy response monitoring, and detection of minimal residual disease.
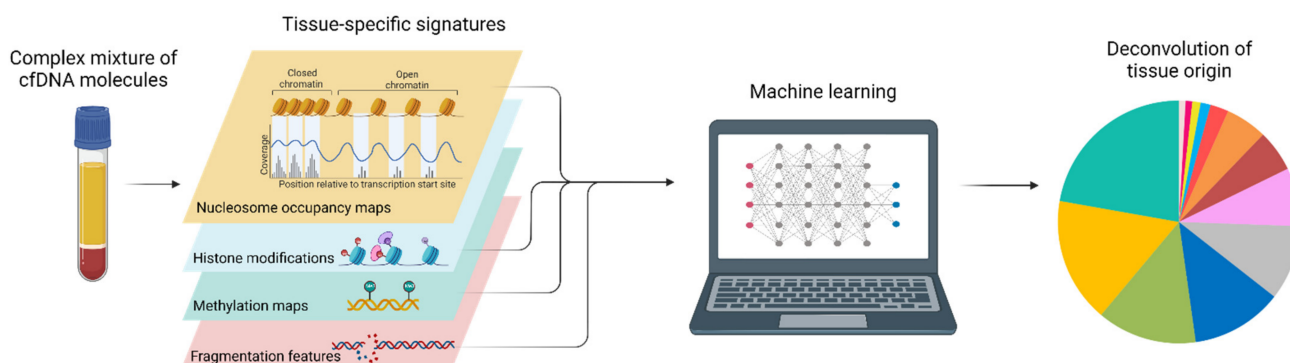
Figure 5. Determining the tissue-of-origin of cfDNA molecules. The cfDNA population in a typical biospecimen is highly complex and derives from numerous different cell and tissue types. However, cfDNA molecules contain multiple layers of cell-type specific epigenetic signatures, such as differentially methylated DNA regions, post-translational histone modifications, nucleosome occupancy, as well as various fragmentation features. Through the use of increasingly sophisticated molecular analysis methods coupled with machine learning algorithms, the epigenetic information carried by cfDNA molecules can be decoded to determine the contribution of different tissue types.

5.1.1. DNA Methylation

For methylation-based tissue-of-origin analyses, genome-wide methylation profiles of reference tissues (mostly based on whole genome bisulfite sequencing) were used to deconvolute cfDNA sequencing data. In 2015, a reference-based methylation deconvolution approach was developed to determine relative contributions of DNA from multiple tissue types to the plasma DNA pool [172]. This approach allowed the identification of differentially methylated regions unique to specific cell types, which enabled the researchers to identify the tissue-of-origin of cfDNA molecules in pregnancy, transplantation, and cancer. They also estimated that ≥70% of the plasma cfDNA was derived from white blood cells (i.e., neutrophils and lymphocytes) [172]. Considering the methylation state of a number of adjacent CpGs instead of a single CpG site significantly reduced background and enhanced the specificity of methylation-based tissue-of-origin analysis and was utilized to detect cell-type specific cell death from plasma samples with pathologies lacking genetic aberrations such as diabetes, multiple sclerosis, and head trauma [173]. Highly coordinated methylation sites, so-called methylation haplotype blocks, were the basis for another methylation-based tissue-of-origin method [174]. This approach investigated tightly coupled CpG sites and observed a reduction in completely coupled CpG pairs in cancer patients, which enabled quantitative estimation of tumor load and tissue-of-origin mapping in cfDNA of patients with lung or colorectal cancer [174]. Immunoprecipitation of methylated DNA followed by sequencing (cfMeDIP-seq) was developed specifically for low-input and already fragmented plasma DNA samples and enabled the classification of cancer in plasma samples from numerous tumor types [148]. Establishing reference methylation atlases of cell types (and not tissues) is very important to further advance methylation deconvolution approaches. One reference atlas of 25 human tissues and cell types covering major organs and cells involved in common diseases [92] and another recently published methylation atlas of 39 cell types sorted from healthy tissue samples [175] are available to further improve tissue-of-origin analyses via methylation patterns. The first commercially available blood-based multi-cancer early detection test was recently introduced by GRAIL [176–178], which utilizes targeted cfDNA bisulfite sequencing followed by ML and is able to detect cancer signals of multiple cancer types and predict cancer origin with high accuracy.

5.1.2. Nucleosome Spacing and Occupancy

Many approaches have been developed to infer nucleosome positioning maps from cfDNA sequencing data. For this purpose, sequenced cfDNA fragments are aligned to the genome and genomic regions with higher number of fragments ending in those regions point to regions not occupied by a nucleosome, whereas regions that are spanned by the majority of sequenced cfDNA fragments are indicative of a genomic region protected by a nucleosome. One metric to quantify this is the windowed protection score (WPS) [179], which was used to determine the nucleosome spacing pattern that, in turn, informed on cfDNA tissues-of-origin. In healthy subjects, these nucleosome occupancy patterns matched hematopoietic lineages and additional contributions were detected in cancer [179]. Another approach employed whole genome sequencing of cfDNA in combination with nucleosome promoter analysis [180]. The read depth coverage at two discrete regions at transcription start sites was determined and enabled distinction of expressed and silent genes. The prediction of the expression status of individual genes can be employed for classification of expressed cancer driver genes [180]. The abovementioned WPS is also informative about the occupancy of transcription factor binding sites when determined for short fragments (35–80 bp) [179]. An alternative approach to assess transcription factor activity is based on cfDNA sequencing data and nucleosome footprint analysis [181]. A bioinformatics pipeline that infers accessibility of transcription factor binding sites from cfDNA fragmentation patterns has also been developed. By determining the activity of lineage-specific transcription factors, patient- and tumor-specific patterns were observed and allowed accurate prediction of tumor subtypes in prostate cancer [181].

5.1.3. Histone Modifications

In one study, post-translational histone modifications have been successfully used for tissue-of-origin analysis. Chromatin immunoprecipitation and sequencing of cell-free nucleosomes directly from human plasma yielded information on DNA-related activities within the cells of origins [182]. Pathology-related changes in transcriptional programs in specific cell types could be identified with this method. In another proof-of-principle study, immunoprecipitation of H3 lysine 36 trimethylation (H3K36me3)-modified nucleosomes, a histone modification associated with active gene transcription, was used as a liquid biopsy marker to identify tumor-specific transcriptional activity of mutated alleles in non-small cell lung cancer [171,183].

5.1.4. CfDNA Fragmentation Profiles

Fragmentomic features of cfDNA molecules can be used in various ways to determine tissue-of-origin and identify cell type contributors to the plasma DNA pool. First, cfDNA fragment size distribution can be employed for tissue-of-origin analysis. Selection for specific cfDNA fragments (90–150 bp) before sequencing enhanced the detection of ctDNA and identified differences in the size distribution of ctDNA and noncancer DNA fragments (i.e., ctDNA fragments are shorter than cfDNA from healthy samples) [184]. This ML-based method was able to detect multiple cancer types in plasma. A large-scale fragmentation pattern analysis approach evaluated cfDNA fragmentation profiles at megabase level across distinct cancer types [185]. This ML model incorporated genome-wide fragmentation features and could be used to identify the tissue-of-origin of multiple cancer types. Combining this fragmentation-based approach with mutation-based cfDNA analysis significantly enhanced detection [185]. Measuring the fragment length diversity at promoter regions of genes of interest via a targeted approach (EPIC-seq) and determining the promoter fragmentation entropy (PFE) allowed the inference of gene expression profiles and tissue-of-origin determination. The PFE approach was used to classify subtypes of lung carcinoma and diffuse large B cell lymphoma [186]. Second, the orientations of cfDNA fragments in open chromatin regions are informative of tissue-of-origin [187]. Determining the orientation in tissue-specific open chromatin regions where the respective tissues contributed DNA into the plasma allowed measurement of relative contributions of various tissues to the

plasma DNA pool in pregnancy, organ transplantation, and cancer [187]. Third, the fragment end sequence motif (commonly the four bases at both ends of a cfDNA fragment) hold information about the cellular origins of cfDNA fragments. Analyzing the plasma DNA end motif via the motif diversity score (MDS, adopted from the normalized Shannon entropy) revealed distinct characteristic sets of plasma DNA end motifs for plasma DNA molecules derived from liver, hepatocellular carcinoma and other cancers, and placenta and hematopoietic cells [188]. A significant increase in the diversity of plasma DNA end motifs in patients with hepatocellular carcinoma was observed, aberrant end motifs were also found in patients with other cancer types [188]. A genome-wide catalogue of cfDNA fragment end sequence patterns of patients with 18 different cancer types demonstrated enhanced cancer detection, monitoring and prognosis [189]. Here, the authors calculated the normalized Shannon index and a Gini index of the 5′trinucleotide and the 5′ and 3′mononucleotide, respectively, to quantify the extent of cfDNA fragment end diversity and demonstrated that the so-called Fragment End Integrated Analysis (FrEIA) score could be employed to quantitatively evaluate the tumor fraction in plasma samples.

While the clinical utility of most of the abovementioned cfDNA features still need to be evaluated and confirmed, sufficient evidence indicates that their analysis may expedite the development of increasingly powerful, clinically meaningful cfDNA-based assays. However, similar to the problems encountered in the development of hotspot mutation-based cfDNA tests, epigenetic profiling of cfDNA is challenged by numerous biological, preanalytical, technical, and analytical issues and limitations. Among many, some of the most significant challenges are (i) similar or identical modifications that arise in both ordinary biological processes and pathologies, (ii) stochastic fluctuations in epigenetics or specific epigenetic biomarkers can induce significant biological noise, and (iii) various biases of different methods can alter the observed epigenetic profile and complicate accurate analysis (reviewed in ref. [140]). To develop clinically meaningful assays, these challenges need to be overcome.

6. Other Sources of Cell-Free DNA

The bulk of cfDNA research to-date is centered on the characterization of DNA originating form the nucleus through cell death. However, increasing evidence indicates that various other sources such as mitochondrial DNA, neutrophil extracellular traps, and extracellular vesicles, which may also originate from non-apoptotic processes, may have significant clinical utility.

6.1. Mitochondrial DNA (mtDNA)

Mitochondria are intracellular organelles that perform a variety of essential functions. They are the primary generators of cellular energy, produce several biosynthetic intermediates (e.g., haem, lipids, and amino acids), and are involved in cellular stress responses, including apoptosis, innate immunity, and hypoxia. Apart from its cell autonomous roles, mitochondria can also influence an organism's physiology by regulating intercellular and interorgan communication [190–192]. Thus, considering its central role in human physiology, it is not surprising that mitochondrial dysfunction is associated with numerous disorders, and that cell-free mitochondrial DNA (cf-mtDNA) is attracting increasing attention as a potentially versatile biomarker for a wide range of diseases and other clinical scenarios. The presence of cf-mtDNA in the human circulatory system was first reported in a study in which a known mtDNA mutation was identified in the DNA isolated from the plasma and serum of type 2 diabetes mellitus patients [193]. Since then, numerous studies have confirmed the presence of cf-mtDNA in the circulation of both healthy and diseased individuals [194].

For example, aberrant cf-mtDNA has been correlated with various cancers [195–197], including breast cancer [198], lung adenocarcinoma [199], squamous cell carcinoma [200,201], Ewing's sarcoma [202], urological malignancies [203], and oral cancer [204]; neurodegenerative diseases [205] such as Friedrich's ataxia [206], multiple sclerosis [207], and Parkinson's

disease [208]; diabetes [193,209]; aging [210]; surgery and trauma [211,212]; sepsis [213,214]; HIV [215–217] and type-2 diabetes [218]-associated cognitive decline and chronic inflammation; exposure to carcinogenic pesticides [219]; adverse effects of spaceflight on the health of astronauts [220,221]; poor outcomes of patients with adult community-acquired bacterial meningitis [222]; psychological issues such as major depressive disorders [223], suicidal behavior [224], and acute psychological stress [225], as well as psychosocial and physical stress [168]. Reports have also shown that cf-mtDNA characteristics can be influenced by exercise [226,227], suggesting a possible use in sports medicine. Another potential use of cf-mtDNA may be minimally invasive haplogroup matching [228].

Cf-mtDNA may also have underappreciated potential as a biomarker for cardiovascular disease (CVD), which is one of the leading causes of death worldwide [229–231]. MtDNA aberrations on the level of mutations, copy number shifts, and methylation changes are associated with a wide range of CVDs [232–235]. Prolonged reduction of blood flow to the heart, as a result of atherosclerosis, for example, decreases the amount of oxygen available for the mitochondria of cardiomyocytes in the territory of the blocked artery. This results in the rapid depletion of cellular ATP, triggering an ischemic cascade, and eventually the induction of cell death through apoptosis or necrosis. If tissue death is severe enough, this can lead to a myocardial infarction (MI) [236,237]. Since mtDNA is released into the bloodstream during this process, cf-mtDNA levels may serve as a potential biomarker for estimating the course and outcome of injury in patients following MI. A number of studies have demonstrated significantly elevated levels of cf-mtDNA in patients with MI compared with healthy subjects [238–241], and cf-mtDNA levels appear to correlate with the degree of myocardial damage and are also higher in transmural MI than in non-transmural MI [238]. Whether cf-mtDNA levels correlate with MRI infarct size is not yet known. Furthermore, cf-mtDNA levels in MI patients decline significantly following percutaneous coronary intervention treatment [239,242]. Apart from MI, elevated levels of cf-mtDNA have also been demonstrated in patients with chronic systolic heart failure [243] and sudden cardiac arrest compared with healthy control subjects and survivors [231]. Furthermore, while there is a paucity of publications on this subject, no study has yet demonstrated a clear correlation between cf-mtDNA levels and most vascular risk factors, such as hypertension, dyslipidemia, and smoking status. However, studies have indicated that cf-mtDNA is significantly increased in patients with diabetes mellitus (DM) [244], and is higher in CVD patients with DM than in CVD patients without DM [245].

MtDNA can be released into body fluids through various modes of cell death [1,246], or by regulated processes, where certain mechanisms actively secrete cf-mtDNA via mitochondria-derived vesicles and neutrophil extracellular traps (NETs). Many cell types such as leukocytes, endothelial cells and platelets, seem to be able to release mtDNA [247], while it was recently also shown that intact respiratory-competent mitochondria circulate in blood plasma, which may also serve as a source of cf-mtDNA [248]. The structure of cf-mtDNA remains poorly characterized; however, it seems that it can be present either in naked form or associated with internal and external mitochondrial membrane fragments [249]. Furthermore, the absence of nucleosome-associated histone proteins and, therefore, the absence of higher-order packaging of mtDNA render "naked" cf-mtDNA exposed to enzymatic cleavage. This suggests that cf-mtDNA molecules should be more fragmented than autosomal cfDNA. Indeed, unlike autosomal cfDNA, which is typically characterized by a three-mode size signature representing apoptotically generated mono-, di-, and tri-nucleosomes, cf-mtDNA generally exhibits a wider range of sizes. Some studies have shown that cf-mtDNA is highly enriched in fragments ranging between 40–80 bp [106,246], while others have observed a broader size distribution of 50–300 bp [250–252]. An important point to bear in mind is that the aforementioned studies have used different methods of cf-mtDNA purification (e.g., QIAamp DSP DNA Blood Mini Kit vs. DNeasy Blood and Tissue Kit) and fragment sizing (e.g., paired-end sequencing vs. qPCR using specific amplicons). Plasma cfDNA obtained with non-hybridization-based extraction methods, which do not filter and capture DNA fragments of a specific size, have

revealed very different size profiles [61]. Moreover, since a fraction of cf-mtDNA fragments can be associated with particles [249], the characteristics of extracted cf-mtDNA can be influenced by other preanalytical processing steps, such as centrifugation. It is therefore possible that these differences in reported cf-mtDNA sizes are merely an artefact of variable methodology and may not be a true reflection of their in vivo counterpart. It is thus clear that there is insufficient data to reach a consensus on the size and exact structure of cf-mtDNA. In order to gain a better understanding, it is necessary to establish a standardized blood processing method and to perform a systematic characterization of both cf-mtDNA and cf-nDNA, perhaps making use of in vitro cell cultures, well established animal models, or samples collected from large clinical cohorts [225,253].

6.2. Neutrophil Extracellular Traps (NETs)

Neutrophils are the most abundant innate immune effector cells, accounting for over 50–60% of white blood cells. Since NETs are composed of a DNA scaffold, they are considered to be important sources of circulating cfDNA [254]. Extracellular traps (ETs) produced by neutrophils were first observed in 2004 [255] and to date it has been shown that other immune cells including macrophages, mast cells, eosinophils, basophils, plasmacytoid dendritic cells, and lymphocytes are all able to produce ETs [256–259]. As neutrophils are present in significant amounts in blood, we will focus on NETs in this review.

NETs consist of a meshwork of DNA fibers bound to histones and cytoplasmic and granular proteins [260]. They are described as three-dimensional net-like [261] or cloud-like [262,263] structures, with differences possibly resulting from varying DNA origins, formation mechanisms, or experimental settings [261,263,264]. Both mitochondrial [265,266] and nuclear DNA [262] have been found to form or at least make up parts of NETs. As reviewed elsewhere [267–269], the details of the different pathways involved in NET formation is still rather controversial and highly debated. With this in mind, we briefly describe what is known to date about the mechanisms of formation (Figure 6). Two principal mechanisms of NET formation have been described, namely (i) suicidal NET formation, or NETosis, which is a unique form of cell death that lasts several hours [270]. After neutrophil activation, nuclear chromatin decondenses and expands, followed by disassembly of nuclear and granule membranes and release of chromatin into cytoplasm, where cytoplasmic and granule proteins are bound [270]. Finally, the plasma membrane disrupts, and the decorated DNA filaments are released. Additionally, mtDNA is released into cytoplasm via a controlled pore forming mechanism during apoptosis. It is unclear whether this is contributing to NET formation [271]; (ii) Vital NET formation or extrusion is a rapid process occurring within 5–60 min. Here it is important to note that these cells fully maintain their viability, and therefore this type of NET formation is described as NET extrusion rather that NETosis, which implies the lysis of the NET-forming cells [272]. Both nuclear and mtDNA have been described as components of these types of ETs [265,273]. Nuclear DNA is released in vesicles by blebbing directly from the nucleus while the nuclear and plasma membrane initially remain intact [273,274]. The nuclear membrane may subsequently rupture, leading to an accumulation of chromatin fibers into the cytosol, while retaining their viability. In 2009 Yousefi et al. demonstrated that NETs from viable neutrophils can also be composed of mtDNA. Two modes of mitochondrial NET formation/extrusion have been suggested so far: (a) mtDNA may be released into cytosol where it is further packed into vesicles and released by fusion with the plasma membrane thereby forming traps [275]; (b) The direct release of mtDNA and granules in a catapult-like manner via fusion of the mitochondrial and plasma membrane [269,271,275].
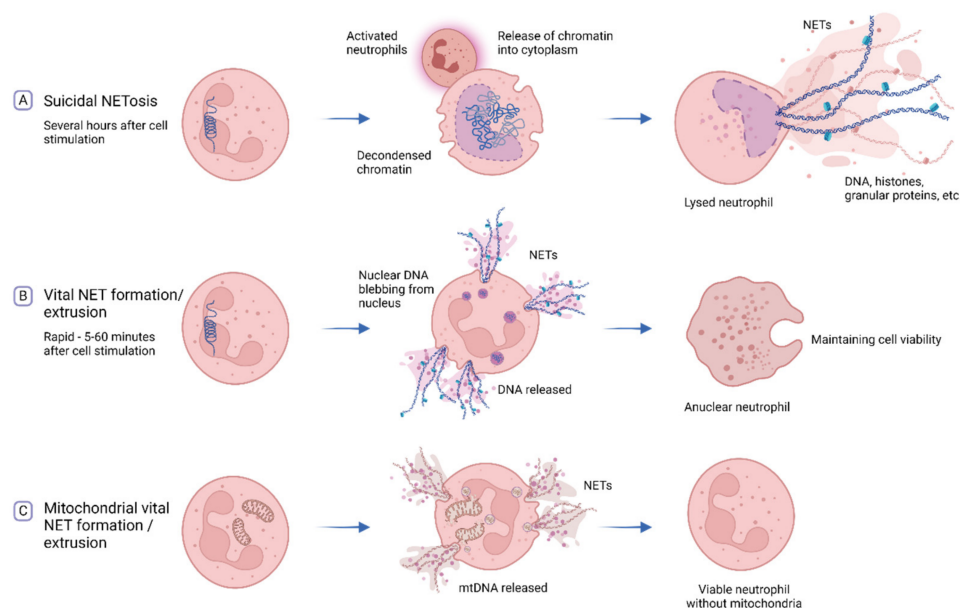
Figure 6. Mechanisms of NET formation. (**A**) Suicidal NETosis occurs over several hours and involves the decondensation of chromatin in activated neutrophils leading to the release of chromatin into the cytoplasm. Subsequently, the cell membrane is ruptured and chromatin is released from the lysed neutrophil into extracellular space. (**B**) Vital NET formation/extrusion is a rapid process occurring within 60 min of cell stimulation where vesicles containing nuclear DNA are fused with the plasma membrane and chromatin is released entirely, leaving anuclear neutrophils. These cells maintain their viability; therefore, this type of NET formation is best categorized as NET extrusion rather than NETosis, which implies that the NET-forming cells are lysed. (**C**) Mitochondrial vital NET formation/extrusion is a secondary form of vital NET formation in which mitochondrial DNA is released as NETs after the rupture of mitochondrial membranes, leaving viable neutrophils lacking mitochondria.

Compared with NET formation, much less is known about their degradation, although the imbalance between the formation and degradation of NETs is relevant to the development of various diseases. It has been shown that DNase I can disrupt the NETs scaffold in vitro [276], but with physiological concentrations of DNase I, it is not capable of fully digesting it. There is evidence that DNase I preprocesses NETs extracellularly, which are then cleared by macrophages [277]. Recently it was shown that proinflammatory polarized macrophages have an increased uptake and boosted further macropinocytosis. Additionally, macrophages secrete DNases, e.g., DNase I and DNase 1L3, which disrupt NETs in preparation for intracellular uptake [278]. DNase 1L3, released by dendritic cells was also found to take part in the extracellular degradation, whereas TREX1 (DNase III) was found to degrade NETs intracellularly within macrophages [279]. In case of insufficient uptake by macrophages, it is possible that partially digested DNA fragments are released. Another factor which contributes to the degradation through macrophages is complement component 1q (C1q), as it has been reported to facilitate the process by opsonizating NETs [277]. There is an ongoing discussion as to whether the antimicrobial peptide LL-37 from neutrophils facilitates the uptake into cells and protects them against degradation by bacterial and cellular nucleases [279]. Overall, additional studies are still needed to identify other nucleases that are potentially involved in NET degradation and to further characterize the mechanisms of extracellular DNA uptake into cells.

6.3. Extracellular Vesicles (EVs)

Extracellular vesicles (EVs) are lipid-bound vesicles that are naturally released by almost all cell types into extracellular space and have been found in all biological fluids tested thus far [280–285]. The different types of EVs are classified according to their

biogenesis, content, function, and size (Table 2). Several in-depth reviews have described the biological characteristics and functions of EVs [281,286–289]. Here our primary focus is to review evidence of EVs as a source of cfDNA.

Table 2. Classifications of different types of extracellular vesicles.

Name	EV Class	Size (nm)	Biogenesis	DNA Content	DNA Localization
Exosome	small EV	40–100	Multivesicular endosome	DNA, mtDNA, viral	outer surface
Microvesicle	large EV	~100–1000	Plasma membrane budding	dsDNA	lumen
Large oncosome	large EV	1000–10,000	Plasma membrane budding	ssDNA/dsDNA	unknown
ARMM	small EV	~40–100	Plasma membrane budding	None found	N/A
Apoptotic body	large EV	500–2000	Apoptosis	dsDNA/mtDNA	lumen
Exomere	non-EV	~35–50	Unknown	DNA detected	unknown
Micronucleus	large non-EV	1000–9000	Mitotic catastrophe	gDNA	lumen
OMV	small EV	20–250	Outer membrane budding	dsDNA	lumen and surface

The most extensively studied small EVs (sEVs) are exosomes. They range in size from 40 to 100 nm and are constitutively generated from late endosomes [290–293]. Numerous studies have reported the presence of single- and double-stranded DNA as well as mtDNA [294–296], transposable elements [297] and viral DNA [298,299] in association with sEVs. The primary localization of exosomal DNA is not clear, as studies have shown DNA to be located in the lumen and on the outer surface [300–302]. Interestingly, a recent study reported that exercise triggered an increase of DNA levels only on the outer surface [301]. While there is ample evidence of exosome-associated DNA, it remains a controversial topic as highly cited studies have reported evidence both for the association of DNA with exosomes [295,303,304] and no association of DNA with exosomes [305]. Apart from biological diversity among different exosome types, which are yet to be fully explored, there may be other reasons for the discrepant findings reported by studies, such as differences in sample handling and exosome isolation protocols [306]. Furthermore, despite the efforts by the International Society of Extracellular Vesicles to provide guidelines for conducting sEV experiments (MISEV2018) [289], it is often the case that simple requirements such as the reporting of the volume of starting material, volume of sEV isolated or number of isolated EVs used for DNA isolation are not met by many publications. This makes it very difficult to reproduce experimental findings or to theorize about possible structures and mechanisms. Other methodological hurdles may include Western blots that lack a size marker, whether reducing conditions were used [298,307,308], or whether fetal bovine serum was included in cell culture experiments [309]. These factors are important because in the context of biofluids the presence of CD81 and CD9 can overlap with human low chain IgG cross-reactivity with the secondary antibody (see [307,310] for light and heavy chain IgG bands) unless non-reducing conditions are used or highly cross-adsorbed secondary antibodies. Despite controversial reports, conflicting data, and an array of preanalytical and analytical hurdles, a growing body of evidence indicates that sEV-DNA may have diagnostic uses and also play an important role in biological and pathological processes. For example, sEV-DNA may serve as cancer biomarkers [309,311–313], act as intercellular messengers [297,314–316], and play a role as a mediator of proinflammatory effects [317,318] and viral responses [298,299,319].

Gram-negative bacteria release outer membrane vesicles (OMVs), which are sEVs (20–250 nm) released from the outer membrane that encapsulate periplasmic contents. Their role and potential are extensively reviewed in ref. [320]. There is evidence that these OMVs can cross the intestinal epithelial barrier and reach the bloodstream where their DNA content have been detected in healthy individuals [320].

ARMMs are a type of sEV (40–100 nm) that bud directly from the plasma membrane and have a size similar to that of exosomes [321]. To our knowledge, ARMMs are the only class of EVs that have not yet been shown to be associated with DNA. However, it is interesting to note that ARMMs are likely to be co-purified with exosomes through

most sEV isolation methods due to their similar size and the fact that they also contain the transmembrane tetraspanin CD9.

Microvesicles (MVs) are the most diverse EV population with regard to their size (0.1–1 μm) and they are generated via direct outward budding of the plasma membrane [322]. Tumor-derived MVs (TMVs) have been shown to contain dsDNA and they could potentially play a role in inhibiting tumor progression [323]. An increase of MVs and dsDNA in MVs is reported in relation to lupus nephritis [322].

Large oncosomes are large EVs (1–10 μm) secreted exclusively by cancer cells [324], and are reported to contain more DNA than sEVs and their DNA cargo includes tumor-specific alterations [308].

Apoptotic bodies (ApoBDs) are the largest of the EVs (0.5–2 μm) and are released during apoptosis [325,326]. They contain a wide variety of cellular components including micronuclei and DNA fragments. When not digested by DNASE1L3, ApoBD-associated DNA can bind directly to antinuclear antibodies, a common feature of autoimmune conditions such as systemic lupus erythematosus [327].

Exomeres are novel extracellular particles (35–50 nm) discovered with the use of asymmetric flow field-flow fractionation (AF4) and although they are not vesicles, we decided to include them as they have been shown to complex with DNA. They are found to be associated with large fragment DNA but their biogenesis, function, and potential in diagnostic, prognostic, and therapeutic applications remain to be elucidated [328].

In summary, all EVs apart from ARMMs have been reported to be associated with DNA [300,308,322,323,327–329], which may have significant implications for the cfDNA research field and currently represents not only an underappreciated source of cfDNA, but also a potentially distinct and biologically active subpopulation of cfDNA. However, there are still many unanswered questions, conflicting data, and obstacles related to the phenomenon of EV-associated DNA that need to be resolved. One major unknown is that it is still unclear what percentage of the total circulating cfDNA population is associated with EVs. This number has, for example, been reported to be as little as 5% [330] and as high as 90% [310]. In line with this, one major obstacle that needs to be addressed is the lack of standardization of methods. A likely reason for the significant difference in the values reported by the two aforementioned studies, for example, may be the use of different sample handling procedures. In one study samples were processed immediately after blood draw [330], whereas samples used in the other study were only processed at a later time [310]. Given the short half-life of unbound cfDNA [75–77], such a delay could significantly impact cfDNA yield and integrity [7]. Other important points that remain to be clarified are (i) the relative contribution of different EV types toward the total cfDNA population in general and specific cases, (ii) whether EV-associated DNA is packaged mainly within the interior of these vesicles or if they are mainly adhered to the exterior surfaces; (iii) the relative proportions of the total EV-associated cfDNA in both these locations; (iv) the mechanisms involved in the binding of cfDNA fragments to the EV surfaces of different types of vesicles; and (v) the effect of other physiological and biological factors that control these mechanisms [254].

7. Multimodal Analysis, Data Integration, and Machine Learning (ML)

Evidence suggests that the sensitivity and specificity of cfDNA assays may not only be augmented by the simultaneous interrogation of multiple cfDNA features, but also by the parallel characterization of cfDNA and other biomarkers, such as proteins [331,332], genomic DNA and RNA from circulating tumor cells (CTCs) [333–340], EVs [338,341], miRNAs [342,343], metabolites [344], or mRNA transcripts [345].

Due to the sheer amount of data points generated per sample by the characterization of multiple cfDNA features or multiple different biomarkers, the trend in recent years has been to extract a "signature" from multiple biomarkers instead of focusing on individual data points. This necessitates a radical change to the traditional way of data analysis and interpretation, and mainly involves the application of ML. Indeed, an increasing number

of cfDNA studies are using ML with great success [154,158,175,180,184,185,332,346–357]. ML is usually accomplished through pre-processing, featurisation, feature selection, model fitting, and evaluation. ML methods range from logistic regression, random forest, and support vector machines to neural networks including their counterparts, deep neural networks. ML methods can capture sophisticated signatures, but as model complexity increases, it also becomes more difficult to interpret data. This is related to the "curse of dimensionality", which increases with the number of features in the ML model. This can be particularly problematic when there are more features than samples in the data set. To make interpretation easier, the number of samples in a data set can be increased. For instance: Deep neural networks can detect signatures in samples with many features, such as flow cytometry images [81], but there is a high probability of overfitting if the number of available samples is less than the number of features used. This configuration is frequent in cfDNA experiments and is one motivation for performing extensive feature selection to incorporate only the most informative bits of each sample. For example, the construction of a reference atlas of tissue samples is often based on selected regions that distinguish a particular tissue from other tissues. One group used fewer than one hundred features per tissue as a reference [175]. This procedure is also known as dimensionality reduction and is performed, e.g., in neural networks or support vector machines. However, even if these precautions are taken, larger cohorts and other mechanisms are still needed to ensure interpretability and generalization of the model, where the latter refers to the ability to find the relevant signals in new samples. Another method to avoid overfitting is regularization, which penalizes overfitting and thus makes the model less specific. Finally, data augmentation can help to artificially increase the sample size by creating new samples from previous samples.

Along with the data processing problems comes the possibility of finding signatures obscured by interacting markers that were not previously visible. In addition, this gives researchers the opportunity to conduct research with shallower sequencing depths. Databases that contain references help researchers to select regions of interest more specifically, and also provide the opportunity to test their algorithms on other datasets and infer different epigenetic features or omics [180]. Table 3 provides an overview of some recent cfDNA papers describing ML methods used for their main objective.

Table 3. Cell-free DNA (cfDNA) studies that employed machine learning.

CfDNA Feature Characterized	Machine Learning Algorithm Applied	Reference
Aneuploidy, amplicons	SVM, LR	[356]
Concentration of cfDNA, fragmentation, methylation	JADBio, SVM, LR, RF (bagged tree)	[353]
Coverage	LR	[358]
CNVs and fragmentation patterns	SVM, LR	[346]
CNVs and fragmentation patterns	SVM	[180]
CtDNA mutations	Hierarchical Bayesian noise model score, spearman's correlation	[355]
CtDNA mutations (loci)	RF	[359]
CtDNA mutations and CNV	Ensemble of 5nn, 3nn, Naive Bayes, LR, Decision Tree	[357]
CtDNA mutations and proteomics	LR	[332]
Fragment ends	RF	[360]
Fragment sizes, coverage	Gradient Tree Boosting	[185]
Fragment sizes, t-MAD, fragment size distribution, fragment profile amplitudes	LR, RF	[184]
Methylation	Clustering UPGMA, dynamic programming	[175]
Methylation	RF	[354]
Methylation	RF	[351]
Methylation	RF, Gaussian model-based Mclust	[154]
Methylation	SVM	[348]
Methylation and fragmentation	SVM	[350]
Methylation, miRNA	RF	[347]
Methylation, top 300 dmr windows of 300 bp	RF	[158]
Somatic point mutations, copy number alterations	SVM. LR, RF	[349]

Abbreviations: SVM: support vector machine; LR: Logistic Regression; RF: Random Forest.

8. Potential Pathological Effects and Biological Functions of cfDNA

As discussed in Section 2, elevated total cfDNA levels have been correlated with a wide range of clinico-pathological data, but an overflow of overlapping variables renders the assessment of total cfDNA inadequate as a stand-alone biomarker. However, the phenomenon of elevated cfDNA levels may be more biologically relevant than previously thought. Many early studies and a growing body of new work indicate an underestimated role of different cfDNA types (e.g., cf-nDNA, cf-mtDNA, NETs, and EV-DNA) in the development and progression of various diseases. Moreover, apart from detrimental effects, several studies and some elegant theoretical work suggests a possible role for cfDNA molecules in immunomodulation, the maintenance of cellular homeostasis, as a mode of intercellular communication, and underappreciated factor in evolutionary processes. In the following section we explore the various detrimental effects and possible biological functions of cfDNA molecules, and the pathways through which they may elicit its effects.

8.1. Oncogenesis, Cancer Progression, and Metastasis

There is some evidence implicating cfDNA in oncogenic transformation, tumor progression, and development of metastases [361–363]. While the molecular mechanisms and cellular circuits by which cfDNA mediate these observed tumor-promoting effects remain mostly unknown, several reports indicate that the lateral transfer of cfDNA between cancer cells and neighboring or distal cells may be sufficient to propagate the genetic alterations required for transformation, and at the same time activate signaling pathways that promote malignant growth. CfDNA or fragmented chromatin from cancer cells have been shown to readily infiltrate surrounding cells and incorporate into host genomes, inducing double-stranded DNA breaks (DSBs), genome instability, cell death, inflammation, and destabilization of the homeostatic capacity of the tumor microenvironment, all of which are potent stimuli for oncogenic transformation and tumor progression [114,364]. The lateral transfer of overexpressed therapy-augmented cfDNA molecules, such as oxidized DNA [365–368] or duplicated oncogenes [369,370], can cause accelerated DNA damage, thereby conferring resistance against radio- and chemotherapy. In line with this, there is evidence that cfDNA released by dead or dying tumor cells can activate TLR9 signaling, which in turn inhibits apoptosis and enhances autophagy, thereby promoting tumor growth [371]. Furthermore, it is possible that cfDNA promotes metastasis in recipient cells by inducing the overexpression of several pro-metastatic genes, which for example enhance cell invasion and migration, through the TLR9/MYD88 independent pathway [365,372,373], while cfDNA may also promote metastasis by altering the expression levels of the inflammatory chemokine CXCL8 [374], or other genes such as MMP9 and CD44 and miRNAs [375]. Other reports suggest that the malignant phenotype of tumor cells may be transferred to neighboring normal cells via the assimilation and transfection of cfDNA and other nucleic acids complexed with EVs, such as apoptotic bodies [326] and exosomes [291,376]. It is not yet clear how cfDNA fragments are integrated into host genomes, but some evidence suggests that it may occur through the non-homologous end-joining (NHEJ) double-strand DNA-repair pathway [113,364,377]. Alternatively, ctDNA enriched in specific retrotransposons (e.g., a hot LINE-1 element) [139,378,379] could, by virtue of their inherent mobility, penetrate recipient cells and initiate tumor formation upon "cut-and-paste" insertion into host tumor suppressor genes [380,381]. While there is no direct evidence, it is interesting to speculate here on the possibility of lateral transfer of therapy resistance mutations from resistant cells to surrounding cells.

In contrast to pro-cancer effects, it has also been demonstrated that the lateral transfer of cfDNA derived from healthy cells can halt the proliferation of cancer cells [382,383]. An early study showed that mouse-derived tumor cell lines cultured in growth medium supplemented with cytosol from non-dividing lymphocytes or hepatocytes was mirrored by a decreased rate of [3H]-thymidine incorporation and inhibition of tumor growth. Despite the molecular complexity of cytosol, this inhibitory effect was ascribed to the DNA present in the cytosol of the lymphocytes or hepatocytes [384]. More recently, in vitro [383] and

in vivo [382] studies have shown that cfDNA from healthy cells can be used to disrupt DNA synthesis in dividing tumors, thereby hampering or completely blocking tumor growth and preventing metastasis. One study also showed that water-soluble nuclear crude extract prepared from salmon soft roe could inhibit the growth of both cancer and non-cancer cell lines through delaying cell cycle progression [385]. While the use of cfDNA as an anti-cancer agent may be an intriguing approach, the research is currently scant, and more evidence is needed. Alternatively, if sufficient evidence indicates the tumor-promoting effects of cfDNA, direct targeting and elimination of cfDNA may constitute a potential therapeutic approach. For example, non-specific degradation of cfDNA in pancreatic cancer cell cultures via DNase I resulted in a reduction of matrix attachment, migration, and invasion, thereby reducing metastatic potential [374]. Similarly, in an in vivo orthotopic xenograft model, DNase I treatment alone suppressed tumor metastasis [374]. Given the uncertainties related to the potential effects or consequences of untargeted or nonspecific DNA digestion in cancer patients, it is interesting to note here that cationic nanoparticles have been successfully used for scavenging cfDNA in a rheumatoid arthritis rat model, which resulted in the inhibition of inflammation and relief of symptoms [386].

8.2. Functions and Effects of NETs

A wide range of bacteria, fungi, parasites, and viruses can induce the formation of NETs (reviewed in refs. [267,387]). Thereby, pathogens can be immobilized, and their elimination is facilitated by antimicrobial compounds and phagocytosis. Whether NETs themselves are capable of killing microorganisms in vivo is not yet clear, but since many of their components are toxic to microorganisms, it might be possible [388,389]. NETs and their components are rapid danger signals, and thus activate necessary immune responses. On the other hand, evidence is accumulating that they are able to amplify inflammation and trigger the formation of additional NETs, which contribute to direct tissue damage, particularly via histones and proteases [267,268,390]. Their possible associations with various diseases are reviewed in detail elsewhere [275,390,391]. Among others, they are described in relation to severe sepsis, cystic fibrosis, asthma, and CVDs, including thrombosis, systemic sclerosis, myocardial infarction, and stroke. In addition, DNA, histones and neutrophil proteins can trigger the production of autoantibodies. This could contribute to the clinical features of SLE, RA, and other autoimmune diseases. There are also indications that NET-derived granule proteins might contribute to the migration of tumor cells [390].

The components of NETs are widely discussed as potential biomarkers and have recently received additional attention due to their correlations with coronavirus disease 2019 (COVID-19). Levels of circulating myeloperoxidase (MPO)-DNA complexes, a marker of NETs levels, were observed to be increased in symptomatic COVID-19 patients. Circulating NETs are thought to be implicated in the pathology of COVID-19 by causing capillary destruction and leakage, and by inducing endothelial cell death and thrombosis. These circumstances can lead to lung epithelial destruction and death [392]. Thus, it has been suggested that the MPO-DNA complex should be further investigated as a biomarker for the early phase of severe acute respiratory syndrome coronavirus type 2 (SARS-CoV-2) infection [393,394]. NETs could also potentially serve as biomarkers in other diseases, such as cancer. In a recent study, researchers were able to distinguish between patients with metastatic colorectal cancer and healthy individuals based on cfDNA, MPO, and neutrophil elastase (NE) concentrations. It was further suggested that cfDNA levels, in combination with MPO-DNA and NE, could serve as potential biomarkers for disease severity, prognosis or treatment guidance of metastatic colorectal cancer and cancer in general [395]. Furthermore, correlation of the markers mentioned above with levels of autoantibodies, e.g., anti-dsDNA, may give insight into autoimmune activity [396].

Not only may NETs and their components serve as biomarkers in the elucidation of diseases, but, due to their involvement in pathogenesis, they also represent potential therapeutic targets. For example: (i) In preclinical studies, targeting NADPH-dependent chromatin decondensation by ROS scavengers, like N-acetyl cysteine, MPO inhibitors and

PAD4 inhibitors, was shown to suppress NET release [268,397]; (ii) An additional target was recently highlighted by Cao et al. They demonstrated an efficient inhibition of NET formation in vitro by a DEK-targeting aptamer (DTA). DEK is a chromatin-associated protein which may play a crucial role in the formation of NETs and serves as an autoantigen in many autoimmune diseases. In addition, they modified DTA chemically and improved its application and achieved an attenuated inflammation in collagen-induced arthritis mice [398]; (iii) another interesting therapeutic approach was proposed by Chiang et. al. [399]. They investigated specialized pro-resolving mediators (SPMs) and encountered a new series that appear to be potent agonists of pro-resolving phagocyte functions. The resolvins, termed 13-series (T-series) resolvins (RvTs), are produced by human cells and blood. Chiang et al. prepared RvTs by total organic synthesis and found a reduction of NETs in PMA-stimulated human whole blood. Furthermore, they found a potent structure of RvTs which activated the cAMP-PKA-AMPK pathway in human macrophages, resulting in improved NET clearance [399]; (iv) Treatment with DNases is an alternative way to accelerate the degradation of NETs. Dornase alpha, a recombinant human DNase I, is already in use for treatment of cystic fibrosis and was tested in a clinical trial for the treatment of acute respiratory distress syndrome secondary to COVID-19. During drug administration, a reduction of MPO-DNA complexes in bronchoalveolar lavage fluid and improved oxygenation and lung compliance were observed. This again illustrates the potential role of NETs in the pathology of COVID-19 [400]. There may be many more approaches to target NETs, such as direct inhibition of granular proteins and enzymes. However, it is necessary to evaluate them not only in vitro but also in vivo and to investigate the more specific pathways of formation in both diseased and healthy conditions.

In summary, while much is still not known about the biology of NETs, it clearly represents an important and biologically active class of cfDNA molecules that deserves more research attention.

### 8.3. Other Potential Functions of cfDNA

Several studies have shown that cfDNA molecules can enter recipient cells and elicit a variety of biological responses, either by integrating into the host genome or by other intracellular activities [4,113,401]. This phenomenon has not only been implicated in the detrimental processes described above, but has also been implicated in benign intercellular messaging functions, such as the synchronization of cell differentiation [4], as well as evolutionary processes, such as the lateral transfer of hereditary information from somatic cells to germ cells or adaptive traits between different cells (reviewed in refs. [5,402,403]). However, the possibility of cfDNA as an intercellular messenger or a factor in evolutionary processes has not yet been taken seriously by the research community.

While it is true that there currently lacks a concrete body of evidence for these functions of cfDNA, it is also true that the research field is significantly biased toward clinically motivated studies, and that there is a general lack of interest in cfDNA as a biological phenomenon in general. Yet there seems to be sufficient evidence to warrant further enquiry into the possible biological functions of cfDNA. A more detailed and structured enquiry into the biological functions of cfDNA may reveal currently unknown processes involved in the mechanisms underlying various pathologies. In line with this, targeted elimination of aberrant cfDNA molecules may represent a potential therapeutic strategy in a variety of pathological scenarios.

9. Biological Factors That Affect cfDNA Measurements

Early studies have focused mainly on the characterization of circulating cfDNA (i.e., blood plasma or serum), but cfDNA molecules have now been detected and are increasingly investigated in all non-circulatory body fluids (e.g., urine, saliva, stool). The composition and fluctuation of cfDNA are modulated by a variety of factors, many of which are unique to or vary depending on the characteristics of specific body fluids. While there are some overlapping factors among the different body fluids, we limit the focus here

to an exploration of the most prominent biological factors that affect the quantitative and qualitative characteristics of cfDNA in relation to its analysis in blood plasma (Figure 7).
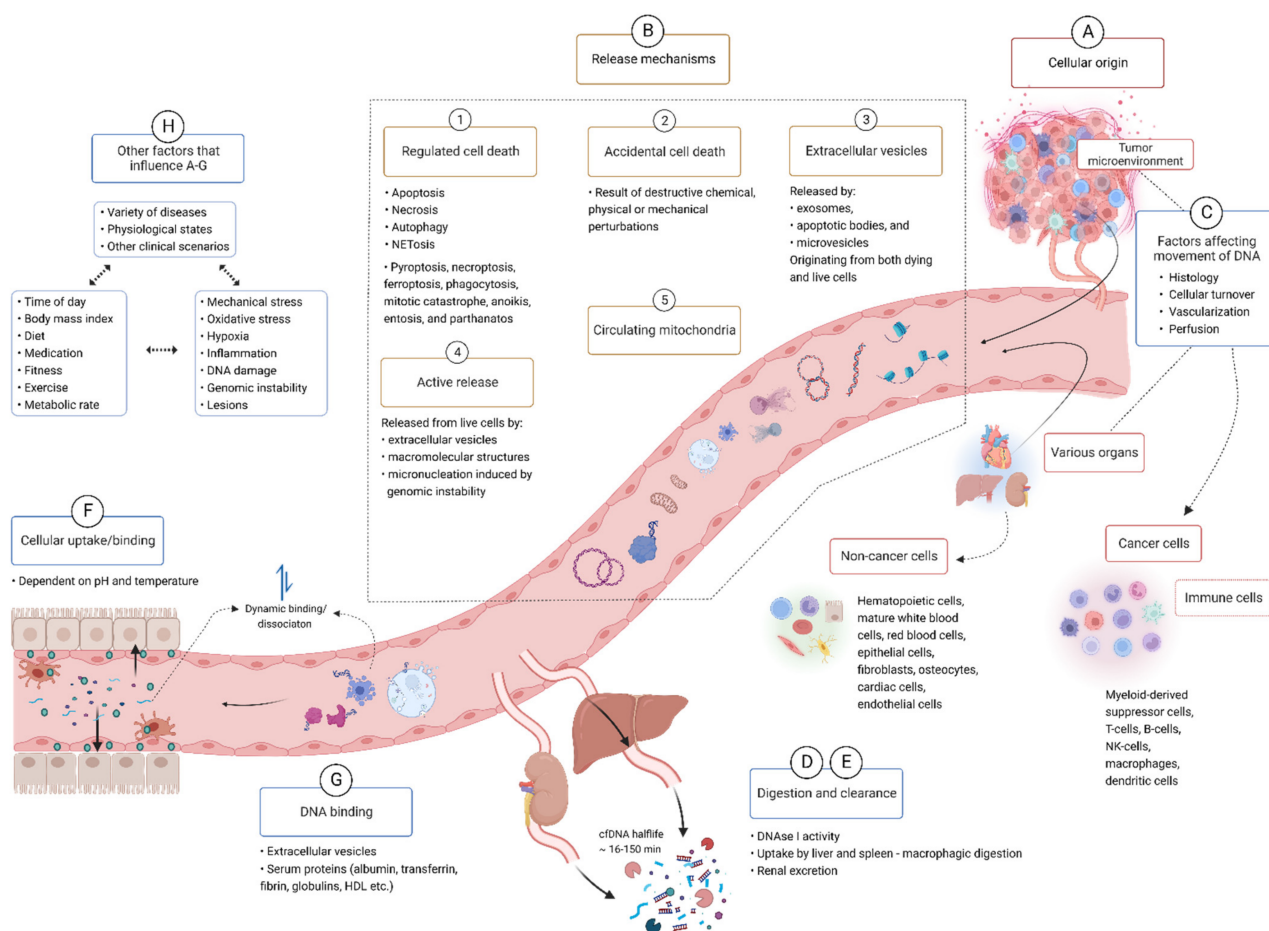
Figure 7. Biological factors that modulate the characteristics of cfDNA. The composition and fluctuation of the cfDNA population in the circulatory system is influenced by numerous, often mutually non-exclusive determinants. The major factors include (**A**) the relative contribution that different cell types make toward the total cfDNA pool; (**B**) the mechanisms by which cfDNA is released from the various contributing cells; (**C**) factors that affect the movement of cfDNA from tissues or cells into circulation; (**D**) modifications and rate of degradation by extracellular nucleases and proteolytic enzymes; (**E**) the rate of uptake and digestion by the liver, spleen or kidneys; (**F**) binding and detachment to circulating or epithelial cells, and (**G**) association with DNA-binding proteins, other macromolecules, or extracellular vesicles. (**H**) All of the former factors are amendable by a web of factors, many of which may interact in known and unknown ways, including (i) a variety of diseases, physiological states, and other clinical scenarios, (ii) phenomena that cause cell death or constitutive DNA release, such as mechanical stress, oxidative stress, hypoxia, inflammation, DNA damage, genomic instability, lesions, and (iii) other factors such as, e.g., time of day, body mass index, diet, medication, fitness, exercise, and metabolic rate, most of which are liable to significant intra- and interindividual variation.

9.1. Relative Contribution of Different Genomes

While it shifts depending on the presence of disease and many other factors, the relative percentage contribution that different origins of cfDNA (e.g., DNA derived from different host-cell types, nuclei, mitochondria, endogenous microbes, pathogens, etc.) make toward the total cfDNA pool has not yet been determined in healthy individuals or in specific pathological or physiological states. However, in cancer patients it is, for example, well understood that the respective contributions of wild-type vs. tumor-derived DNA

depends on cancer stage but exhibits significant intra- and interindividual variation [9,404]. Moreover, regarding wild-type cfDNA of host-cell origin, there is no scientific consensus on the relative percentage contributions of various tissue and cell types, but there are two important observations. First, several studies indicate that the majority of cfDNA originates from hematopoietic cells [173,405,406] and mature white blood cells [172,407–409]. Second, despite a major contribution from blood cells, cfDNA has been found to originate from many different cell types with significant heterogeneity in the relative contributions observed among different studies [81]. A better understanding of this variation can only be gained through increasingly accurate estimations of the cellular origin of cfDNA, facilitated by (i) methods that allow total cfDNA extraction and (ii) studies that systematically map the landscape of cell- and tissue specific features of cfDNA [92,174,179,347,348,354,410–413].

9.2. Mechanisms of Release and Intracellular Modifications

Apoptosis is normally referenced as the main and most relevant mechanism involved in the release of DNA into extracellular space. However, this biased view is slowly being replaced by the understanding that there are several different pathways for the generation of cfDNA, many of which may contribute significantly towards the total pool of cfDNA. The major contributors include: (i) regulated, rapid or gradual cell death mediated by a variety of dedicated molecular mechanisms, such as necrosis, autophagy, and many other cell death subroutines, including NETosis, which results in the release of NETs, pyroptosis, necroptosis, ferroptosis, phagocytosis, mitotic catastrophe, anoikis, entosis, and parthanatos; (ii) accidental, rapid cell death caused by destructive chemical, physical, or mechanical perturbations; (iii) DNA-containing extracellular vesicles that can be released by both dying cells and live cells; (iv) active release of DNA from live cells [4,414–422], although evidence on the latter remains scant and is still under debate/investigation, and (v) release of mtDNA from cell-free respiratory competent mitochondria present in circulation [248].

As discussed earlier, the various cfDNA subtypes present in a typical biospecimen is genetically, epigenetically, and structurally diverse. While the above-mentioned cell death mechanisms are, for example, very well described in the literature, there are still very few studies on the correlation between the respective mechanisms of release from different contributing cells and their impact on the physico-chemical features of cfDNA. However, the limited research done to date suggests a strong correlation. It is generally reported that apoptosis generates mostly mono-nucleosomes with a smaller fraction of di- and tri-nucleosomes in some cases [423,424], while necrosis is reported to generate HMW cfDNA fragments (~10 Kbp) [425–428]. However, there is evidence that apoptosis can also result in the release of HMW cfDNA [429], while necrosis can result in the release of DNA populations that mirror apoptotic laddering and produce an abundant population of mono-nucleosomes [430]. The relative contribution of different mechanisms toward the pool of cf-mtDNA is not known; however, it is clear that cf-mtDNA fragments range in size between 40 and 300 bp [53–56]. This broad distribution is likely the result of enzymatic degradation coupled with a lack of protection by histone proteins, but it is not clear how much of this digestion takes place prior to release intracellularly or extracellularly after release. Concerning regulated cfDNA release, some reports suggest that DNA may be released from specific chromosomal regions during the division of genomically unstable cells. This DNA is postulated to be released by at least two different modes: (i) 1000–3000 bp fragments derived from micronuclei [378,379], and (ii) free-floating extrachromosomal circular DNA ranging between 30 and 20,000 bp [431–434]. Furthermore, as discussed earlier, there is also evidence of actively released EVs that are associated with DNA fragments ranging between 150 and 6000 bp [303,310,401].

9.3. Movement of cfDNA

The characteristics of cfDNA can be influenced by several conditions surrounding its movement from cells/tissues into body fluids. Taking cancer as an example, the level of

cfDNA released by a tumor, as well as the rate at which cfDNA moves from the tumor into the circulatory system may depend on the unique histology, cellular turnover, vascularization, and perfusion of the tumor. These factors can also alter the pathway of cfDNA release. For example, hypoxia due to poor vascularization can result in increased rates of cell-death and the generation of more cfDNA [435]. Hypoxia may also shift the relative contributions of different modes of cell death, thereby altering the physico-chemical features of the cfDNA. Furthermore, the movement of cfDNA can also be influenced by biological barriers. For example, the blood-brain barrier may restrict the movement of cfDNA from brain tumors into the circulatory system. Indeed, higher levels of tumor-derived cfDNA has been found in cerebrospinal fluid (CSF) vs. plasma [79,80,117]. Similarly, a higher ratio of tumor-derived vs. background cfDNA may be found in body fluids that are in closer proximity to specific tumors, such as stool in colorectal cancers [436], cervical smear [437], or uterine lavage [438] in gynecological cancer, saliva in oral cancers [439], or urine in bladder cancers [440–442].

9.4. Extracellular Modifications, Stability, Clearance, and Half-Life

A variety of extracellular factors can affect the quantitative and qualitative properties of cfDNA. (i) While it is difficult to determine the physical properties of cfDNA at the instance of cellular release, several lines of evidence indicate that cfDNA fragments may be rapidly shortened by the action of different enzymes present in blood, such as DNase I [443,444] and DNase1L3 [445]. The most studied cfDNA size population in blood plasma is the mono-nucleosome with the most commonly reported size of 167–168 bp, representing 145–147 bp of DNA wrapped around the nucleosome core particle (NCP) plus linker DNA bound to histone H1 [415,417,446]. This size is likely due to Caspase activated DNase (CAD) digesting linker DNA 10 bp up- and downstream form the NCP [447]. The prevalence of the 167 bp mono-nucleosome in cfDNA studies suggests that the chromatosome is a stabilizing structure that protects DNA against enzymatic cleavage. However, CAD can also slide its digestion sites in increments of 5 bp in some cases [447], resulting in mono-nucleosome sizes of approximately 158, 163, 173, 178, 183, and 188, which are also observed in studies [430]. Among these, the 177–178 bp population is also often observed in studies [430,446], which is a size-population that has a higher probability of forming [448]. In addition to mono-nucleosomes, multiples of nucleosomal repeats are also often observed, e.g., di-nucleosomes, tri-nucleosomes, tetra-nucleosomes, and penta-nucleosomes. These cfDNA size populations also display modal sizes (i.e., 356 bp, 534 bp, 712 bp, and 890 bp, respectively), which seems to represent the size that is most likely to form under stochastic digestion of linker DNA [448]. The presence of these size populations is generally ascribed to apoptotic cleavage of chromatin. However, there is a growing number of studies reporting on the presence of HMW cfDNA present in body fluids [449–457] and cell cultures [305,414–417,430,458,459]. These HMW fragments may originate from apoptosis but may also be derived from many other release mechanisms, such as accidental cell lysis, necrosis, NETosis, regulated release through extracellular vesicles, and so forth. There is increasing evidence suggesting that these HMW cfDNA fragments may be digested into shorter fragments. While there may be some selectivity involved in cleavage locations, a generally stochastic inter-nucleosomal DNA digestion scheme seems likely. In such a scheme, the digestion of all longer fragments feeds all shorter fragments, which ultimately results in the rapid accumulation of mono-nucleosomal fragments. While more research is needed, this represents an alternative view to the view in which the presence of mono-nucleosomes is ascribed mainly to apoptosis. In addition to HMW cfDNA and mono-nucleosomes, a growing number of studies are demonstrating the presence of sub-nucleosomal and ultrashort, single-stranded cfDNA fragments in body fluids [106,184,252,460–464]. Whether these shortened cfDNA fragments represent short fragments released from cells or extracellularly digested products of longer fragments is not yet clear. However, given the presence of proteolytic enzymes in blood, it is likely that a large proportion of these short fragments represent DNA derived from destructed

nucleosomes. (ii) In addition to enzymatic or proteolytic degradation, cfDNA fragments can either be captured and digested by the liver and spleen [465,466], or be absorbed by the kidneys followed by urinary elimination [467–469]; (iii) CfDNA molecules can also attach to the membranes of circulating cells (e.g., red and white blood cells) or endothelial cells, where they may either (a) remain attached for prolonged periods or be digested by enzymes, (b) be assimilated into the cells, or (c) attach and detach dynamically depending on pH, temperature, and the influence of various substances (reviewed in ref. [470]); (iv) the enzymatic/proteolytic degradation, uptake by organs for digestion or excretion, and cellular internalization of cfDNA may depend significantly on its physico-chemical features, many of which may be altered by the binding of cfDNA molecules to EVs, circulating DNA-binding proteins (e.g., high density lipoprotein, argonaute 2, albumin, fibrin, fibrinogen, prothrombin, transferrin, globulins, C-reactive protein, and serum amyloid A), and other macromolecules. It is important to note here that (1) most of the factors discussed above have not yet been studied extensively, (2) the relative contribution of each factor towards the stability and clearance of cfDNA is not known, (3) we still have a poor understanding of the processes involved in the generation of cfDNA, and (4) many of these factors are non-mutually exclusive. This makes it very difficult to establish the dynamics and baseline characteristics of cfDNA. For example, while the half-life of cfDNA is estimated between 16 min and 2.5 h [75–77], there is no actual consensus, as this half-life refers to the stability of mono-nucleosomal cfDNA and not the total cfDNA population.

9.5. Summary

As discussed earlier, a wide range of disorders, physiological states and factors, and clinical scenarios result in increased cfDNA levels. Among these, numerous factors (e.g., mechanical stress, oxidative stress, hypoxia, inflammation, DNA damage, genomic instability, lesions, etc.) may trigger different types of cellular demise or alter constitutive DNA release. Similarly, many cfDNA-modifying factors such as the extracellular concentration of enzymes and DNA-binding proteins, or the rate of cfDNA uptake by the liver, spleen, and kidneys are augmentable by various factors (e.g., time of day, body mass index, diet, medication, fitness, exercise, metabolic rate, etc.) many of which are liable to significant intra- and interindividual variation. As many of these factors may not only occur simultaneously but are also inextricably linked in a specific pathological or physiological state, it has to-date been virtually impossible to make concrete correlations between a specific factor and the dynamics or characteristics of one cfDNA subtype.

10. Characterization of cfDNA in Animals

Beyond the role of cfDNA in humans, cfDNA has been investigated in various animals with an overrepresentation of studies on dogs. While cfDNA research on non-human animals is not as developed as in humans, there is increasing interest in the role of cfDNA as a biomarker as well as a tool to study pathophysiology in animals, especially companion animals. For example, several studies indicate the potential of implementing cfDNA tests in veterinary clinics for the minimally-invasive diagnosis and monitoring of cancer in dogs [471–477]. Furthermore, cfDNA may also serve as a potential marker of gastric dilatation–volvulus (GDV) [478] and immune-mediated hemolytic anemia (IMHA) [479], tissue injury [480], and sepsis [481–483] in dogs. Additionally, similar to humans, strenuous exercise has been shown to correlate with elevated levels of cfDNA in dogs [484]. Besides dogs, one study has measured cfDNA in cats with diffuse iris melanomas and concluded that cfDNA levels and integrity are not sufficient markers for diagnosis and prognosis [485]. Interestingly, the characterization of cfDNA derived from the microbiome of non-human primates may allow the discovery and monitoring of taxa that cause zoonotic diseases [108,486]. Furthermore, various animal models have been used to study correlations between different pathological factors and corresponding changes in cfDNA, which may have implications in human biology and pathology, such as the study of traumatic brain injury in rats [487], carbon tetrachloride-induced acute liver injury in rats [488], perinatal asphyxia in piglets [489],

tumor progression and metastases in rats [490,491], the onset of parturition in mice [492], and gateways regulating the release of tumor-derived cfDNA in mice [493].

11. Perspectives on the Importance of an Improved Understanding of cfDNA Biology

In this review, we showed how the composition of cfDNA in blood is both highly complex and changeable. Several overlapping phenomena contribute to this (Figure 8).

Diverse origins

i.   cfDNA may originate from diverse genomes, e.g., healthy and damaged tissue, nuclei, mitochondria, fetuses, tumors, various host-cell and tissue types, organ transplants, the microbiome, pathogenic microbes, and parasites.

Cause of release

ii.   A variety of causes can trigger the release of DNA from these sources. While there are reports of constitutive and regulated release from live cells, the bulk of DNA is released into blood through various pathways of cell death. The modes of cell death are dictated by various processes, such as mechanical stress, oxidative stress, hypoxia, inflammation, DNA damage, and genomic instability. These detrimental processes may either give rise to *or* arise due to a wide range of diseases, physiological states, environmental exposures, and other clinical scenarios.

Mode of release

iii.   The mode of cell death in turn dictates the genetic and physico-chemical features of the DNA released, in at least three different ways. First, cells may harbor several DNA structures formed by processes unrelated to cell death, e.g., extrachromosomal circular DNA, mtDNA, vesicle-associated, or DNA associated with macromolecular structures. These forms of DNA may be conserved by some modes of cell death but degraded by others. Second, different modes of cell death are characterized by different schemes of DNA digestion, resulting in different cfDNA types, e.g., apoptosis may generally produce short fragments while necrosis generates longer fragments. Third, the unique genetic and epigenetic characteristics and gene expression programs of different cell types may result in the generation of unique cfDNA types during cell death. For example, cell or tissue-specific methylation or nucleosome-spacing patterns may protect some genomic sites from cleavage by apoptotic enzymes while exposing others, resulting in tissue-specific fragmentation patterns, fragment endpoints, etc.

Physico-chemical diversity

iv.   cfDNA fragments thus carry unique genetic and epigenetic information that informs on (a) the source from which they originate (b) the type of cell death that caused its release, and (c) the condition that dictated the mode of cell death, e.g., anticancer agents may induce apoptosis in cancer cells resulting in the release of mutated cfDNA fragments with a modal size of 167 bp.

Movement in the body

v.   The abundance and characteristics of cfDNA molecules originating from different regions of the body can then be influenced by factors related to its movement from cells/tissues into blood. The movement of cfDNA from different tissue types, for example, may depend on their unique histology, cellular turnover, vascularization, and perfusion. These features may, for example, differ between tumor types and change over the course of disease.

Interactions in blood

vi.   Once cfDNA fragments are present in the blood, their abundance and physico-chemical feature can be changed by several extracellular factors, e.g., degradation by nucleases or proteases, clearance by the liver, spleen or kidneys, or urinary excretion. This is in turn influenced by the association of cfDNA with cell membranes, proteins, and other macromolecular structures. Both the degradation/clearance and binding of cfDNA in turn depends on the initial structure of cfDNA molecules (upon first entry into blood).

Physiological variables

vii.   The presence, abundance, and activity of extracellular factors that modify or eliminate cfDNA molecules may in turn be influenced by various factors, e.g., time of day, food intake, diet, medication, body-mass-index, fitness, physical activity or exercise, and metabolic rate, many of which demonstrate intra- and interindividual variability.

Methodological variables

viii.   Lastly, given the structural diversity of cfDNA molecules, the measured quantitative and qualitative characteristics of cfDNA in a biospecimen are significantly influenced by the efficiency and biases of purification methods, and several other preanalytical steps that are biased toward the preservation, degradation, elimination, or capture of specific cfDNA structural subtypes.

Figure 8. Factors that contribute to the complexity and fluctuation of cfDNA composition.

From the points outlined in Figure 8, a picture of immense complexity emerges. The factors that modulate the observed properties of cfDNA are vast, inextricably linked, and are riddled with overlapping characteristics and circular causation. Not only do we currently have a weak grasp on the outlines of this problem, but also a very poor understanding of most of the individual factors. Yet, it is increasingly recognized that a holistic understanding of cfDNA biology is vital [1,3,6,59,130,137,253,494]. A deep and structured enquiry into the origin, physico-chemical features, and functions of cfDNA as it relates to the various biological, physiological, pathological, environmental, and lifestyle factors as well as preanalytical factors will provide new insights that impact many aspects of cfDNA research.

11.1. Increasing the Sensitivity and Specificity of Existing cfDNA Assays

In a highly heterogeneous cfDNA population, both the absolute amount and relative proportions of most target molecules are often low. However, elucidation of the various factors that respectively affect the concentration of target molecules and background cfDNA molecules in a biospecimen will on the one hand inform the selection of optimal preanalytical procedures and patient conditions, and on the other hand facilitate the development of methodologies that either maximizes the absolute number of target molecules or increases the ratio of target-to-background cfDNA molecules, thereby significantly increasing the chances of detection.

For example, the amount of target molecules in a sample may be increased by (a) selecting the right biospecimen type (e.g., target molecules may be enriched in body fluids that are in closer proximity to the source of the target molecules of interest, such as ctDNA

in the CSF of patients with brain tumors); (b) selecting patient conditions that (i) favor the release of target molecules, (ii) limit the release of background molecules, or more likely (iii) enhance the release of total cfDNA. In line with this, it can be conjectured that an improved understanding of the mechanisms involved in the release of cfDNA may permit the use of compounds to enhance or limit cfDNA release. However, this approach may be offset by the potential detrimental effects of dramatically shifting cfDNA levels (as discussed in Section 8); (c) the use of tailored extraction procedures developed for the capture of specific target molecules or elimination of non-target molecules, as informed by knowledge of the exact structural features or chemical properties of specific cfDNA molecules. The efficacy of cfDNA biology-informed methods is demonstrated by (1) a study that has achieved greater cancer assay sensitivity and specificity through experimental capture or in silico selection of mutant-enriched short cfDNA fragments [184]; (2) a study that demonstrated the possibility of selectively capturing mutant molecules based on cancer-specific DNA methylation aberrations with enhanced affinity for gold nanoparticles [495]; (3) studies that have shown that exosomes often carry cargo enriched in tumor material. Besides the aforementioned examples, cfDNA molecules come in a variety of shapes and sizes, many of which are affected in different ways by various preanalytical steps. An exploration of these differences is not only likely to reveal the ideal preanalytical workflows for specific cfDNA molecules, but will also inform the development of new and more effective products and methods.

11.2. Expanding the Repertoire of Disease-Specific cfDNA Markers

As outlined in Section 5, a growing body of clinical studies shows strong correlations between epigenetic features of cfDNA and disease dynamics in various pathologies, especially cancer. By virtue of the increased proportionality of epigenetic modifications vs. single-gene mutations, epigenetic cfDNA assays often show increased diagnostic sensitivity and specificity, especially for the characterization of early-stage disease. While there is currently limited evidence of its clinical validity, and many preanalytical obstacles to overcome, there is little doubt among researchers and clinicians that cfDNA testing will be integrated into standard patient care in the near-future. While the majority of cfDNA applications in oncology are still centered on the detection of single-gene mutations in cfDNA, the focus of cfDNA research is slowly shifting towards epigenetic characterization. Indeed, the major efforts that are currently being made to characterize tissue- and disease-specific epigenetic markers of cfDNA will lead to the development of new and more powerful assays that significantly expand the liquid biopsy toolbox. This represents an entirely new modality in the application of cfDNA assays and will play a significant part in the transformation of personal molecular medicine through cfDNA profiling.

11.3. Enable the Characterization and Study of Temporal Genome Dynamics

An expanded knowledge of cfDNA biology will enable increasingly high-fidelity reconstruction of the physiological, pathological, and cell- and tissue-specific information harbored by cfDNA molecules. Robust longitudinal profiling of cfDNA may allow minimally invasive characterization of temporal genomic changes in specific regions of interest in response to a wide variety of factors. In contrast to traditional methods of genome analysis (e.g., cell cultures, in vivo models, and tissue biopsies), which have been valuable but limited by the inherent restraints of reductive approaches (e.g., sampling bias, loss of contextual logic, snapshot information) (reviewed in ref. [130]), data from serially profiled cfDNA represent a richer, more dynamic and much more realistic source of actual genome function, composition, and alterations.

Therefore, the genomic information accessible through serial cfDNA analysis can be mined for (i) the discovery of new and increasingly powerful combinations of surrogate markers for various disease indications, and (ii) unprecedented analysis of genome function and systematic mapping of both benign and malignant genetic and epigenetic alterations caused by a variety of biological, physiological, pathological, and environmental

factors. Research on temporal genomic characterization through cfDNA is still in its infancy, with many challenges ahead. However, the successes achieved in temporal analyses of tumor genomes have moved theoretical deliberations from the realm of fantasy and speculation into concrete evidence and practical utility. For example, serial cfDNA assays have been used to detect and monitor minimum residual disease, metastases, intratumor genetic heterogeneity, shifting mutational landscapes, genetic responses to chemo- and radiotherapy, and mechanisms that underlie the emergence of therapy resistance. There is strong evidence that this broad information, which is virtually unattainable through tissue biopsies, can be leveraged to improve diagnoses, prognoses, guide and improve therapeutic regimes, limit overtreatment and risk of recurrence, and ultimately improve patient quality-of-life and overall survival. Another example of the promising results achieved by serial analyses of cfDNA is the monitoring of donor-derived cfDNA in organ transplant patients to detect signs of rejection or injury of the transplanted organ. While much clinical validation is needed before these cfDNA assays will be widely implemented, the progress made in the field of oncology and organ transplant monitoring indicates the possibility of developing cfDNA assays for monitoring a potentially wide range of pathologies and other clinical scenarios.

Serial cfDNA analyses may have many other potential uses. (i) They may provide new mechanistic insights into the pathological events underpinning many slowly progressive or chronic illnesses, especially those that have been correlated with cell death and aberrant cfDNA profiles, such as CVDs, diabetes, autoimmunity, and neurodegenerative disease; (ii) they may be especially useful for monitoring progressive diseases or clinical scenarios that cause rapid genomic alterations at specific time-points, such as Parkinson's disease, Alzheimer's disease, sepsis, stroke, traumatic injuries, and malfunctioning gene therapy; (iii) they may be used to monitor the safety and efficacy of various kinds of therapy; (iv) they may be useful for studying the diversity and fluctuation of the microbiome as well as its role in human health and disease; (v) they can be used to trace the biological footprint and effects of assimilated environmental DNA; (vi) they can be used to study complex biological phenomena such as genomic mosaicism; (vii) they can be used to investigate the dynamic response of the genome in general or the genome of specific organs to the diet (e.g., long-term consumption of inflammatory foods) or dramatic dietary changes; and (viii) they can be used to determine body-wide changes of the genome as a result of aging. In addition to structural genomic changes, an increasing number of studies indicate that cfDNA can be used to infer gene expression patterns in specific tissues. Refinement of such approaches in concurrence with an improved understanding of cfDNA biology may, in the future, allow the use of cfDNA for studying benign and detrimental gene expression in various contexts, e.g., disease, exercise, and poorly understood biological processes (e.g., embryogenesis).

While the use of cfDNA for these purposes is mostly speculative at this stage and depends highly on the rate at which we expand our knowledge of cfDNA biology, its potential to transform our understanding of genome biology and pathology is undeniable and should, therefore, be provoking more comment than it currently is.

12. Concluding Remarks

While considered trivial at the time of its discovery and even decades thereafter, cfDNA is now emerging as one of the most interesting classes of molecules in human biology. In this review we showed how cfDNA is uniquely poised to transform clinical diagnostics as well as genomics research in various ways: (i) it may serve as highly specific and versatile biomarkers for a wide range of pathologies; (ii) it may serve as surrogate markers for monitoring or studying numerous physiological states; (iii) it may grant an unprecedented window of access to characterize temporal genomic alterations in numerous contexts; and lastly (iv) as cfDNA molecules have biological activity and may contribute to both pathology and normal biological functions, it may possibly shed new light on some of the unknown or poorly understood mechanisms underlying various pathologies and

important biological processes. However, several challenges need to be overcome if we want to harness the full potential of cfDNA for these various purposes (Figure 9).
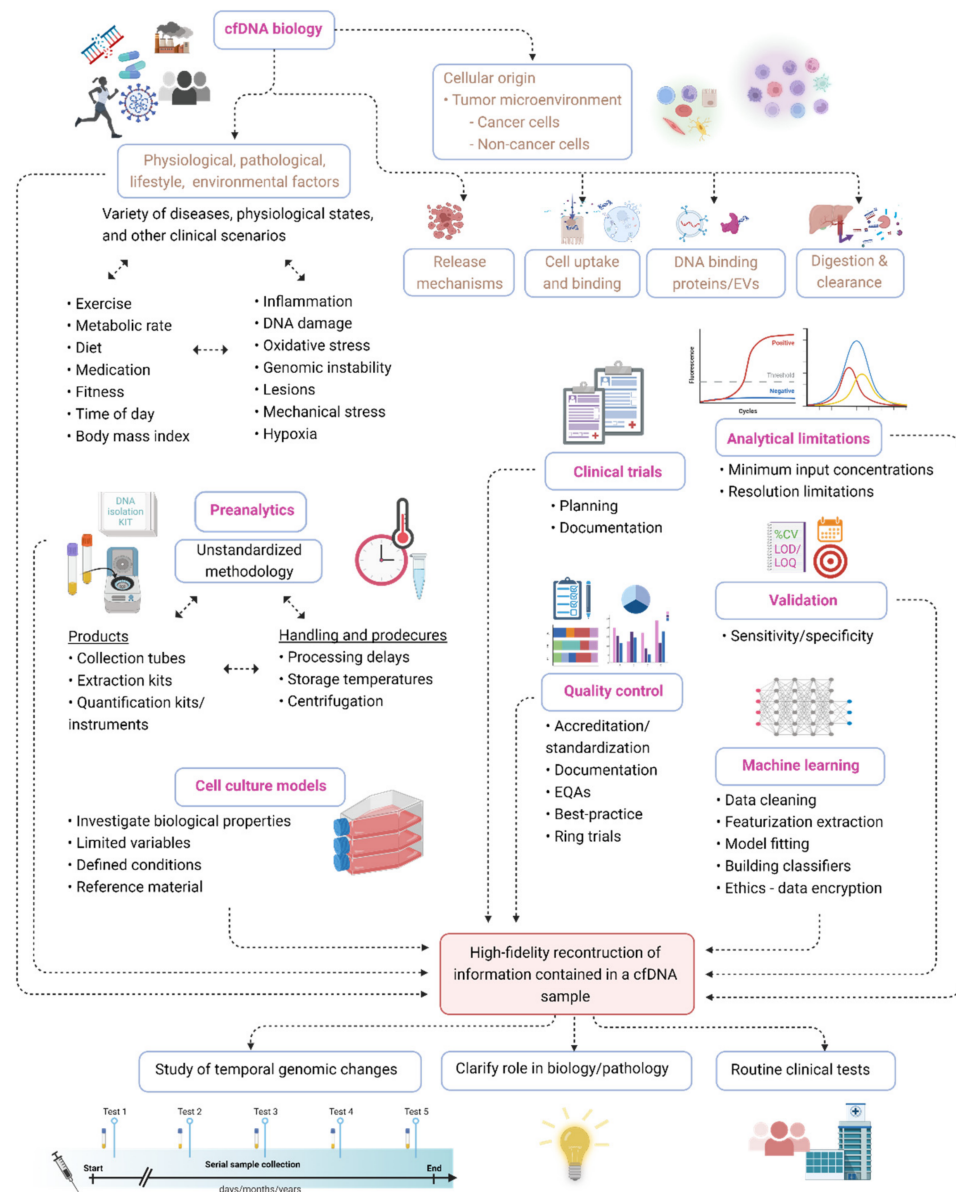
Figure 9. Factors that need to be addressed in order to increase the fidelity of cfDNA analysis. In order to (i) use cfDNA for studying temporal genomic changes, (ii) investigate the role of cfDNA in human health and disease, and (iii) develop increasingly powerful clinical cfDNA assays, the quantitative and qualitative information contained in cfDNA samples needs to be reconstructed with high fidelity. Here we briefly summarize various factors that need to be considered and steps that can be taken towards increasingly accurate cfDNA measurements.

Among the potential applications of cfDNA, its perusal as a biomarker has received the most attention. However, despite the spectacular results that have been demonstrated by clinical studies in various domains of medical research, only a small number of cfDNA assays have to date been validated for clinical use, and only in the fields of oncology and prenatal testing. The factors that challenge the development and clinical implementation of cfDNA assays are multifarious and overlapping:

As it is a neglected factor in cfDNA research, we, in this review, shed some light on how the immense heterogeneity and fluctuation of the cfDNA population in vivo, and by extension in biospecimens, is the unique factor that concomitantly makes cfDNA such

a valuable biomarker class and tool in basic biological and genomic research, but also a very challenging analyte. High-fidelity reconstruction of the information encoded into the aggregate cfDNA population, or specific cfDNA target molecules, requires a significantly improved understanding of the genetic and physico-chemical features of cfDNA as it relates to various biological, physiological, pathological, lifestyle, environmental, and preanalytical factors. This may prove to be very challenging, but useful insights may also be achieved using in vitro methods. For example, in studying the biological factors that affect the properties of cfDNA, many of the inherent complexities of the in vivo milieu may be circumvented by using cell culture models. While much context about the in vivo setting may be lost in cell culture investigations, significantly diminished variables may allow unique insights into the biological properties of cfDNA in vivo. Another approach may be to monitor the physico-chemical properties and fluctuation of a purified cfDNA subtype spiked into plasma biospecimens or into artificial/simulated plasma.

Nevertheless, the heterogeneity and fluctuation of cfDNA is only part of the problem. While an in-depth enquiry into these factors were beyond the scope of this review, it is worth noting that the developmental timeline for clinically meaningful tests is extended by several persistent obstacles that need to be overcome, including a lack of universal preanalytical standards, an ever-expanding menu of preanalytical products, limited best practice guidelines, analytical limitations, no standard reference materials, insufficient analytical validation, and poorly-designed clinical trials. In addressing each of these obstacles, it would be beneficial to develop strategies for assessing the influence of the biological factors of cfDNA.

Moreover, it is becoming increasingly clear that new bioinformatics approaches, such as the incorporation of ML algorithms, are desperately needed to interpret and understand the sheer amount of data generated in cfDNA research, especially studies in which numerous parameters are assessed.

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References

1. Aucamp, J.; Bronkhorst, A.J.; Badenhorst, C.P.S.; Pretorius, P.J. The diverse origins of circulating cell-free DNA in the human body: A critical re-evaluation of the literature. *Biol. Rev. Camb. Philos. Soc.* **2018**, *93*, 1649–1683. [CrossRef] [PubMed]
2. Bronkhorst, A.J.; Ungerer, V.; Holdenrieder, S. The emerging role of cell-free DNA as a molecular marker for cancer management. *Biomol. Detect. Quantif.* **2019**, *17*, 100087. [CrossRef] [PubMed]
3. Kustanovich, A.; Schwartz, R.; Peretz, T.; Grinshpun, A. Life and death of circulating cell-free DNA. *Cancer Biol. Ther.* **2019**, *20*, 1057–1067. [CrossRef] [PubMed]
4. Gahan, P.B.; Stroun, M. The virtosome-a novel cytosolic informative entity and intercellular messenger. *Cell Biochem. Funct.* **2010**, *28*, 529–538. [CrossRef] [PubMed]
5. Aucamp, J.; Bronkhorst, A.J.; Badenhorst, C.P.; Pretorius, P.J. A historical and evolutionary perspective on the biological significance of circulating DNA and extracellular vesicles. *Cell. Mol. Life Sci.* **2016**, *73*, 4355–4381. [CrossRef] [PubMed]
6. Odenheimer-Bergman, A.; Markus, H.; Murtaza, M. Biology of Circulating DNA in Health and Disease. In *Cell-Free Circulating DNA: Purification and Analysis Techniques*; World Scientific: Singapore, 2022; pp. 1–20.
7. Ungerer, V.; Bronkhorst, A.J.; Holdenrieder, S. Preanalytical variables that affect the outcome of cell-free DNA measurements. *Crit. Rev. Clin. Lab. Sci.* **2020**, *57*, 484–507. [CrossRef] [PubMed]
8. Bronkhorst, A.J.; Ungerer, V.; Holdenrieder, S. Early detection of cancer using circulating tumor DNA: Biological, physiological and analytical considerations. *Crit. Rev. Clin. Lab. Sci.* **2019**, *57*, 1–17. [CrossRef]
9. Fleischhacker, M.; Schmidt, B. Circulating nucleic acids (CNAs) and cancer—A survey. *Biochim. Biophys. Acta (BBA)-Rev. Cancer* **2007**, *1775*, 181–232. [CrossRef]
10. Duvvuri, B.; Lood, C. Cell-free DNA as a biomarker in autoimmune rheumatic diseases. *Front. Immunol.* **2019**, *10*, 502. [CrossRef]
11. Tan, E.M.; Schur, P.H.; Carr, R.I.; Kunkel, H.G. Deoxybonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. *J. Clin. Investig.* **1966**, *45*, 1732–1740. [CrossRef]

12. Rumore, P.M.; Steinman, C.R. Endogenous circulating DNA in systemic lupus erythematosus. Occurrence as multimeric complexes bound to histone. *J. Clin. Invest.* **1990**, *86*, 69–74. [CrossRef]

13. Hashimoto, T.; Yoshida, K.; Hashimoto, N.; Nakai, A.; Kaneshiro, K.; Suzuki, K.; Kawasaki, Y.; Shibanuma, N.; Hashiramoto, A. Circulating cell free DNA: A marker to predict the therapeutic response for biological DMARDs in rheumatoid arthritis. *Int. J. Rheum. Dis.* **2017**, *20*, 722–730. [CrossRef]

14. Mosca, M.; Giuliano, T.; Cuomo, G.; Doveri, M.; Tani, C.; Curcio, M.; Abignano, G.; De Feo, F.; Bazzichi, L.; Della Rossa, A.; et al. Cell-free DNA in the plasma of patients with systemic sclerosis. *Clin. Rheumatol.* **2009**, *28*, 1437–1440. [CrossRef]

15. Gögenur, M.; Burcharth, J.; Gögenur, I. The role of total cell-free DNA in predicting outcomes among trauma patients in the intensive care unit: A systematic review. *Critical Care* **2017**, *21*, 14. [CrossRef]

16. Macher, H.; Egea-Guerrero, J.J.; Revuelto-Rey, J.; Gordillo-Escobar, E.; Enamorado-Enamorado, J.; Boza, A.; Rodriguez, A.; Molinero, P.; Guerrero, J.M.; Dominguez-Roldán, J.M. Role of early cell-free DNA levels decrease as a predictive marker of fatal outcome after severe traumatic brain injury. *Clin. Chim. Acta* **2012**, *414*, 12–17. [CrossRef] [PubMed]

17. Regner, A.; Meirelles, L.D.S.; Ikuta, N.; Cecchini, A.; Simon, D. Prognostic utility of circulating nucleic acids in acute brain injuries. *Expert Rev. Mol. Diagn.* **2018**, *18*, 925–938. [CrossRef]

18. Chiu, T.W.; Young, R.; Chan, L.Y.S.; Burd, A.; Lo, D.Y.M. Plasma cell-free DNA as an indicator of severity of injury in burn patients. *Clin. Chem. Lab. Med.* **2006**, *44*, 13–17. [CrossRef]

19. Lippi, G.; Sanchis-Gomar, F.; Cervellin, G. Cell-free DNA for diagnosing myocardial infarction: Not ready for prime time. *Clin. Chem. Lab. Med.* **2015**, *53*, 1895–1901. [CrossRef]

20. Balta, S.; Demirkol, S.; Cakar, M.; Karaman, M.; Ay, S.A.; Arslan, Z. Cell-free circulating DNA as a novel biomarker in patients with the acute coronary syndrome. *Cardiology* **2013**, *126*, 122–123. [CrossRef]

21. Outinen, T.K.; Kuparinen, T.; Jylhava, J.; Leppanen, S.; Mustonen, J.; Makela, S.; Porsti, I.; Syrjanen, J.; Vaheri, A.; Hurme, M. Plasma cell-free DNA levels are elevated in acute Puumala hantavirus infection. *PLoS ONE* **2012**, *7*, e31455. [CrossRef]

22. Bakir, M.; Engin, A.; Kuskucu, M.A.; Bakir, S.; Gundag, O.; Midilli, K. Relationship of plasma cell-free DNA level with mortality and prognosis in patients with Crimean-Congo hemorrhagic fever. *J. Med. Virol.* **2016**, *88*, 1152–1158. [CrossRef]

23. Shapiro, B.; Chakrabarty, M.; Cohn, E.M.; Leon, S.A. Determination of circulating DNA levels in patients with benign or malignant gastrointestinal disease. *Cancer* **1983**, *51*, 2116–2120. [CrossRef]

24. Netz, U.; Perry, Z.; Mizrahi, S.; Kirshtein, B.; Czeiger, D.; Sebbag, G.; Reshef, A.; Douvdevani, A. Cell-free deoxyribonucleic acid as a prognostic marker of bowel ischemia in patients with small bowel obstruction. *Surgery* **2017**, *162*, 1063–1070. [CrossRef]

25. Arnalich, F.; Maldifassi, M.C.; Ciria, E.; Quesada, A.; Codoceo, R.; Herruzo, R.; Garcia-Cerrada, C.; Montoya, F.; Vazquez, J.J.; López-Collazo, E. Association of cell-free plasma DNA with perioperative mortality in patients with suspected acute mesenteric ischemia. *Clin. Chim. Acta* **2010**, *411*, 1269–1274. [CrossRef] [PubMed]

26. Celec, P.; Vlkova, B.; Laukova, L.; Babickova, J.; Boor, P. Cell-free DNA: The role in pathophysiology and as a biomarker in kidney diseases. *Expert Rev. Mol. Med.* **2018**, *20*, e1. [CrossRef] [PubMed]

27. Korabecna, M.; Opatrna, S.; Wirth, J.; Rulcova, K.; Eiselt, J.; Sefrna, F.; Horinek, A. Cell-free plasma DNA during peritoneal dialysis and hemodialysis and in patients with chronic kidney disease. *Ann. N. Y. Acad. Sci.* **2008**, *1137*, 296–301. [CrossRef] [PubMed]

28. Avriel, A.; Rozenberg, D.; Raviv, Y.; Heimer, D.; Bar-Shai, A.; Gavish, R.; Sheynin, J.; Douvdevani, A. Prognostic utility of admission cell-free DNA levels in patients with chronic obstructive pulmonary disease exacerbations. *Int. J. Chron. Obstruct. Pulmon. Dis.* **2016**, *11*, 3153–3161. [CrossRef] [PubMed]

29. Arnalich, F.; Maldifassi, M.C.; Ciria, E.; Codoceo, R.; Renart, J.; Fernández-Capitán, C.; Herruzo, R.; Garcia-Rio, F.; López-Collazo, E.; Montiel, C. Plasma levels of mitochondrial and nuclear DNA in patients with massive pulmonary embolism in the emergency department: A prospective cohort study. *Crit. Care* **2013**, *17*, R90. [CrossRef]

30. Caglar, O.; Cilgin, B.; Eroglu, M.; Cayir, A. Evaluation of circulating cell free DNA in plasma as a biomarker of different thyroid diseases. *Braz. J. Otorhinolaryngol.* **2020**, *86*, 321–326. [CrossRef]

31. Taglauer, E.S.; Wilkins-Haug, L.; Bianchi, D.W. Review: Cell-free fetal DNA in the maternal circulation as an indication of placental health and disease. *Placenta* **2014**, *35*, S64–S68. [CrossRef]

32. Vlkova, B.; Kalousova, M.; Germanova, A.; Parizek, A.; Hajek, Z.; Zima, T.; Celec, P. Cell-free DNA is higher and more fragmented in intrahepatic cholestasis of pregnancy. *Prenat. Diagn.* **2016**, *36*, 1156–1158. [CrossRef]

33. Kashiwada-Nakamura, K.; Myangat, T.M.; Kajihara, I.; Kanemaru, H.; Sawamura, S.; Makino, K.; Aoi, J.; Masuguchi, S.; Fukushima, S. Circulating Janus kinase family DNA levels in psoriasis: Elevated JAK2 DNA copy number in cell-free DNA. *J. Dermatol.* **2021**, *49*, e106–e107. [CrossRef]

34. Beranek, M.; Fiala, Z.; Kremlacek, J.; Andrys, C.; Krejsek, J.; Hamakova, K.; Chmelarova, M.; Palicka, V.; Borska, L. Changes in circulating cell-free DNA and nucleosomes in patients with exacerbated psoriasis. *Arch. Dermatol. Res.* **2017**, *309*, 815–821. [CrossRef]

35. Glebova, K.V.; Veiko, N.N.; Nikonov, A.A.; Porokhovnik, L.N.; Kostuyk, S.V. Cell-free DNA as a biomarker in stroke: Current status, problems and perspectives. *Crit. Rev. Clin. Lab. Sci.* **2018**, *55*, 55–70. [CrossRef]

36. Lam, N.Y.L.; Rainer, T.H.; Wong, L.K.S.; Lam, W.; Lo, Y.M.D. Plasma DNA as a prognostic marker for stroke patients with negative neuroimaging within the first 24 h of symptom onset. *Resuscitation* **2006**, *68*, 71–78. [CrossRef]

37. Jiang, J.; Chen, X.; Sun, L.; Qing, Y.; Yang, X.; Hu, X.; Yang, C.; Xu, T.; Wang, J.; Wang, P.; et al. Analysis of the concentrations and size distributions of cell-free DNA in schizophrenia using fluorescence correlation spectroscopy. *Transl. Psychiatry* **2018**, *8*, 104. [CrossRef]

38. Alapirtti, T.; Jylhava, J.; Raitanen, J.; Makinen, R.; Peltola, J.; Hurme, M.A.; Liimatainen, S. The concentration of cell-free DNA in video-EEG patients is dependent on the epilepsy syndrome and duration of epilepsy. *Neurol. Res.* **2016**, *38*, 45–50. [CrossRef]

39. Rule, K.N.; Chosed, R.J.; Chang, T.A.; Robinson, R.D.; Wininger, J.D.; Roudebush, W. Blastocoel cell-free DNA, a marker of embryonic quality. *Fertil. Steril.* **2017**, *108*, E106. [CrossRef]

40. Czamanski-Cohen, J.; Sarid, O.; Cwikel, J.; Lunenfeld, E.; Douvdevani, A.; Levitas, E.; Har-Vardi, I. Increased plasma cell-free DNA is associated with low pregnancy rates among women undergoing IVF-embryo transfer. *Reprod. Biomed. Online* **2013**, *26*, 36–41. [CrossRef]

41. Czamanski-Cohen, J.; Sarid, O.; Cwikel, J.; Levitas, E.; Lunenfeld, E.; Douvdevani, A.; Har-Vardi, I. Decrease in cell free DNA levels following participation in stress reduction techniques among women undergoing infertility treatment. *Arch. Womens Ment. Health* **2014**, *17*, 251–253. [CrossRef]

42. Li, H.G.; Huang, S.Y.; Zhou, H.; Liao, A.H.; Xiong, C.L. Quick recovery and characterization of cell-free DNA in seminal plasma of normozoospermia and azoospermia: Implications for non-invasive genetic utilities. *Asian J. Androl.* **2009**, *11*, 703–709. [CrossRef]

43. Boissiere, A.; Gala, A.; Ferrieres-Hoa, A.; Mullet, T.; Baillet, S.; Petiton, A.; Torre, A.; Hamamah, S. Cell-free and intracellular nucleic acids: New non-invasive biomarkers to explore male infertility. *Basic. Clin. Androl.* **2017**, *27*, 7. [CrossRef] [PubMed]

44. Davis, G.L., Jr.; Davis, J.S., IV. Detection of circulating DNA by counterimmunoelectrophoresis (CIE). *Arthritis Rheum. Off. J. Am. Coll. Rheumatol.* **1973**, *16*, 52–58. [CrossRef]

45. Hughes, G.R.; Cohen, S.A.; Lightfoot, R.W., Jr.; Meltzer, J.I.; Christian, C.L. The release of DNA into serum and synovial fluid. *Arthritis Rheum.* **1971**, *14*, 259–266. [CrossRef] [PubMed]

46. Atamaniuk, J.; Kopecky, C.; Skoupy, S.; Saemann, M.D.; Weichhart, T. Apoptotic cell-free DNA promotes inflammation in haemodialysis patients. *Nephrol. Dial. Transplant.* **2012**, *27*, 902–905. [CrossRef] [PubMed]

47. Tovbin, D.; Novack, V.; Wiessman, M.P.; Abd Elkadir, A.; Zlotnik, M.; Douvdevani, A. Circulating cell-free DNA in hemodialysis patients predicts mortality. *Nephrol. Dial. Transplant.* **2012**, *27*, 3929–3935. [CrossRef]

48. Wijeratne, S.; Butt, A.; Burns, S.; Sherwood, K.; Boyd, O.; Swaminathan, R. Cell-free plasma DNA as a prognostic marker in intensive treatment unit patients. *Ann. N. Y. Acad. Sci.* **2004**, *1022*, 232–238. [CrossRef] [PubMed]

49. Okkonen, M.; Lakkisto, P.; Korhonen, A.-M.; Parviai-nen, I.; Reinikainen, M.; Varpula, T.; Pettilä, V. Plasma cell-free DNA in patients needing mechanical ventilation. *Crit. Care* **2011**, *15*, R196. [CrossRef]

50. Jylhava, J.; Kotipelto, T.; Raitala, A.; Jylha, M.; Hervonen, A.; Hurme, M. Aging is associated with quantitative and qualitative changes in circulating cell-free DNA: The Vitality 90+ study. *Mech. Ageing Dev.* **2011**, *132*, 20–26. [CrossRef]

51. Teo, Y.V.; Capri, M.; Morsiani, C.; Pizza, G.; Faria, A.M.C.; Franceschi, C.; Neretti, N. Cell-free DNA as a biomarker of aging. *Aging Cell* **2019**, *18*, e12890. [CrossRef]

52. Haghiac, M.; Vora, N.L.; Basu, S.; Johnson, K.L.; Presley, L.; Bianchi, D.W.; Hauguel-de Mouzon, S. Increased death of adipose cells, a path to release cell-free DNA into systemic circulation of obese women. *Obes. Silver Spring* **2012**, *20*, 2213–2219. [CrossRef] [PubMed]

53. Livergood, M.C.; LeChien, K.A.; Trudell, A.S. Obesity and cell-free DNA "no calls": Is there an optimal gestational age at time of sampling? *Am. J. Obstet. Gynecol.* **2017**, *216*, 413.e1–413.e9. [CrossRef] [PubMed]

54. Fatouros, I.G.; Jamurtas, A.Z.; Nikolaidis, M.G.; Destouni, A.; Michailidis, Y.; Vrettou, C.; Douroudos, I.I.; Avloniti, A.; Chatziniko-laou, A.; Taxildaris, K. Time of sampling is crucial for measurement of cell-free plasma DNA following acute aseptic inflammation induced by exercise. *Clin. Biochem.* **2010**, *43*, 1368–1370. [CrossRef]

55. Madsen, A.T.; Hojbjerg, J.A.; Sorensen, B.S.; Winther-Larsen, A. Day-to-day and within-day biological variation of cell-free DNA. *EBioMedicine* **2019**, *49*, 284–290. [CrossRef]

56. Korabecna, M.; Horinek, A.; Bila, N.; Opatrna, S. *Circadian Rhythmicity and Clearance of Cell-Free DNA in Human Plasma*; Springer: Berlin/Heidelberg, Germany, 2010; pp. 195–198.

57. Meddeb, R.; Dache, Z.A.A.; Thezenas, S.; Otandault, A.; Tanos, R.; Pastor, B.; Sanchez, C.; Azzi, J.; Tousch, G.; Azan, S.; et al. Quantifying circulating cell-free DNA in humans. *Sci. Rep.* **2019**, *9*, 5220. [CrossRef]

58. Meddeb, R.; Pisareva, E.; Thierry, A.R. Guidelines for the Preanalytical Conditions for Analyzing Circulating Cell-Free DNA. *Clin. Chem.* **2019**, *65*, 623–633. [CrossRef]

59. Yuwono, N.L.; Warton, K.; Ford, C.E. The influence of biological and lifestyle factors on circulating cell-free DNA in blood plasma. *Elife* **2021**, *10*, e69679. [CrossRef]

60. Brodbeck, K.; Schick, S.; Bayer, B.; Anslinger, K.; Krüger, K.; Mayer, Z.; Holdenrieder, S.; Peldschus, S. Biological variability of cell-free DNA in healthy females at rest within a short time course. *Int. J. Leg. Med.* **2020**, *134*, 911–919. [CrossRef]

61. Breitbach, S.; Tug, S.; Helmig, S.; Zahn, D.; Kubiak, T.; Michal, M.; Gori, T.; Ehlert, T.; Beiter, T.; Simon, P. Direct quantification of cell-free, circulating DNA from unpurified plasma. *PLoS ONE* **2014**, *9*, e87838. [CrossRef]

62. Breitbach, S.; Sterzing, B.; Magallanes, C.; Tug, S.; Simon, P. Direct measurement of cell-free DNA from serially collected capillary plasma during incremental exercise. *J. Appl. Physiol.* **2014**, *117*, 119–130. [CrossRef]

63. Yuwono, N.L.; Henry, C.E.; Ford, C.E.; Warton, K. Total and endothelial cell-derived cell-free DNA in blood plasma does not change during menstruation. *PLoS ONE* **2021**, *16*, e0250561. [CrossRef] [PubMed]

64. Pölcher, M.; Ellinger, J.; Willems, S.; El-Maarri, O.; Höller, T.; Amann, C.; Wolfgarten, M.; Rudlowski, C.; Kuhn, W.; Braun, M. Impact of the menstrual cycle on circulating cell-free DNA. *Anticancer. Res.* **2010**, *30*, 2235–2240.
65. Schmidt, S.; Taenny, P.; Petry, J.; Haller, N.; Simon, P.; Helmig, S. Circulating, cell-free DNA as a marker for exercise load in intermittent sports. *PLoS ONE* **2018**, *13*, e0191915. [CrossRef]
66. Atamaniuk, J.; Vidotto, C.; Kinzlbauer, M.; Bachl, N.; Tiran, B.; Tschan, H. Cell-free plasma DNA and purine nucleotide degradation markers following weightlifting exercise. *Eur. J. Appl. Physiol.* **2010**, *110*, 695–701. [CrossRef]
67. Atamaniuk, J.; Stuhlmeier, K.M.; Vidotto, C.; Tschan, H.; Dossenbach-Glaninger, A.; Mueller, M.M. Effects of ultra-marathon on circulating DNA and mRNA expression of pro- and anti-apoptotic genes in mononuclear cells. *Eur. J. Appl. Physiol.* **2008**, *104*, 711–717. [CrossRef]
68. Beiter, T.; Fragasso, A.; Hudemann, J.; Nieß, A.M.; Simon, P. Short-term treadmill running as a model for studying cell-free DNA kinetics in vivo. *Clin. Chem.* **2011**, *57*, 633–636. [CrossRef]
69. Breitbach, S.; Tug, S.; Simon, P. Circulating cell-free DNA. *Sports Med.* **2012**, *42*, 565–586. [CrossRef]
70. Hsieh, C.-C.; Hsu, H.-S.; Chang, S.-C.; Chen, Y.-J. Circulating Cell-Free DNA Levels Could Predict Oncological Outcomes of Patients Undergoing Esophagectomy for Esophageal Squamous Cell Carcinoma. *Int. J. Mol. Sci.* **2016**, *17*, 2131. [CrossRef]
71. Kim, K.; Shin, D.G.; Park, M.K.; Baik, S.H.; Kim, T.H.; Kim, S.; Lee, S. Circulating cell-free DNA as a promising biomarker in patients with gastric cancer: Diagnostic validity and significant reduction of cfDNA after surgical resection. *Ann. Surg. Treat. Res.* **2014**, *86*, 136–142. [CrossRef]
72. Cayir, A.; Coskun, M.; Coskun, M.; Cobanoglu, H. DNA damage and circulating cell free DNA in greenhouse workers exposed to pesticides. *Environ. Mol. Mutagen.* **2018**, *59*, 161–169. [CrossRef]
73. Villalba-Campos, M.; Ramirez-Clavijo, S.R.; Sanchez-Corredor, M.C.; Rondon-Lagos, M.; Ibanez-Pinilla, M.; Palma, R.M.; Varona-Uribe, M.E.; Chuaire-Noack, L. Quantification of cell-free DNA for evaluating genotoxic damage from occupational exposure to car paints. *J. Occup. Med. Toxicol.* **2016**, *11*, 33. [CrossRef] [PubMed]
74. Korzeneva, I.B.; Kostuyk, S.V.; Ershova, L.S.; Osipov, A.N.; Zhuravleva, V.F.; Pankratova, G.V.; Porokhovnik, L.N.; Veiko, N.N. Human circulating plasma DNA significantly decreases while lymphocyte DNA damage increases under chronic occupational exposure to low-dose gamma-neutron and tritium β-radiation. *Mutat. Res. Fundam. Mol. Mech. Mutagen.* **2015**, *779*, 1–15. [CrossRef] [PubMed]
75. Yao, W.; Mei, C.; Nan, X.; Hui, L. Evaluation and comparison of in vitro degradation kinetics of DNA in serum, urine and saliva: A qualitative study. *Gene* **2016**, *590*, 142–148. [CrossRef]
76. Diehl, F.; Schmidt, K.; Choti, M.A.; Romans, K.; Goodman, S.; Li, M.; Thornton, K.; Agrawal, N.; Sokoll, L.; Szabo, S.A.; et al. Circulating mutant DNA to assess tumor dynamics. *Nat. Med.* **2008**, *14*, 985–990. [CrossRef]
77. To, E.W.; Chan, K.C.; Leung, S.F.; Chan, L.Y.; To, K.F.; Chan, A.T.; Johnson, P.J.; Lo, Y.M. Rapid clearance of plasma Epstein-Barr virus DNA after surgical treatment of nasopharyngeal carcinoma. *Clin. Cancer Res.* **2003**, *9*, 3254–3259.
78. Cook, R.T. Alcohol abuse, alcoholism, and damage to the immune system—A review. *Alcohol. Clin. Exp. Res.* **1998**, *22*, 1927–1942.
79. Jones, M.R.; Hall, O.M.; Kaye, A.M.; Kaye, A.D. Drug-induced acute pancreatitis: A review. *Ochsner J.* **2015**, *15*, 45–51.
80. Zhong, X.Y.; Hahn, S.; Kiefer, V.; Holzgreve, W. Is the quantity of circulatory cell-free DNA in human plasma and serum samples associated with gender, age and frequency of blood donations? *Ann. Hematol.* **2007**, *86*, 139–143. [CrossRef]
81. Heitzer, E.; Haque, I.S.; Roberts, C.E.S.; Speicher, M.R. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat. Rev. Genet.* **2019**, *20*, 71–88. [CrossRef]
82. Wan, J.C.M.; Massie, C.; Garcia-Corbacho, J.; Mouliere, F.; Brenton, J.D.; Caldas, C.; Pacey, S.; Baird, R.; Rosenfeld, N. Liquid biopsies come of age: Towards implementation of circulating tumour DNA. *Nat. Rev. Cancer* **2017**, *17*, 223–238. [CrossRef]
83. Holdenrieder, S.; Ungerer, V.; Oberhofer, A.; Bronkhorst, A.J. Pan-cancer screening by circulating tumor DNA (ctDNA)–recent breakthroughs and chronic pitfalls. *LaboratoriumsMedizin* **2022**. [CrossRef]
84. Pons-Belda, O.D.; Fernandez-Uriarte, A.; Diamandis, E.P. Can circulating tumor DNA support a successful screening test for early cancer detection? The Grail paradigm. *Diagnostics* **2021**, *11*, 2171. [CrossRef]
85. Klein, E.A.; Beer, T.M.; Seiden, M. The Promise of Multicancer Early Detection. Comment on Pons-Belda et al. Can Circulating Tumor DNA Support a Successful Screening Test for Early Cancer Detection? The Grail Paradigm. Diagnostics 2021, 11, 2171. *Diagnostics* **2022**, *12*, 1243. [CrossRef]
86. Devaney, S.A.; Palomaki, G.E.; Scott, J.A.; Bianchi, D.W. Noninvasive fetal sex determination using cell-free fetal DNA: A systematic review and meta-analysis. *JAMA* **2011**, *306*, 627–636. [CrossRef]
87. Benn, P.; Cuckle, H.; Pergament, E. Non-invasive prenatal testing for aneuploidy: Current status and future prospects. *Ultrasound Obstet. Gynecol.* **2013**, *42*, 15–33. [CrossRef]
88. Skrzypek, H.; Hui, L. Noninvasive prenatal testing for fetal aneuploidy and single gene disorders. *Best Pract. Res. Clin. Obstet. Gynaecol.* **2017**, *42*, 26–38. [CrossRef]
89. Allyse, M.; Minear, M.A.; Berson, E.; Sridhar, S.; Rote, M.; Hung, A.; Chandrasekharan, S. Non-invasive prenatal testing: A review of international implementation and challenges. *Int. J. Women's Health* **2015**, *7*, 113. [CrossRef]
90. Bloom, R.D.; Bromberg, J.S.; Poggio, E.D.; Bunnapradist, S.; Langone, A.J.; Sood, P.; Matas, A.J.; Mehta, S.; Mannon, R.B.; Sharfuddin, A. Cell-free DNA and active rejection in kidney allografts. *J. Am. Soc. Nephrol.* **2017**, *28*, 2221–2232. [CrossRef]

91. De Vlaminck, I.; Valantine, H.A.; Snyder, T.M.; Strehl, C.; Cohen, G.; Luikart, H.; Neff, N.F.; Okamoto, J.; Bernstein, D.; Weisshaar, D. Circulating cell-free DNA enables noninvasive diagnosis of heart transplant rejection. *Sci. Transl. Med.* **2014**, *6*, 241ra277. [CrossRef]

92. Moss, J.; Magenheim, J.; Neiman, D.; Zemmour, H.; Loyfer, N.; Korach, A.; Samet, Y.; Maoz, M.; Druid, H.; Arner, P.; et al. Comprehensive human cell-type methylation atlas reveals origins of circulating cell-free DNA in health and disease. *Nat. Commun.* **2018**, *9*, 5068. [CrossRef] [PubMed]

93. Schütz, E.; Fischer, A.; Beck, J.; Harden, M.; Koch, M.; Wuensch, T.; Stockmann, M.; Nashan, B.; Kollmar, O.; Matthaei, J. Graft-derived cell-free DNA, a noninvasive early rejection and graft damage marker in liver transplantation: A prospective, observational, multicenter cohort study. *PLoS Med.* **2017**, *14*, e1002286. [CrossRef]

94. Sharon, E.; Shi, H.; Kharbanda, S.; Koh, W.; Martin, L.R.; Khush, K.K.; Valantine, H.; Pritchard, J.K.A. Quantification of transplant-derived circulating cell-free DNA in absence of a donor genotype. *PLoS Comput. Biol.* **2017**, *13*, e1005629. [CrossRef] [PubMed]

95. Sigdel, T.K.; Archila, F.A.; Constantin, T.; Prins, S.A.; Liberto, J.; Damm, I.; Towfighi, P.; Navarro, S.; Kirkizlar, E.; Demko, Z.P. Optimizing detection of kidney transplant injury by assessment of donor-derived cell-free DNA via massively multiplex PCR. *J. Clin. Med.* **2019**, *8*, 19. [CrossRef] [PubMed]

96. Snyder, T.M.; Khush, K.K.; Valantine, H.A.; Quake, S.R. Universal noninvasive detection of solid organ transplant rejection. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 6229–6234. [CrossRef] [PubMed]

97. Yamamoto, M.; Ushio, R.; Watanabe, H.; Tachibana, T.; Tanaka, M.; Yokose, T.; Tsukiji, J.; Nakajima, H.; Kaneko, T. Detection of Mycobacterium tuberculosis-derived DNA in circulating cell-free DNA from a patient with disseminated infection using digital PCR. *Int. J. Infect. Dis.* **2018**, *66*, 80–82. [CrossRef] [PubMed]

98. Langelier, C.; Fung, M.; Caldera, S.; Deiss, T.; Lyden, A.; Prince, B.C.; Serpa, P.H.; Moazed, F.; Chin-Hong, P.; DeRisi, J.L. Detection of pneumonia pathogens from plasma cell-free DNA. *Am. J. Respir. Crit. Care Med.* **2020**, *201*, 491–495. [CrossRef]

99. Weerakoon, K.G.; McManus, D.P. Cell-Free DNA as a Diagnostic Tool for Human Parasitic Infections. *Trends. Parasitol.* **2016**, *32*, 378–391. [CrossRef]

100. Ullrich, E.; Heidinger, P.; Soh, J.; Villanova, L.; Grabuschnig, S.; Bachler, T.; Hirschböck, E.; Sanchez-Heredero, S.; Ford, B.; Sensen, M.; et al. Evaluation of host-based molecular markers for the early detection of human sepsis. *J. Biotechnol.* **2020**, *310*, 80–88. [CrossRef]

101. Chan, K.C.A.; Woo, J.K.S.; King, A.; Zee, B.C.Y.; Lam, W.K.J.; Chan, S.L.; Chu, S.W.I.; Mak, C.; Tse, I.O.L.; Leung, S.Y.M.; et al. Analysis of Plasma Epstein-Barr Virus DNA to Screen for Nasopharyngeal Cancer. *N. Engl. J. Med.* **2017**, *377*, 513–522. [CrossRef]

102. Lo, Y.D.; Chan, L.Y.; Lo, K.-W.; Leung, S.-F.; Zhang, J.; Chan, A.T.; Lee, J.C.; Hjelm, N.M.; Johnson, P.J.; Huang, D.P. Quantitative analysis of cell-free Epstein-Barr virus DNA in plasma of patients with nasopharyngeal carcinoma. *Cancer Res.* **1999**, *59*, 1188–1191.

103. Wuerdemann, N.; Jain, R.; Adams, A.; Speel, E.M.; Wagner, S.; Joosse, S.A.; Klussmann, J.P. Cell-Free HPV-DNA as a Biomarker for Oropharyngeal Squamous Cell Carcinoma-A Step Towards Personalized Medicine? *Cancers* **2020**, *12*, 2997. [CrossRef]

104. Hanna, G.J.; Supplee, J.G.; Kuang, Y.; Mahmood, U.; Lau, C.J.; Haddad, R.I.; Janne, P.A.; Paweletz, C.P. Plasma HPV cell-free DNA monitoring in advanced HPV-associated oropharyngeal cancer. *Ann. Oncol.* **2018**, *29*, 1980–1986. [CrossRef]

105. Kang, Z.; Stevanovic, S.; Hinrichs, C.S.; Cao, L. Circulating Cell-free DNA for Metastatic Cervical Cancer Detection, Genotyping, and Monitoring. *Clin. Cancer Res.* **2017**, *23*, 6856–6862. [CrossRef]

106. Burnham, P.; Kim, M.S.; Agbor-Enoh, S.; Luikart, H.; Valantine, H.A.; Khush, K.K.; De Vlaminck, I. Single-stranded DNA library preparation uncovers the origin and diversity of ultrashort cell-free DNA in plasma. *Sci. Rep.* **2016**, *6*, 27859. [CrossRef]

107. Chiu, K.-P.; Alice, L.Y. Application of cell-free DNA sequencing in characterization of bloodborne microbes and the study of microbe-disease interactions. *PeerJ* **2019**, *7*, e7426. [CrossRef]

108. Kowarsky, M.; Camunas-Soler, J.; Kertesz, M.A. Numerous uncharacterized and highly divergent microbes which colonize humans are revealed by circulating cell-free DNA. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 9623–9628. [CrossRef]

109. Spisák, S.; Solymosi, N.; Ittzés, P.; Bodor, A.; Kondor, D.; Vattay, G.; Barták, B.K.; Sipos, F.; Galamb, O.; Tulassay, Z. Complete genes may pass from food to human blood. *PLoS ONE* **2013**, *8*, e69805. [CrossRef]

110. Woegerbauer, M.; Bellanger, X.; Merlin, C. Cell-Free DNA: An Underestimated Source of Antibiotic Resistance Gene Dissemination at the Interface Between Human Activities and Downstream Environments in the Context of Wastewater Reuse. *Front. Microbiol.* **2020**, *11*, 671. [CrossRef]

111. Raghuram, G.V.; Gupta, D.; Subramaniam, S.; Gaikwad, A.; Khare, N.K.; Nobre, M.; Nair, N.K.; Mittra, I. Physical shearing imparts biological activity to DNA and ability to transmit itself horizontally across species and kingdom boundaries. *BMC Mol. Biol.* **2017**, *18*, 21. [CrossRef] [PubMed]

112. Basak, R.; Nair, N.K.; Mittra, I. Evidence for cell-free nucleic acids as continuously arising endogenous DNA mutagens. *Mutat. Res./Fundam. Mol. Mech. Mutagen.* **2016**, *793*, 15–21. [CrossRef]

113. Mittra, I.; Khare, N.K.; Raghuram, G.V.; Chaubal, R.; Khambatti, F.; Gupta, D.; Gaikwad, A.; Prasannan, P.; Singh, A.; Iyer, A.; et al. Circulating nucleic acids damage DNA of healthy cells by integrating into their genomes. *J. Biosci.* **2015**, *40*, 91–111. [CrossRef] [PubMed]

114. Mittra, I.; Samant, U.; Sharma, S.; Raghuram, G.V.; Saha, T.; Tidke, P.; Pancholi, N.; Gupta, D.; Prasannan, P.; Gaikwad, A.; et al. Cell-free chromatin from dying cancer cells integrate into genomes of bystander healthy cells to induce DNA damage and inflammation. *Cell Death Discov.* **2017**, *3*, 17015. [CrossRef] [PubMed]

115. Albano, M.S.; Scaradavou, A.; Stevens, C.E.; Rubinstein, P. Extracellular DNA in cord blood plasma and applications in cord blood banking for sample identification. *Transfusion* **2009**, *49*, 1685–1691. [CrossRef]

116. Quinones, I.; Daniel, B. Cell free DNA as a component of forensic evidence recovered from touched surfaces. *Forensic Sci. Int. Genet.* **2012**, *6*, 26–30. [CrossRef]

117. Vandewoestyne, M.; Van Hoofstat, D.; Franssen, A.; Van Nieuwerburgh, F.; Deforce, D. Presence and potential of cell free DNA in different types of forensic samples. *Forensic Sci. Int. Genet.* **2013**, *7*, 316–320. [CrossRef]

118. Sewell, J.; Quinones, I.; Ames, C.; Multaney, B.; Curtis, S.; Seeboruth, H.; Moore, S.; Daniel, B. Recovery of DNA and fingerprints from touched documents. *Forensic Sci. Int. Genet.* **2008**, *2*, 281–285. [CrossRef]

119. Kita, T.; Yamaguchi, H.; Yokoyama, M.; Tanaka, T.; Tanaka, N. Morphological study of fragmented DNA on touched objects. *Forensic Sci. Int. Genet.* **2008**, *3*, 32–36. [CrossRef]

120. Kleinman, S.; Strong, D.; Tegtmeier, G.; Holland, P.; Gorlin, J.; Cousins, C.; Chiacchierini, R.; Pietrelli, L. Hepatitis B virus (HBV) DNA screening of blood donations in minipools with the COBAS AmpliScreen HBV test. *Transfusion* **2005**, *45*, 1247–1257. [CrossRef]

121. Yang, L.; Yang, D.; Yang, Q.; Cheng, F.; Huang, Y. Extracellular DNA in blood products and its potential effects on transfusion. *Biosci. Rep.* **2020**, *40*, BSR20192770. [CrossRef]

122. Greytak, S.R.; Engel, K.B.; Parpart-Li, S.; Murtaza, M.; Bronkhorst, A.J.; Pertile, M.D.; Moore, H.M. Harmonizing cell-free DNA collection and processing practices through evidence-based guidance. *Clin. Cancer Res.* **2020**, *26*, 3104–3109. [CrossRef]

123. Devonshire, A.S.; Whale, A.S.; Gutteridge, A.; Jones, G.; Cowen, S.; Foy, C.A.; Huggett, J.F. Towards standardisation of cell-free DNA measurement in plasma: Controls for extraction efficiency, fragment size bias and quantification. *Anal. Bioanal. Chem.* **2014**, *406*, 6499–6512. [CrossRef]

124. Tsui, D.W.; Barnett, E.; Scher, H.I. Toward Standardization of Preanalytical Procedures for Cell-Free DNA Profiling. *Clin. Chem.* **2019**, *66*, 3–5. [CrossRef]

125. Cavallone, L.; Aldamry, M.; Lafleur, J.; Lan, C.; Ginestet, P.G.; Alirezaie, N.; Ferrario, C.; Aguilar-Mahecha, A.; Basik, M. A study of pre-analytical variables and optimization of extraction method for circulating tumor DNA measurements by digital droplet PCR. *Cancer Epidemiol. Biomark. Prev.* **2019**, *28*, 909–916. [CrossRef]

126. Lampignano, R.; Neumann, M.H.D.; Weber, S.; Kloten, V.; Herdean, A.; Voss, T.; Groelz, D.; Babayan, A.; Tibbesma, M.; Schlumpberger, M.; et al. Multicenter Evaluation of Circulating Cell-Free DNA Extraction and Downstream Analyses for the Development of Standardized (Pre)analytical Work Flows. *Clin. Chem.* **2020**, *66*, 149–160. [CrossRef]

127. Bronkhorst, A.J.; Aucamp, J.; Pretorius, P.J. Cell-free DNA: Preanalytical variables. *Clin. Chim. Acta* **2015**, *450*, 243–253. [CrossRef]

128. Johansson, G.; Andersson, D.; Filges, S.; Li, J.; Muth, A.; Godfrey, T.E.; Ståhlberg, A. Considerations and quality controls when analyzing cell-free tumor DNA. *Biomol. Detect. Quantif.* **2019**, *17*, 100078. [CrossRef]

129. Nikolaev, S.; Lemmens, L.; Koessler, T.; Blouin, J.L.; Nouspikel, T. Circulating tumoral DNA: Preanalytical validation and quality control in a diagnostic laboratory. *Anal. Biochem.* **2018**, *542*, 34–39. [CrossRef]

130. Bronkhorst, A.J.; Ungerer, V.; Oberhofer, A.; Holdenrieder, S. The rising tide of cell-free DNA profiling: From snapshot to temporal genome analysis. *LaboratoriumsMedizin* **2022**. [CrossRef]

131. Hu, Y.; Ulrich, B.C.; Supplee, J.; Kuang, Y.; Lizotte, P.H.; Feeney, N.B.; Guibert, N.M.; Awad, M.M.; Wong, K.K.; Janne, P.A.; et al. False-Positive Plasma Genotyping Due to Clonal Hematopoiesis. *Clin. Cancer Res.* **2018**, *24*, 4437–4443. [CrossRef] [PubMed]

132. Anglesio, M.S.; Papadopoulos, N.; Ayhan, A.; Nazeran, T.M.; Noe, M.; Horlings, H.M.; Lum, A.; Jones, S.; Senz, J.; Seckin, T.; et al. Cancer-Associated Mutations in Endometriosis without Cancer. *N. Engl. J. Med.* **2017**, *376*, 1835–1848. [CrossRef] [PubMed]

133. Shaw, J.A.; Guttery, D.S.; Hills, A.; Fernandez-Garcia, D.; Page, K.; Rosales, B.M.; Goddard, K.S.; Hastings, R.K.; Luo, J.; Ogle, O.; et al. Mutation analysis of cell-free DNA and single circulating tumor cells in metastatic breast cancer patients with high circulating tumor cell counts. *Clin. Cancer Res.* **2017**, *23*, 88–96. [CrossRef] [PubMed]

134. Genovese, G.; Kahler, A.K.; Handsaker, R.E.; Lindberg, J.; Rose, S.A.; Bakhoum, S.F.; Chambert, K.; Mick, E.; Neale, B.M.; Fromer, M.; et al. Clonal hematopoiesis and blood-cancer risk inferred from blood DNA sequence. *N. Engl. J. Med.* **2014**, *371*, 2477–2487. [CrossRef] [PubMed]

135. Gormally, E.; Vineis, P.; Matullo, G.; Veglia, F.; Caboux, E.; Le Roux, E.; Peluso, M.; Garte, S.; Guarrera, S.; Munnia, A.; et al. TP53 and KRAS2 mutations in plasma DNA of healthy subjects and subsequent cancer occurrence: A prospective study. *Cancer Res.* **2006**, *66*, 6871–6876. [CrossRef] [PubMed]

136. Marass, F.; Stephens, D.; Ptashkin, R.; Zehir, A.; Berger, M.F.; Solit, D.B.; Diaz, L.A., Jr.; Tsui, D.W. Fragment Size Analysis May Distinguish Clonal Hematopoiesis from Tumor-Derived Mutations in Cell-Free DNA. *Clin. Chem.* **2020**, *66*, 616–618. [CrossRef]

137. Lo, Y.D.; Han, D.S.; Jiang, P.; Chiu, R.W. Epigenetics, fragmentomics, and topology of cell-free DNA in liquid biopsies. *Science* **2021**, *372*, eaaw3616. [CrossRef]

138. Ding, S.C.; Lo, Y.D. Cell-Free DNA Fragmentomics in Liquid Biopsy. *Diagnostics* **2022**, *12*, 978. [CrossRef]

139. Gezer, U.; Bronkhorst, A.J.; Holdenrieder, S. The Utility of Repetitive Cell-Free DNA in Cancer Liquid Biopsies. *Diagnostics* **2022**, *12*, 1363. [CrossRef]

140. Van der Pol, Y.; Mouliere, F. Toward the Early Detection of Cancer by Decoding the Epigenetic and Environmental Fingerprints of Cell-Free DNA. *Cancer Cell* **2019**, *36*, 350–368. [CrossRef]

141. Lamb, Y.N.; Dhillon, S. Epi proColon® 2.0 CE: A blood-based screening test for colorectal cancer. *Mol. Diagn. Ther.* **2017**, *21*, 225–232. [CrossRef]

142. Cheng, N.; Skead, K.; Ouellette, T.; Bratman, S.; De Carvalho, D.; Soave, D.; Awadalla, P. Early signatures of breast cancer up to seven years prior to clinical diagnosis in plasma cell-free DNA methylomes. *Res. Sq.* **2022**. [CrossRef]

143. Chan, K.C.; Jiang, P.; Chan, C.W.; Sun, K.; Wong, J.; Hui, E.P.; Chan, S.L.; Chan, W.C.; Hui, D.S.; Ng, S.S.; et al. Noninvasive detection of cancer-associated genome-wide hypomethylation and copy number aberrations by plasma DNA bisulfite sequencing. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 18761–18768. [CrossRef]

144. Hulbert, A.; Jusue-Torres, I.; Stark, A.; Chen, C.; Rodgers, K.; Lee, B.; Griffin, C.; Yang, A.; Huang, P.; Wrangle, J.; et al. Early detection of lung cancer using DNA promoter hypermethylation in plasma and sputum. *Clin. Cancer Res.* **2017**, *23*, 1998–2005. [CrossRef]

145. Zeng, H.; He, B.; Yi, C.; Peng, J. Liquid biopsies: DNA methylation analyses in circulating cell-free DNA. *J. Genet. Genom.* **2018**, *45*, 185–192. [CrossRef]

146. Lubotzky, A.; Zemmour, H.; Neiman, D.; Gotkine, M.; Loyfer, N.; Piyanzin, S.; Ochana, B.L.; Lehmann-Werman, R.; Cohen, D.; Moss, J.; et al. Liquid biopsy reveals collateral tissue damage in cancer. *JCI Insight* **2022**, *7*, e153559. [CrossRef]

147. Korshunova, Y.; Maloney, R.K.; Lakey, N.; Citek, R.W.; Bacher, B.; Budiman, A.; Ordway, J.M.; McCombie, W.R.; Leon, J.; Jeddeloh, J.A.; et al. Massively parallel bisulphite pyrosequencing reveals the molecular complexity of breast cancer-associated cytosine-methylation patterns obtained from tissue and serum DNA. *Genome Res.* **2008**, *18*, 19–29. [CrossRef]

148. Shen, S.Y.; Singhania, R.; Fehringer, G.; Chakravarthy, A.; Roehrl, M.H.A.; Chadwick, D.; Zuzarte, P.C.; Borgida, A.; Wang, T.T.; Li, T.; et al. Sensitive tumour detection and classification using plasma cell-free DNA methylomes. *Nature* **2018**, *563*, 579–583. [CrossRef]

149. Brock, M.V.; Hooker, C.M.; Ota-Machida, E.; Han, Y.; Guo, M.; Ames, S.; Glöckner, S.; Piantadosi, S.; Gabrielson, E.; Pridham, G. DNA methylation markers and early recurrence in stage I lung cancer. *N. Engl. J. Med.* **2008**, *358*, 1118–1128. [CrossRef]

150. Gezer, U.; Üstek, D.; Yörüker, E.E.; Cakiris, A.; Abaci, N.; Leszinski, G.; Dalay, N.; Holdenrieder, S. Characterization of H3K9me3- and H4K20me3-associated circulating nucleosomal DNA by high-throughput sequencing in colorectal cancer. *Tumour Biol.* **2013**, *34*, 329–336. [CrossRef]

151. Gezer, U.; Yörüker, E.E.; Keskin, M.; Kulle, C.B.; Dharuman, Y.; Holdenrieder, S. Histone methylation marks on circulating nucleosomes as novel blood-based biomarker in colorectal cancer. *Int. J. Mol. Sci.* **2015**, *16*, 29654–29662. [CrossRef]

152. Kneip, C.; Schmidt, B.; Seegebarth, A.; Weickmann, S.; Fleischhacker, M.; Liebenberg, V.; Field, J.K.; Dietrich, D. SHOX2 DNA methylation is a biomarker for the diagnosis of lung cancer in plasma. *J. Thorac. Oncol.* **2011**, *6*, 1632–1638. [CrossRef]

153. Li, M.; Chen, W.-D.; Papadopoulos, N.; Goodman, S.N.; Bjerregaard, N.C.; Laurberg, S.; Levin, B.; Juhl, H.; Arber, N.; Moinova, H. Sensitive digital quantification of DNA methylation in clinical samples. *Nat. Biotechnol.* **2009**, *27*, 858. [CrossRef] [PubMed]

154. Song, C.X.; Yin, S.; Ma, L.; Wheeler, A.; Chen, Y.; Zhang, Y.; Liu, B.; Xiong, J.; Zhang, W.; Hu, J.; et al. 5-Hydroxymethylcytosine signatures in cell-free DNA provide information about tumor types and stages. *Cell Res.* **2017**, *27*, 1231–1242. [CrossRef]

155. Li, W.; Zhang, X.; Lu, X.; You, L.; Song, Y.; Luo, Z.; Zhang, J.; Nie, J.; Zheng, W.; Xu, D. 5-Hydroxymethylcytosine signatures in circulating cell-free DNA as diagnostic biomarkers for human cancers. *Cell Res.* **2017**, *27*, 1243–1257. [CrossRef] [PubMed]

156. McAnena, P.; Brown, J.A.; Kerin, M.J. Circulating nucleosomes and nucleosome modifications as biomarkers in cancer. *Cancers* **2017**, *9*, 5. [CrossRef] [PubMed]

157. Müller, H.M.; Widschwendter, A.; Fiegl, H.; Ivarsson, L.; Goebel, G.; Perkmann, E.; Marth, C.; Widschwendter, M. DNA methylation in serum of breast cancer patients: An independent prognostic marker. *Cancer Res.* **2003**, *63*, 7641–7645.

158. Nassiri, F.; Chakravarthy, A.; Feng, S.; Shen, S.Y.; Nejad, R.; Zuccato, J.A.; Voisin, M.R.; Patil, V.; Horbinski, C.; Aldape, K. Detection and discrimination of intracranial tumors using plasma cell-free DNA methylomes. *Nat. Med.* **2020**, *26*, 1044–1047. [CrossRef]

159. Schröck, A.; Leisse, A.; de Vos, L.; Gevensleben, H.; Dröge, F.; Franzen, A.; Wachendörfer, M.; Schröck, F.; Ellinger, J.; Teschke, M. Free-circulating methylated DNA in blood for diagnosis, staging, prognosis, and monitoring of head and neck squamous cell carcinoma patients: An observational prospective cohort study. *Clin. Chem.* **2017**, *63*, 1288–1296. [CrossRef]

160. Thålin, C.; Lundström, S.; Seignez, C.; Daleskog, M.; Lundström, A.; Henriksson, P.; Helleday, T.; Phillipson, M.; Wallén, H.; Demers, M. Citrullinated histone H3 as a novel prognostic blood marker in patients with advanced cancer. *PLoS ONE* **2018**, *13*, e0191231. [CrossRef]

161. Xu, R.H.; Wei, W.; Krawczyk, M.; Wang, W.; Luo, H.; Flagg, K.; Yi, S.; Shi, W.; Quan, Q.; Li, K.; et al. Circulating tumour DNA methylation markers for diagnosis and prognosis of hepatocellular carcinoma. *Nat. Mater.* **2017**, *16*, 1155–1161. [CrossRef]

162. De Castro Cuadrat, R.R.; Kratzer, A.; Arnal, H.G.; Wreczycka, K.; Blume, A.; Ebenal, V.; Mauno, T.; Osberg, B.; Moobed, M.; Hartung, J. Cardiovascular disease biomarkers derived from circulating cell-free DNA methylation. *medRxiv* **2021**. [CrossRef]

163. Hardy, T.; Zeybel, M.; Day, C.P.; Dipper, C.; Masson, S.; McPherson, S.; Henderson, E.; Tiniakos, D.; White, S.; French, J.; et al. Plasma DNA methylation: A potential biomarker for stratification of liver fibrosis in non-alcoholic fatty liver disease. *Gut* **2017**, *66*, 1321–1328. [CrossRef]

164. Lehmann-Werman, R.; Magenheim, J.; Moss, J.; Neiman, D.; Abraham, O.; Piyanzin, S.; Zemmour, H.; Fox, I.; Dor, T.; Grompe, M.; et al. Monitoring liver damage using hepatocyte-specific methylation markers in cell-free circulating DNA. *JCI Insight* **2018**, *3*, e120687. [CrossRef]

165. Akirav, E.M.; Lebastchi, J.; Galvan, E.M.; Henegariu, O.; Akirav, M.; Ablamunits, V.; Lizardi, P.M.; Herold, K.C. Detection of β cell death in diabetes using differentially methylated circulating DNA. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 19018–19023. [CrossRef] [PubMed]

166. Zhang, K.; Lin, G.; Han, Y.; Xie, J.; Li, J. Circulating unmethylated insulin DNA as a potential non-invasive biomarker of beta cell death in type 1 Diabetes: A review and future prospect. *Clin. Epigenetics* **2017**, *9*, 44. [CrossRef]

167. Dunaeva, M.; Derksen, M.; Pruijn, G.J. LINE-1 hypermethylation in serum cell-free DNA of relapsing remitting multiple sclerosis patients. *Mol. Neurobiol.* **2018**, *55*, 4681–4688. [CrossRef]

168. Hummel, E.; Hessas, E.; Müller, S.; Beiter, T.; Fisch, M.; Eibl, A.; Wolf, O.T.; Giebel, B.; Platen, P.; Kumsta, R. Cell-free DNA release under psychosocial and physical stress conditions. *Transl. Psychiatry* **2018**, *8*, 236. [CrossRef]

169. Lubotzky, A.; Pelov, I.; Teplitz, R.; Neiman, D.; Smadja, A.; Zemmour, H.; Piyanzin, S.; Ochana, B.L.; Spalding, K.L.; Glaser, B. Elevated brain-derived cell-free DNA among patients with first psychotic episode-a proof-of-concept study. *eLife* **2022**, *11*, e76391. [CrossRef]

170. Oberhofer, A.; Bronkhorst, A.J.; Ungerer, V.; Holdenrieder, S. Profiling disease and tissue-specific epigenetic signatures in cell-free DNA. *LaboratoriumsMedizin* **2022**. [CrossRef]

171. Oberhofer, A.; Bronkhorst, A.J.; Uhlig, C.; Ungerer, V.; Holdenrieder, S. Tracing the Origin of Cell-Free DNA Molecules through Tissue-Specific Epigenetic Signatures. *Diagnostics* **2022**, *12*, 1834. [CrossRef]

172. Sun, K.; Jiang, P.; Chan, K.C.; Wong, J.; Cheng, Y.K.; Liang, R.H.; Chan, W.K.; Ma, E.S.; Chan, S.L.; Cheng, S.H.; et al. Plasma DNA tissue mapping by genome-wide methylation sequencing for noninvasive prenatal, cancer, and transplantation assessments. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E5503–E5512. [CrossRef]

173. Lehmann-Werman, R.; Neiman, D.; Zemmour, H.; Moss, J.; Magenheim, J.; Vaknin-Dembinsky, A.; Rubertsson, S.; Nellgard, B.; Blennow, K.; Zetterberg, H.; et al. Identification of tissue-specific cell death using methylation patterns of circulating DNA. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, E1826–E1834. [CrossRef] [PubMed]

174. Guo, S.; Diep, D.; Plongthongkum, N.; Fung, H.L.; Zhang, K.; Zhang, K. Identification of methylation haplotype blocks aids in deconvolution of heterogeneous tissue samples and tumor tissue-of-origin mapping from plasma DNA. *Nat. Genet.* **2017**, *49*, 635–642. [CrossRef] [PubMed]

175. Loyfer, N.; Magenheim, J.; Peretz, A.; Cann, G.; Bredno, J.; Klochendler, A.; Fox-Fisher, I.; Shabi-Porat, S.; Hecht, M.; Pelet, T.; et al. A human DNA methylation atlas reveals principles of cell type-specific methylation and identifies thousands of cell type-specific regulatory elements. *bioRxiv* **2022**. [CrossRef]

176. Klein, E.A.; Richards, D.; Cohn, A.; Tummala, M.; Lapham, R.; Cosgrove, D.; Chung, G.; Clement, J.; Gao, J.; Hunkapiller, N.; et al. Clinical validation of a targeted methylation-based multi-cancer early detection test using an independent validation set. *Ann. Oncol.* **2021**, *32*, 1167–1177. [CrossRef]

177. Chen, X.; Dong, Z.; Hubbell, E.; Kurtzman, K.N.; Oxnard, G.R.; Venn, O.; Melton, C.; Clarke, C.A.; Shaknovich, R.; Ma, T.; et al. Prognostic Significance of Blood-Based Multi-cancer Detection in Plasma Cell-Free DNA. *Clin. Cancer Res.* **2021**, *27*, 4221–4229. [CrossRef]

178. Liu, M.C.; Oxnard, G.R.; Klein, E.A.; Swanton, C.; Seiden, M.V.; Consortium, C. Sensitive and specific multi-cancer detection and localization using methylation signatures in cell-free DNA. *Ann. Oncol.* **2020**, *31*, 745–759. [CrossRef]

179. Snyder, M.W.; Kircher, M.; Hill, A.J.; Daza, R.M.; Shendure, J. Cell-free DNA Comprises an In Vivo Nucleosome Footprint that Informs Its Tissues-Of-Origin. *Cell* **2016**, *164*, 57–68. [CrossRef]

180. Ulz, P.; Thallinger, G.G.; Auer, M.; Graf, R.; Kashofer, K.; Jahn, S.W.; Abete, L.; Pristauz, G.; Petru, E.; Geigl, J.B.; et al. Inferring expressed genes by whole-genome sequencing of plasma DNA. *Nat. Genet.* **2016**, *48*, 1273–1278. [CrossRef]

181. Ulz, P.; Perakis, S.; Zhou, Q.; Moser, T.; Belic, J.; Lazzeri, I.; Wolfler, A.; Zebisch, A.; Gerger, A.; Pristauz, G.; et al. Inference of transcription factor binding from cell-free DNA enables tumor subtype prediction and early detection. *Nat. Commun.* **2019**, *10*, 4666. [CrossRef]

182. Sadeh, R.; Sharkia, I.; Fialkoff, G.; Rahat, A.; Gutin, J.; Chappleboim, A.; Nitzan, M.; Fox-Fisher, I.; Neiman, D.; Meler, G.; et al. ChIP-seq of plasma cell-free nucleosomes identifies gene expression programs of the cells of origin. *Nat. Biotechnol.* **2021**, *39*, 586–598. [CrossRef]

183. Månsson, C.T.; Vad-Nielsen, J.; Meldgaard, P.; Nielsen, A.L.; Sorensen, B.S. EGFR transcription in non-small-cell lung cancer tumours can be revealed in ctDNA by cell-free chromatin immunoprecipitation (cfChIP). *Mol. Oncol.* **2021**, *15*, 2868–2876. [CrossRef] [PubMed]

184. Mouliere, F.; Chandrananda, D.; Piskorz, A.M.; Moore, E.K.; Morris, J.; Ahlborn, L.B.; Mair, R.; Goranova, T.; Marass, F.; Heider, K.; et al. Enhanced detection of circulating tumor DNA by fragment size analysis. *Sci. Transl. Med.* **2018**, *10*, eaat4921. [CrossRef] [PubMed]

185. Cristiano, S.; Leal, A.; Phallen, J.; Fiksel, J.; Adleff, V.; Bruhm, D.C.; Jensen, S.O.; Medina, J.E.; Hruban, C.; White, J.R.; et al. Genome-wide cell-free DNA fragmentation in patients with cancer. *Nature* **2019**, *570*, 385–389. [CrossRef] [PubMed]

186. Esfahani, M.S.; Hamilton, E.G.; Mehrmohamadi, M.; Nabet, B.Y.; Alig, S.K.; King, D.A.; Steen, C.B.; Macaulay, C.W.; Schultz, A.; Nesselbush, M.C.; et al. Inferring gene expression from cell-free DNA fragmentation profiles. *Nat. Biotechnol.* **2022**, *40*, 585–597. [CrossRef]

187. Sun, K.; Jiang, P.; Cheng, S.H.; Cheng, T.H.T.; Wong, J.; Wong, V.W.S.; Ng, S.S.M.; Ma, B.B.Y.; Leung, T.Y.; Chan, S.L.; et al. Orientation-aware plasma cell-free DNA fragmentation analysis in open chromatin regions informs tissue of origin. *Genome Res.* **2019**, *29*, 418–427. [CrossRef]

188. Jiang, P.; Sun, K.; Peng, W.; Cheng, S.H.; Ni, M.; Yeung, P.C.; Heung, M.M.S.; Xie, T.; Shang, H.; Zhou, Z.; et al. Plasma DNA End-Motif Profiling as a Fragmentomic Marker in Cancer, Pregnancy, and Transplantation. *Cancer Discov.* **2020**, *10*, 664–673. [CrossRef]

189. Moldovan, N.; van der Pol, Y.; van den Ende, T.; Boers, D.; Verkuijlen, S.; Creemers, A.; Ramaker, J.; Vu, T.; Fransen, M.F.; Pegtel, M.; et al. Genome-wide cell-free DNA termini in patients with cancer. *medRxiv* **2021**. [CrossRef]

190. Oka, T.; Hikoso, S.; Yamaguchi, O.; Taneike, M.; Takeda, T.; Tamai, T.; Oyabu, J.; Murakawa, T.; Nakayama, H.; Nishida, K. Mitochondrial DNA that escapes from autophagy causes inflammation and heart failure. *Nature* **2012**, *485*, 251–255. [CrossRef]

191. Nunnari, J.; Suomalainen, A. Mitochondria: In sickness and in health. *Cell* **2012**, *148*, 1145–1159. [CrossRef]

192. West, A.P.; Shadel, G.S.; Ghosh, S. Mitochondria in innate immune responses. *Nat. Rev. Immunol.* **2011**, *11*, 389–402. [CrossRef]

193. Zhong, S.; Ng, M.C.; Lo, Y.M.; Chan, J.C.; Johnson, P.J. Presence of mitochondrial tRNA(Leu(UUR)) A to G 3243 mutation in DNA extracted from serum and plasma of patients with type 2 diabetes mellitus. *J. Clin. Pathol.* **2000**, *53*, 466–469. [CrossRef]

194. Boyapati, R.K.; Tamborska, A.; Dorward, D.A.; Ho, G.T. Advances in the understanding of mitochondrial DNA as a pathogenic factor in inflammatory diseases. *F1000Res* **2017**, *6*, 169. [CrossRef]

195. Yu, M. Circulating cell-free mitochondrial DNA as a novel cancer biomarker: Opportunities and challenges. *Mitochondrial DNA* **2012**, *23*, 329–332. [CrossRef]

196. An, Q.; Hu, Y.; Li, Q.; Chen, X.; Huang, J.; Pellegrini, M.; Zhou, X.J.; Rettig, M.; Fan, G. The size of cell-free mitochondrial DNA in blood is inversely correlated with tumor burden in cancer patients. *Precis. Clin. Med.* **2019**, *2*, 131–139. [CrossRef] [PubMed]

197. Randeu, H.; Bronkhorst, A.J.; Mayer, Z.; Oberhofer, A.; Polatoglou, E.; Heinemann, V.; Haas, M.; Boeck, S.; Holdenrieder, S. Preanalytical Variables in the Analysis of Mitochondrial DNA in Whole Blood and Plasma from Pancreatic Cancer Patients. *Diagnostics* **2022**, *12*, 1905. [CrossRef]

198. Kohler, C.; Radpour, R.; Barekati, Z.; Asadollahi, R.; Bitzer, J.; Wight, E.; Bürki, N.; Diesch, C.; Holzgreve, W.; Zhong, X.Y. Levels of plasma circulating cell free nuclear and mitochondrial DNA as potential biomarkers for breast tumors. *Mol. Cancer* **2009**, *8*, 105. [CrossRef]

199. Huang, C.Y.; Chen, Y.M.; Wu, C.H.; Tsai, C.M.; Lee, Y.C.; Perng, R.P.; Whang-Peng, J. Circulating free mitochondrial DNA concentration and its association with erlotinib treatment in patients with adenocarcinoma of the lung. *Oncol. Lett.* **2014**, *7*, 2180–2184. [CrossRef]

200. Uzawa, K.; Baba, T.; Uchida, F.; Yamatoji, M.; Kasamatsu, A.; Sakamoto, Y.; Ogawara, K.; Shiiba, M.; Bukawa, H.; Tanzawa, H. Circulating tumor-derived mutant mitochondrial DNA: A predictive biomarker of clinical prognosis in human squamous cell carcinoma. *Oncotarget* **2012**, *3*, 670. [CrossRef]

201. Kumar, M.; Srivastava, S.; Singh, S.A.; Das, A.K.; Das, G.C.; Dhar, B.; Ghosh, S.K.; Mondal, R. Cell-free mitochondrial DNA copy number variation in head and neck squamous cell carcinoma: A study of non-invasive biomarker from Northeast India. *Tumour Biol.* **2017**, *39*, 1010428317736643. [CrossRef]

202. Yu, M.; Wan, Y.-F.; Zou, Q.-H. Cell-free circulating mitochondrial DNA in the serum: A potential non-invasive biomarker for Ewing's sarcoma. *Arch. Med. Res.* **2012**, *43*, 389–394. [CrossRef]

203. Ellinger, J.; Müller, D.C.; Müller, S.C.; Hauser, S.; Heukamp, L.C.; von Ruecker, A.; Bastian, P.J.; Walgenbach-Brunagel, G. Circulating mitochondrial DNA in serum: A universal diagnostic biomarker for patients with urological malignancies. *Urol. Oncol. Semin. Orig. Investig.* **2012**, *30*, 509–515. [CrossRef] [PubMed]

204. Kandel, E.S. Mutations in circulating mitochondrial DNA: Cassandra of oral cancer? *Oncotarget* **2012**, *3*, 664. [CrossRef] [PubMed]

205. Lowes, H.; Kurzawa-Akanbi, M.; Pyle, A.; Hudson, G. Post-mortem ventricular cerebrospinal fluid cell-free-mtDNA in neurode-generative disease. *Sci. Rep.* **2020**, *10*, 15253. [CrossRef] [PubMed]

206. Dantham, S.; Srivastava, A.K.; Gulati, S.; Rajeswari, M.R. Plasma circulating cell-free mitochondrial DNA in the assessment of Friedreich's ataxia. *J. Neurol. Sci.* **2016**, *365*, 82–88. [CrossRef]

207. Varhaug, K.N.; Vedeler, C.A.; Myhr, K.-M.; Aarseth, J.H.; Tzoulis, C.; Bindoff, L.A. Increased levels of cell-free mitochondrial DNA in the cerebrospinal fluid of patients with multiple sclerosis. *Mitochondrion* **2017**, *34*, 32–35. [CrossRef]

208. Lowes, H.; Pyle, A.; Santibanez-Koref, M.; Hudson, G. Circulating cell-free mitochondrial DNA levels in Parkinson's disease are influenced by treatment. *Mol. Neurodegener.* **2020**, *15*, 10. [CrossRef]

209. Silzer, T.; Barber, R.; Sun, J.; Pathak, G.; Johnson, L.; O'Bryant, S.; Phillips, N. Circulating mitochondrial DNA: New indices of type 2 diabetes-related cognitive impairment in Mexican Americans. *PLoS ONE* **2019**, *14*, e0213527. [CrossRef]

210. Pinti, M.; Cevenini, E.; Nasi, M.; De Biasi, S.; Salvioli, S.; Monti, D.; Benatti, S.; Gibellini, L.; Cotichini, R.; Stazi, M.A. Circulating mitochondrial DNA increases with age and is a familiar trait: Implications for "inflamm-aging". *Eur. J. Immunol.* **2014**, *44*, 1552–1562. [CrossRef]

211. Nakahira, K.; Kyung, S.Y.; Rogers, A.J.; Gazourian, L.; Youn, S.; Massaro, A.F.; Quintana, C.; Osorio, J.C.; Wang, Z.; Zhao, Y.; et al. Circulating mitochondrial DNA in patients in the ICU as a marker of mortality: Derivation and validation. *PLoS Med.* **2013**, *10*, e1001577. [CrossRef]

212. Thurairajah, K.; Briggs, G.D.; Balogh, Z.J. The source of cell-free mitochondrial DNA in trauma and potential therapeutic strategies. *Eur. J. Trauma Emerg. Surg.* **2018**, *44*, 325–334. [CrossRef]

213. Timmermans, K.; Kox, M.; Scheffer, G.J.; Pickkers, P. Plasma Nuclear and Mitochondrial DNA Levels, and Markers of Inflammation, Shock, and Organ Damage in Patients with Septic Shock. *Shock* **2016**, *45*, 607–612. [CrossRef]

214. Yan, H.P.; Li, M.; Lu, X.L.; Zhu, Y.M.; Ou-Yang, W.X.; Xiao, Z.H.; Qiu, J.; Li, S.J. Use of plasma mitochondrial DNA levels for determining disease severity and prognosis in pediatric sepsis: A case control study. *BMC Pediatr.* **2018**, *18*, 267. [CrossRef]

215. Arshad, O.; Gadawska, I.; Sattha, B.; Cote, H.C.F.; Hsieh, A.Y.Y.; CARMA. Elevated Cell-Free Mitochondrial DNA in Filtered Plasma Is Associated with HIV Infection and Inflammation. *J. Acquir. Immune Defic. Syndr.* **2018**, *78*, 111–118. [CrossRef]

216. Mehta, S.R.; Perez-Santiago, J.; Hulgan, T.; Day, T.R.; Barnholtz-Sloan, J.; Gittleman, H.; Letendre, S.; Ellis, R.; Heaton, R.; Patton, S.; et al. Cerebrospinal fluid cell-free mitochondrial DNA is associated with HIV replication, iron transport, and mild HIV-associated neurocognitive impairment. *J. Neuroinflamm.* **2017**, *14*, 72. [CrossRef]

217. Perez-Santiago, J.; Schrier, R.D.; de Oliveira, M.F.; Gianella, S.; Var, S.R.; Day, T.R.; Ramirez-Gaona, M.; Suben, J.D.; Murrell, B.; Massanella, M.; et al. Cell-free mitochondrial DNA in CSF is associated with early viral rebound, inflammation, and severity of neurocognitive deficits in HIV infection. *J. Neurovirol.* **2016**, *22*, 191–200. [CrossRef]

218. Bae, J.H.; Jo, S.I.; Kim, S.J.; Lee, J.M.; Jeong, J.H.; Kang, J.S.; Cho, N.J.; Kim, S.S.; Lee, E.Y.; Moon, J.S. Circulating Cell-Free mtDNA Contributes to AIM2 Inflammasome-Mediated Chronic Inflammation in Patients with Type 2 Diabetes. *Cells* **2019**, *8*, 328. [CrossRef]

219. Budnik, L.T.; Kloth, S.; Baur, X.; Preisser, A.M.; Schwarzenbach, H. Circulating mitochondrial DNA as biomarker linking environmental chemical exposure to early preclinical lesions elevation of mtDNA in human serum after exposure to carcinogenic halo-alkane-based pesticides. *PLoS ONE* **2013**, *8*, e64413. [CrossRef]

220. Bisserier, M.; Shanmughapriya, S.; Rai, A.K.; Gonzalez, C.; Brojakowska, A.; Garikipati, V.N.S.; Madesh, M.; Mills, P.J.; Walsh, K.; Arakelyan, A.; et al. Cell-Free Mitochondrial DNA as a Potential Biomarker for Astronauts' Health. *J. Am. Heart Assoc.* **2021**, *10*, e022055. [CrossRef]

221. Bezdan, D.; Grigorev, K.; Meydan, C.; Pelissier Vatter, F.A.; Cioffi, M.; Rao, V.; MacKay, M.; Nakahira, K.; Burnham, P.; Afshinnekoo, E.; et al. Cell-free DNA (cfDNA) and Exosome Profiling from a Year-Long Human Spaceflight Reveals Circulating Biomarkers. *iScience* **2020**, *23*, 101844. [CrossRef]

222. Lu, C.H.; Chang, W.N.; Tsai, N.W.; Chuang, Y.C.; Huang, C.R.; Wang, H.C. The value of serial plasma nuclear and mitochondrial DNA levels in adult community-acquired bacterial meningitis. *QJM* **2010**, *103*, 169–175. [CrossRef]

223. Lindqvist, D.; Wolkowitz, O.M.; Picard, M.; Ohlsson, L.; Bersani, F.S.; Fernstrom, J.; Westrin, A.; Hough, C.M.; Lin, J.; Reus, V.I.; et al. Circulating cell-free mitochondrial DNA, but not leukocyte mitochondrial DNA copy number, is elevated in major depressive disorder. *Neuropsychopharmacology* **2018**, *43*, 1557–1564. [CrossRef]

224. Lindqvist, D.; Fernstrom, J.; Grudet, C.; Ljunggren, L.; Traskman-Bendz, L.; Ohlsson, L.; Westrin, A. Increased plasma levels of circulating cell-free mitochondrial DNA in suicide attempters: Associations with HPA-axis hyperactivity. *Transl. Psychiatry* **2016**, *6*, e971. [CrossRef]

225. Trumpff, C.; Marsland, A.L.; Basualto-Alarcón, C.; Martin, J.L.; Carroll, J.E.; Sturm, G.; Vincent, A.E.; Mosharov, E.V.; Gu, Z.; Kaufman, B.A. Acute psychological stress increases serum circulating cell-free mitochondrial DNA. *Psychoneuroendocrinology* **2019**, *106*, 268–276. [CrossRef] [PubMed]

226. Ohlsson, L.; Hall, A.; Lindahl, H.; Danielsson, R.; Gustafsson, A.; Lavant, E.; Ljunggren, L. Increased level of circulating cell-free mitochondrial DNA due to a single bout of strenuous physical exercise. *Eur. J. Appl. Physiol.* **2020**, *120*, 897–905. [CrossRef]

227. Shockett, P.E.; Khanal, J.; Sitaula, A.; Oglesby, C.; Meachum, W.A.; Castracane, V.D.; Kraemer, R.R. Plasma cell-free mitochondrial DNA declines in response to prolonged moderate aerobic exercise. *Physiol. Rep.* **2016**, *4*, e12672. [CrossRef] [PubMed]

228. Newell, C.; Hume, S.; Greenway, S.C.; Podemski, L.; Shearer, J.; Khan, A. Plasma-derived cell-free mitochondrial DNA: A novel non-invasive methodology to identify mitochondrial DNA haplogroups in humans. *Mol. Genet. Metab.* **2018**, *125*, 332–337. [CrossRef] [PubMed]

229. Berezin, A.E. Circulating Cell-Free Mitochondrial DNA as Biomarker of Cardiovascular risk: New Challenges of Old Findings. *Angiol. Open Access* **2015**, *3*, 4. [CrossRef]

230. Berezin, A.E. The Cell-Free Mitochondrial DNA: A Novel Biomarker of Cardiovascular Risk? *Transl. Biomed.* **2016**, *7*, 68. [CrossRef]

231. Arnalich, F.; Codoceo, R.; Lopez-Collazo, E.; Montiel, C. Circulating cell-free mitochondrial DNA: A better early prognostic marker in patients with out-of-hospital cardiac arrest. *Resuscitation* **2012**, *83*, e162–e163. [CrossRef] [PubMed]

232. Dabravolski, S.A.; Khotina, V.A.; Sukhorukov, V.N.; Kalmykov, V.A.; Mikhaleva, L.M.; Orekhov, A.N. The role of mitochondrial DNA mutations in cardiovascular diseases. *Int. J. Mol. Sci.* **2022**, *23*, 952. [CrossRef]

233. Yue, P.; Jing, S.; Liu, L.; Ma, F.; Zhang, Y.; Wang, C.; Duan, H.; Zhou, K.; Hua, Y.; Wu, G. Association between mitochondrial DNA copy number and cardiovascular disease: Current evidence based on a systematic review and meta-analysis. *PLoS ONE* **2018**, *13*, e0206003. [CrossRef] [PubMed]

234. Ballinger, S.W. Mitochondrial dysfunction in cardiovascular disease. *Free. Radic. Biol. Med.* **2005**, *38*, 1278–1295. [CrossRef]

235. Baccarelli, A.A.; Byun, H.-M. Platelet mitochondrial DNA methylation: A potential new marker of cardiovascular disease. *Clin. Epigenetics* **2015**, *7*, 44. [CrossRef]

236. Giordano, F.J. Oxygen, oxidative stress, hypoxia, and heart failure. *J. Clin. Investig.* **2005**, *115*, 500–508. [CrossRef] [PubMed]

237. Thygesen, K.; Alpert, J.S.; White, H.D.; Joint ESC/ACCF/AHA/WHF Task Force for the Redefinition of Myocardial Infarction. Universal definition of myocardial infarction. *J. Am. Coll. Cardiol.* **2007**, *50*, 2173–2195. [CrossRef]

238. Bliksøen, M.; Mariero, L.H.; Ohm, I.K.; Haugen, F.; Yndestad, A.; Solheim, S.; Seljeflot, I.; Ranheim, T.; Andersen, G.Ø.; Aukrust, P. Increased circulating mitochondrial DNA after myocardial infarction. *Int. J. Cardiol.* **2012**, *158*, 132–134. [CrossRef]

239. Qin, C.; Gu, J.; Liu, R.; Xu, F.; Qian, H.; He, Q.; Meng, W. Release of mitochondrial DNA correlates with peak inflammatory cytokines in patients with acute myocardial infarction. *Anatol. J. Cardiol.* **2017**, *17*, 224.
240. Sudakov, N.; Popkova, T.; Katyshev, A.; Goldberg, O.; Nikiforov, S.; Pushkarev, B.; Klimenkov, I.; Lepekhova, S.; Apartsin, K.; Nevinsky, G. Level of blood cell-free circulating mitochondrial DNA as a novel biomarker of acute myocardial ischemia. *Biochemistry* **2015**, *80*, 1387–1392. [CrossRef]
241. Sudakov, N.P.; Apartsin, K.A.; Lepekhova, S.A.; Nikiforov, S.B.; Katyshev, A.I.; Lifshits, G.I.; Vybivantseva, A.V.; Konstantinov, Y.M. The level of free circulating mitochondrial DNA in blood as predictor of death in case of acute coronary syndrome. *Eur. J. Med. Res.* **2017**, *22*, 1. [CrossRef]
242. Wang, L.; Xie, L.; Zhang, Q.; Cai, X.; Tang, Y.; Wang, L.; Hang, T.; Liu, J.; Gong, J. Plasma nuclear and mitochondrial DNA levels in acute myocardial infarction patients. *Coron. Artery Dis.* **2015**, *26*, 296. [CrossRef]
243. Dhondup, Y.; Ueland, T.; Dahl, C.P.; Askevold, E.T.; Sandanger, Ø.; Fiane, A.; Ohm, I.K.; Sjaastad, I.; Finsen, A.V.; Wæhre, A. Low circulating levels of mitochondrial and high levels of nuclear DNA predict mortality in chronic heart failure. *J. Card. Fail.* **2016**, *22*, 823–828. [CrossRef] [PubMed]
244. Cataño Cañizalez, Y.; Uresti Rivera, E.; Garcia Jacobo, R.; Portales Perez, D.; Bastian, Y.; Rodriguez Rivera, J.; Gonzalez Amaro, R.; Enciso Moreno, J.; Garcia Hernandez, M. Increased levels of AIM2 and circulating mitochondrial DNA in type 2 diabetes. *Iran. J. Immunol.* **2018**, *15*, 142–155.
245. Liu, J.; Zou, Y.; Tang, Y.; Xi, M.; Xie, L.; Zhang, Q.; Gong, J. Circulating cell-free mitochondrial deoxyribonucleic acid is increased in coronary heart disease patients with diabetes mellitus. *J. Diabetes Investig.* **2016**, *7*, 109–114. [CrossRef]
246. Zhang, R.; Nakahira, K.; Guo, X.; Choi, A.M.K.; Gu, Z. Very Short Mitochondrial DNA Fragments and Heteroplasmy in Human Plasma. *Sci. Rep.* **2016**, *6*, 36097. [CrossRef]
247. Trumpff, C.; Michelson, J.; Lagranha, C.J.; Taleon, V.; Karan, K.R.; Sturm, G.; Lindqvist, D.; Fernström, J.; Moser, D.; Kaufman, B.A. Stress and circulating cell-free mitochondrial DNA: A systematic review of human studies, physiological considerations, and technical recommendations. *Mitochondrion* **2021**, *59*, 225–245. [CrossRef]
248. Al Amir Dache, Z.; Otandault, A.; Tanos, R.; Pastor, B.; Meddeb, R.; Sanchez, C.; Arena, G.; Lasorsa, L.; Bennett, A.; Grange, T.; et al. Blood contains circulating cell-free respiratory competent mitochondria. *FASEB J.* **2020**, *34*, 3616–3630. [CrossRef] [PubMed]
249. Chiu, R.W.K.; Chan, L.Y.S.; Lam, N.Y.L.; Tsui, N.B.Y.; Ng, E.K.O.; Rainer, T.H.; Lo, Y.M.D. Quantitative analysis of circulating mitochondrial DNA in plasma. *Clin. Chem.* **2003**, *49*, 719–726. [CrossRef]
250. Chandrananda, D.; Thorne, N.P.; Bahlo, M. High-resolution characterization of sequence signatures due to non-random cleavage of cell-free DNA. *BMC Med Genom.* **2015**, *8*, 29. [CrossRef] [PubMed]
251. Ellinger, J.; Albers, P.; Muller, S.C.; von Ruecker, A.; Bastian, P.J. Circulating mitochondrial DNA in the serum of patients with testicular germ cell cancer as a novel noninvasive diagnostic biomarker. *BJU Int.* **2009**, *104*, 48–52. [CrossRef] [PubMed]
252. Jiang, P.; Chan, C.W.; Chan, K.C.; Cheng, S.H.; Wong, J.; Wong, V.W.; Wong, G.L.; Chan, S.L.; Mok, T.S.; Chan, H.L.; et al. Lengthening and shortening of plasma DNA in hepatocellular carcinoma patients. *Proc. Natl. Acad. Sci. USA* **2015**, *112*, E1317–E1325. [CrossRef]
253. Thierry, A.R.; El Messaoudi, S.; Gahan, P.B.; Anker, P.; Stroun, M. Origins, structures, and functions of circulating DNA in oncology. *Cancer Metastasis Rev.* **2016**, *35*, 347–376. [CrossRef]
254. Grabuschnig, S.; Bronkhorst, A.J.; Holdenrieder, S.; Rosales Rodriguez, I.; Schliep, K.P.; Schwendenwein, D.; Ungerer, V.; Sensen, C.W. Putative Origins of Cell-Free DNA in Humans: A Review of Active and Passive Nucleic Acid Release Mechanisms. *Int. J. Mol. Sci.* **2020**, *21*, 8062. [CrossRef]
255. Brinkmann, V.; Reichard, U.; Goosmann, C.; Fauler, B.; Uhlemann, Y.; Weiss, D.S.; Weinrauch, Y.; Zychlinsky, A. Neutrophil extracellular traps kill bacteria. *Science* **2004**, *303*, 1532–1535. [CrossRef] [PubMed]
256. Goldmann, O.; Medina, E. The expanding world of extracellular traps: Not only neutrophils but much more. *Front. Immunol.* **2013**, *3*, 420. [CrossRef]
257. Morshed, M.; Hlushchuk, R.; Simon, D.; Walls, A.F.; Obata-Ninomiya, K.; Karasuyama, H.; Djonov, V.; Eggel, A.; Kaufmann, T.; Simon, H.-U. NADPH oxidase–independent formation of extracellular DNA traps by basophils. *J. Immunol.* **2014**, *192*, 5314–5323. [CrossRef]
258. Loures, F.V.; Röhm, M.; Lee, C.K.; Santos, E.; Wang, J.P.; Specht, C.A.; Calich, V.L.; Urban, C.F.; Levitz, S.M. Recognition of Aspergillus fumigatus hyphae by human plasmacytoid dendritic cells is mediated by dectin-2 and results in formation of extracellular traps. *PLoS Pathog.* **2015**, *11*, e1004643. [CrossRef]
259. Rocha Arrieta, Y.C.; Rojas, M.; Vasquez, G.; Lopez, J. The Lymphocytes Stimulation Induced DNA Release, a Phenomenon Similar to NET osis. *Scand. J. Immunol.* **2017**, *86*, 229–238. [CrossRef]
260. Urban, C.F.; Ermert, D.; Schmid, M.; Abu-Abed, U.; Goosmann, C.; Nacken, W.; Brinkmann, V.; Jungblut, P.R.; Zychlinsky, A. Neutrophil extracellular traps contain calprotectin, a cytosolic protein complex involved in host defense against Candida albicans. *PLoS Pathog.* **2009**, *5*, e1000639. [CrossRef]
261. Yousefi, S.; Simon, H.-U. NETosis—Does it really represent nature's "suicide bomber"? *Front. Immunol.* **2016**, *7*, 328. [CrossRef]
262. Brinkmann, V.; Zychlinsky, A. Beneficial suicide: Why neutrophils die to make NETs. *Nat. Rev. Microbiol.* **2007**, *5*, 577–582. [CrossRef]
263. Brinkmann, V.; Zychlinsky, A. Neutrophil extracellular traps: Is immunity the second function of chromatin? *J. Cell Biol.* **2012**, *198*, 773–783. [CrossRef] [PubMed]

264. Boeltz, S.; Amini, P.; Anders, H.-J.; Andrade, F.; Bilyy, R.; Chatfield, S.; Cichon, I.; Clancy, D.M.; Desai, J.; Dumych, T. To NET or not to NET: Current opinions and state of the science regarding the formation of neutrophil extracellular traps. *Cell Death Differ.* **2019**, *26*, 395–408. [CrossRef]

265. Yousefi, S.; Mihalache, C.; Kozlowski, E.; Schmid, I.; Simon, H.-U. Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps. *Cell Death Differ.* **2009**, *16*, 1438–1444. [CrossRef]

266. Lood, C.; Blanco, L.P.; Purmalek, M.M.; Carmona-Rivera, C.; De Ravin, S.S.; Smith, C.K.; Malech, H.L.; Ledbetter, J.A.; Elkon, K.B.; Kaplan, M.J. Neutrophil extracellular traps enriched in oxidized mitochondrial DNA are interferogenic and contribute to lupus-like disease. *Nat. Med.* **2016**, *22*, 146–153. [CrossRef] [PubMed]

267. Papayannopoulos, V. Neutrophil extracellular traps in immunity and disease. *Nat. Rev. Immunol.* **2018**, *18*, 134–147. [CrossRef] [PubMed]

268. Jorch, S.K.; Kubes, P. An emerging role for neutrophil extracellular traps in noninfectious disease. *Nat. Med.* **2017**, *23*, 279–287. [CrossRef] [PubMed]

269. Stojkov, D.; Gigon, L.; Peng, S.; Lukowski, R.; Ruth, P.; Karaulov, A.; Rizvanov, A.; Barlev, N.A.; Yousefi, S.; Simon, H.-U. Physiological and pathophysiological roles of metabolic pathways for NET formation and other neutrophil functions. *Front. Immunol.* **2022**, *13*, 826515. [CrossRef]

270. Fuchs, T.A.; Abed, U.; Goosmann, C.; Hurwitz, R.; Schulze, I.; Wahn, V.; Weinrauch, Y.; Brinkmann, V.; Zychlinsky, A. Novel cell death program leads to neutrophil extracellular traps. *J. Cell Biol.* **2007**, *176*, 231–241. [CrossRef]

271. Yousefi, S.; Stojkov, D.; Germic, N.; Simon, D.; Wang, X.; Benarafa, C.; Simon, H.U. Untangling "NETosis" from NETs. *Eur. J. Immunol.* **2019**, *49*, 221–227. [CrossRef]

272. Galluzzi, L.; Vitale, I.; Aaronson, S.A.; Abrams, J.M.; Adam, D.; Agostinis, P.; Alnemri, E.S.; Altucci, L.; Amelio, I.; Andrews, D.W.; et al. Molecular mechanisms of cell death: Recommendations of the Nomenclature Committee on Cell Death 2018. *Cell Death Differ.* **2018**, *25*, 486–541. [CrossRef]

273. Pilsczek, F.H.; Salina, D.; Poon, K.K.; Fahey, C.; Yipp, B.G.; Sibley, C.D.; Robbins, S.M.; Green, F.H.; Surette, M.G.; Sugai, M. A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus. *J. Immunol.* **2010**, *185*, 7413–7425. [CrossRef]

274. Yipp, B.G.; Kubes, P. NETosis: How vital is it? *Blood J. Am. Soc. Hematol.* **2013**, *122*, 2784–2794. [CrossRef]

275. Conceição-Silva, F.; Reis, C.S.; De Luca, P.M.; Leite-Silva, J.; Santiago, M.A.; Morrot, A.; Morgado, F.N. The immune system throws its traps: Cells and their extracellular traps in disease and protection. *Cells* **2021**, *10*, 1891. [CrossRef]

276. Caudrillier, A.; Kessenbrock, K.; Gilliss, B.M.; Nguyen, J.X.; Marques, M.B.; Monestier, M.; Toy, P.; Werb, Z.; Looney, M.R. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. *J. Clin. Investig.* **2012**, *122*, 2661–2671. [CrossRef]

277. Farrera, C.; Fadeel, B. Macrophage clearance of neutrophil extracellular traps is a silent process. *J. Immunol.* **2013**, *191*, 2647–2656. [CrossRef]

278. Haider, P.; Kral-Pointner, J.B.; Mayer, J.; Richter, M.; Kaun, C.; Brostjan, C.; Eilenberg, W.; Fischer, M.B.; Speidl, W.S.; Hengstenberg, C. Neutrophil extracellular trap degradation by differently polarized macrophage subsets. *Arterioscler. Thromb. Vasc. Biol.* **2020**, *40*, 2265–2278. [CrossRef]

279. Lazzaretto, B.; Fadeel, B. Intra-and extracellular degradation of neutrophil extracellular traps by macrophages and dendritic cells. *J. Immunol.* **2019**, *203*, 2276–2290. [CrossRef]

280. Zaborowski, M.P.; Balaj, L.; Breakefield, X.O.; Lai, C.P. Extracellular vesicles: Composition, biological relevance, and methods of study. *Bioscience* **2015**, *65*, 783–797. [CrossRef]

281. Yáñez-Mó, M.; Siljander, P.R.-M.; Andreu, Z.; Bedina Zavec, A.; Borràs, F.E.; Buzas, E.I.; Buzas, K.; Casal, E.; Cappello, F.; Carvalho, J. Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. Vesicles* **2015**, *4*, 27066. [CrossRef]

282. Doyle, L.M.; Wang, M.Z. Overview of extracellular vesicles, their origin, composition, purpose, and methods for exosome isolation and analysis. *Cells* **2019**, *8*, 727. [CrossRef]

283. Hessvik, N.P.; Llorente, A. Current knowledge on exosome biogenesis and release. *Cell. Mol. Life Sci.* **2018**, *75*, 193–208. [CrossRef]

284. Pegtel, D.M.; Gould, S.J. Exosomes. *Annu. Rev. Biochem.* **2019**, *88*, 487–514. [CrossRef]

285. Kalluri, R.; LeBleu, V.S. The biology, function, and biomedical applications of exosomes. *Science* **2020**, *367*, eaau6977. [CrossRef]

286. Aucamp, J.; Bronkhorst, A.J.; Pretorius, P.J. A Historical and Evolutionary Perspective on Circulating Nucleic Acids and Extracellular Vesicles: Circulating Nucleic Acids as Homeostatic Genetic Entities. *Adv. Exp. Med. Biol.* **2016**, *924*, 91–95. [CrossRef]

287. Van der Pol, E.; Böing, A.N.; Harrison, P.; Sturk, A.; Nieuwland, R. Classification, functions, and clinical relevance of extracellular vesicles. *Pharmacol. Rev.* **2012**, *64*, 676–705. [CrossRef]

288. Van Niel, G.; d'Angelo, G.; Raposo, G. Shedding light on the cell biology of extracellular vesicles. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 213–228. [CrossRef]

289. Théry, C.; Witwer, K.W.; Aikawa, E.; Alcaraz, M.J.; Anderson, J.D.; Andriantsitohaina, R.; Antoniou, A.; Arab, T.; Archer, F.; Atkin-Smith, G.K. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* **2018**, *7*, 1535750. [CrossRef] [PubMed]

290. Sharma, A.; Johnson, A. Exosome DNA: Critical regulator of tumor immunity and a diagnostic biomarker. *J. Cell. Physiol.* **2020**, *235*, 1921–1932. [CrossRef] [PubMed]

291. Azmi, A.S.; Bao, B.; Sarkar, F.H. Exosomes in cancer development, metastasis, and drug resistance: A comprehensive review. *Cancer Metastasis Rev.* **2013**, *32*, 623–642. [CrossRef]

292. Soung, Y.H.; Ford, S.; Zhang, V.; Chung, J. Exosomes in cancer diagnostics. *Cancers* **2017**, *9*, 8. [CrossRef] [PubMed]

293. Vlassov, A.V.; Magdaleno, S.; Setterquist, R.; Conrad, R. Exosomes: Current knowledge of their composition, biological functions, and diagnostic and therapeutic potentials. *Biochim. Biophys. Acta (BBA)-Gen. Subj.* **2012**, *1820*, 940–948. [CrossRef]

294. Lazo, S.; Noren Hooten, N.; Green, J.; Eitan, E.; Mode, N.A.; Liu, Q.R.; Zonderman, A.B.; Ezike, N.; Mattson, M.P.; Ghosh, P. Mitochondrial DNA in extracellular vesicles declines with age. *Aging Cell* **2021**, *20*, e13283. [CrossRef]

295. Marcatti, M.; Saada, J.; Okereke, I.; Wade, C.E.; Bossmann, S.H.; Motamedi, M.; Szczesny, B. Quantification of Circulating Cell Free Mitochondrial DNA in Extracellular Vesicles with PicoGreen™ in Liquid Biopsies: Fast Assessment of Disease/Trauma Severity. *Cells* **2021**, *10*, 819. [CrossRef]

296. Guescini, M.; Guidolin, D.; Vallorani, L.; Casadei, L.; Gioacchini, A.M.; Tibollo, P.; Battistelli, M.; Falcieri, E.; Battistin, L.; Agnati, L. C2C12 myoblasts release micro-vesicles containing mtDNA and proteins involved in signal transduction. *Exp. Cell Res.* **2010**, *316*, 1977–1984. [CrossRef]

297. Balaj, L.; Lessard, R.; Dai, L.; Cho, Y.-J.; Pomeroy, S.L.; Breakefield, X.O.; Skog, J. Tumour microvesicles contain retrotransposon elements and amplified oncogene sequences. *Nat. Commun.* **2011**, *2*, 180. [CrossRef] [PubMed]

298. O'Hara, B.A.; Morris-Love, J.; Gee, G.V.; Haley, S.A.; Atwood, W.J. JC Virus infected choroid plexus epithelial cells produce extracellular vesicles that infect glial cells independently of the virus attachment receptor. *PLoS Pathog.* **2020**, *16*, e1008371. [CrossRef]

299. Scribano, S.; Guerrini, M.; Arvia, R.; Guasti, D.; Nardini, P.; Romagnoli, P.; Giannecchini, S. Archetype JC polyomavirus DNA associated with extracellular vesicles circulates in human plasma samples. *J. Clin. Virol.* **2020**, *128*, 104435. [CrossRef]

300. Tutanov, O.; Shtam, T.; Grigor'eva, A.; Tupikin, A.; Tsentalovich, Y.; Tamkovich, S. Blood Plasma Exosomes Contain Circulating DNA in Their Crown. *Diagnostics* **2022**, *12*, 854. [CrossRef]

301. Neuberger, E.W.; Hillen, B.; Mayr, K.; Simon, P.; Krämer-Albers, E.-M.; Brahmer, A. Kinetics and topology of DNA associated with circulating extracellular vesicles released during exercise. *Genes* **2021**, *12*, 522. [CrossRef]

302. Lázaro-Ibáñez, E.; Lässer, C.; Shelke, G.V.; Crescitelli, R.; Jang, S.C.; Cvjetkovic, A.; García-Rodríguez, A.; Lötvall, J. DNA analysis of low-and high-density fractions defines heterogeneous subpopulations of small extracellular vesicles based on their DNA cargo and topology. *J. Extracell. Vesicles* **2019**, *8*, 1656993. [CrossRef]

303. Thakur, B.K.; Zhang, H.; Becker, A.; Matei, I.; Huang, Y.; Costa-Silva, B.; Zheng, Y.; Hoshino, A.; Brazier, H.; Xiang, J.; et al. Double-stranded DNA in exosomes: A novel biomarker in cancer detection. *Cell Res.* **2014**, *24*, 766–769. [CrossRef]

304. Kahlert, C.; Melo, S.A.; Protopopov, A.; Tang, J.; Seth, S.; Koch, M.; Zhang, J.; Weitz, J.; Chin, L.; Futreal, A. Identification of double-stranded genomic DNA spanning all chromosomes with mutated KRAS and p53 DNA in the serum exosomes of patients with pancreatic cancer. *J. Biol. Chem.* **2014**, *289*, 3869–3875. [CrossRef] [PubMed]

305. Jeppesen, D.K.; Fenix, A.M.; Franklin, J.L.; Higginbotham, J.N.; Zhang, Q.; Zimmerman, L.J.; Liebler, D.C.; Ping, J.; Liu, Q.; Evans, R. Reassessment of exosome composition. *Cell* **2019**, *177*, 428–445.e418. [CrossRef] [PubMed]

306. Liangsupree, T.; Multia, E.; Riekkola, M.-L. Modern isolation and separation techniques for extracellular vesicles. *J. Chromatogr. A* **2021**, *1636*, 461773. [CrossRef]

307. Liu, H.; Tian, Y.; Xue, C.; Niu, Q.; Chen, C.; Yan, X. Analysis of extracellular vesicle DNA at the single-vesicle level by nano-flow cytometry. *J. Extracell. Vesicles* **2022**, *11*, e12206. [CrossRef]

308. Vagner, T.; Spinelli, C.; Minciacchi, V.R.; Balaj, L.; Zandian, M.; Conley, A.; Zijlstra, A.; Freeman, M.R.; Demichelis, F.; De, S. Large extracellular vesicles carry most of the tumour DNA circulating in prostate cancer patient plasma. *J. Extracell. Vesicles* **2018**, *7*, 1505403. [CrossRef]

309. Zocco, D.; Bernardi, S.; Novelli, M.; Astrua, C.; Fava, P.; Zarovni, N.; Carpi, F.M.; Bianciardi, L.; Malavenda, O.; Quaglino, P. Isolation of extracellular vesicles improves the detection of mutant DNA from plasma of metastatic melanoma patients. *Sci. Rep.* **2020**, *10*, 15745. [CrossRef]

310. Fernando, M.R.; Jiang, C.; Krzyzanowski, G.D.; Ryan, W.L. New evidence that a large proportion of human blood plasma cell-free DNA is localized in exosomes. *PLoS ONE* **2017**, *12*, e0183915. [CrossRef]

311. Galbiati, S.; Damin, F.; Brambilla, D.; Ferraro, L.; Soriani, N.; Ferretti, A.M.; Burgio, V.; Ronzoni, M.; Vago, R.; Sola, L. Small evs-associated dna as complementary biomarker to circulating tumor dna in plasma of metastatic colorectal cancer patients. *Pharmaceuticals* **2021**, *14*, 128. [CrossRef]

312. Sina, A.A.; Carrascosa, L.G.; Liang, Z.; Grewal, Y.S.; Wardiana, A.; Shiddiky, M.J.A.; Gardiner, R.A.; Samaratunga, H.; Gandhi, M.K.; Scott, R.J.; et al. Epigenetically reprogrammed methylation landscape drives the DNA self-assembly and serves as a universal cancer biomarker. *Nat. Commun.* **2018**, *9*, 4915. [CrossRef]

313. Ghanam, J.; Chetty, V.K.; Barthel, L.; Reinhardt, D.; Hoyer, P.-F.; Thakur, B.K. DNA in extracellular vesicles: From evolution to its current application in health and disease. *Cell Biosci.* **2022**, *12*, 37. [CrossRef]

314. Sansone, P.; Savini, C.; Kurelac, I.; Chang, Q.; Amato, L.B.; Strillacci, A.; Stepanova, A.; Iommarini, L.; Mastroleo, C.; Daly, L. Packaging and transfer of mitochondrial DNA via exosomes regulate escape from dormancy in hormonal therapy-resistant breast cancer. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, E9066–E9075. [CrossRef]

315. Cai, J.; Han, Y.; Ren, H.; Chen, C.; He, D.; Zhou, L.; Eisner, G.M.; Asico, L.D.; Jose, P.A.; Zeng, C. Extracellular vesicle-mediated transfer of donor genomic DNA to recipient cells is a novel mechanism for genetic influence between cells. *J. Mol. Cell Biol.* **2013**, *5*, 227–238. [CrossRef]

316. Waldenström, A.; Gennebäck, N.; Hellman, U.; Ronquist, G. Cardiomyocyte microvesicles contain DNA/RNA and convey biological messages to target cells. *PLoS ONE* **2012**, *7*, e34653. [CrossRef]

317. Li, Y.; Bax, C.; Patel, J.; Vazquez, T.; Ravishankar, A.; Bashir, M.M.; Grinnell, M.; Diaz, D.; Werth, V.P. Plasma-derived DNA containing-extracellular vesicles induce STING-mediated proinflammatory responses in dermatomyositis. *Theranostics* **2021**, *11*, 7144. [CrossRef]

318. Allen, E.R.; Lempke, S.L.; Miller, M.M.; Bush, D.M.; Braswell, B.G.; Estes, C.L.; Benedict, E.L.; Mahon, A.R.; Sabo, S.L.; Greenlee-Wacker, M.C. Effect of extracellular vesicles from S. aureus-challenged human neutrophils on macrophages. *J. Leukoc. Biol.* **2020**, *108*, 1841–1850. [CrossRef]

319. Martins, S.d.T.; Alves, L.R. Extracellular vesicles in viral infections: Two sides of the same coin? *Front. Cell. Infect. Microbiol.* **2020**, *10*, 593170. [CrossRef]

320. Stentz, R.; Carvalho, A.L.; Jones, E.J.; Carding, S.R. Fantastic voyage: The journey of intestinal microbiota-derived microvesicles through the body. *Biochem. Soc. Trans.* **2018**, *46*, 1021–1027. [CrossRef]

321. Nabhan, J.F.; Hu, R.; Oh, R.S.; Cohen, S.N.; Lu, Q. Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 4146–4151. [CrossRef]

322. Rasmussen, N.; Nielsen, C.; Nielsen, C.; Jacobsen, S. Microvesicles in active lupus nephritis show Toll-like receptor 9-dependent co-expression of galectin-3 binding protein and double-stranded DNA. *Clin. Exp. Immunol.* **2021**, *204*, 64–77. [CrossRef]

323. Clancy, J.W.; Sheehan, C.S.; Boomgarden, A.C.; D'Souza-Schorey, C. Recruitment of DNA to tumor-derived microvesicles. *Cell Rep.* **2022**, *38*, 110443. [CrossRef] [PubMed]

324. Ciardiello, C.; Migliorino, R.; Leone, A.; Budillon, A. Large extracellular vesicles: Size matters in tumor progression. *Cytokine Growth Factor Rev.* **2020**, *51*, 69–74. [CrossRef] [PubMed]

325. Holmgren, L.; Szeles, A.; Rajnavölgyi, E.; Folkman, J.; Klein, G.; Ernberg, I.; Falk, K.I. Horizontal transfer of DNA by the uptake of apoptotic bodies. *Blood J. Am. Soc. Hematol.* **1999**, *93*, 3956–3963.

326. Bergsmedh, A.; Szeles, A.; Henriksson, M.; Bratt, A.; Folkman, M.J.; Spetz, A.-L.; Holmgren, L. Horizontal transfer of oncogenes by uptake of apoptotic bodies. *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 6407–6411. [CrossRef]

327. Sisirak, V.; Sally, B.; d'Agati, V.; Martinez-Ortiz, W.; Özçakar, Z.B.; David, J.; Rashidfarrokhi, A.; Yeste, A.; Panea, C.; Chida, A.S. Digestion of chromatin in apoptotic cell microparticles prevents autoimmunity. *Cell* **2016**, *166*, 88–101. [CrossRef] [PubMed]

328. Zhang, Q.; Higginbotham, J.N.; Jeppesen, D.K.; Yang, Y.-P.; Li, W.; McKinley, E.T.; Graves-Deal, R.; Ping, J.; Britain, C.M.; Dorsett, K.A. Transfer of functional cargo in exomeres. *Cell Rep.* **2019**, *27*, 940–954.e946. [CrossRef]

329. Ruhen, O.; Mirzai, B.; Clark, M.E.; Nguyen, B.; Salomon, C.; Erber, W.; Meehan, K. Comparison of circulating tumour dna and extracellular vesicle dna by low-pass whole-genome sequencing reveals molecular drivers of disease in a breast cancer patient. *Biomedicines* **2020**, *9*, 14. [CrossRef] [PubMed]

330. Helmig, S.; Frühbeis, C.; Krämer-Albers, E.-M.; Simon, P.; Tug, S. Release of bulk cell free DNA during physical exercise occurs independent of extracellular vesicles. *Eur. J. Appl. Physiol.* **2015**, *115*, 2271–2280. [CrossRef] [PubMed]

331. Cohen, J.D.; Javed, A.A.; Thoburn, C.; Wong, F.; Tie, J.; Gibbs, P.; Schmidt, C.M.; Yip-Schneider, M.T.; Allen, P.J.; Schattner, M.; et al. Combined circulating tumor DNA and protein biomarker-based liquid biopsy for the earlier detection of pancreatic cancers. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 10202–10207. [CrossRef] [PubMed]

332. Cohen, J.D.; Li, L.; Wang, Y.; Thoburn, C.; Afsari, B.; Danilova, L.; Douville, C.; Javed, A.A.; Wong, F.; Mattox, A.; et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. *Science* **2018**, *359*, 926–930. [CrossRef] [PubMed]

333. Alix-Panabières, C.; Schwarzenbach, H.; Pantel, K. Circulating tumor cells and circulating tumor DNA. *Annu. Rev. Med.* **2012**, *63*, 199–215. [CrossRef]

334. Alix-Panabières, C.; Pantel, K. Circulating tumor cells: Liquid biopsy of cancer. *Clin. Chem.* **2013**, *59*, 110–118. [CrossRef]

335. Alix-Panabières, C.; Pantel, K. Clinical applications of circulating tumor cells and circulating tumor DNA as liquid biopsy. *Cancer Discov.* **2016**, *6*, 479–491. [CrossRef]

336. Keup, C.; Storbeck, M.; Hauch, S.; Hahn, P.; Sprenger-Haussels, M.; Hoffmann, O.; Kimmig, R.; Kasimir-Bauer, S. Multimodal targeted deep sequencing of circulating tumor cells and matched cell-free DNA provides a more comprehensive tool to identify therapeutic targets in metastatic breast cancer patients. *Cancers* **2020**, *12*, 1084. [CrossRef]

337. Keup, C.; Kimmig, R.; Kasimir-Bauer, S. Multimodality in liquid biopsy: Does a combination uncover insights undetectable in individual blood analytes? *LaboratoriumsMedizin* **2022**. [CrossRef]

338. Keup, C.; Suryaprakash, V.; Storbeck, M.; Hoffmann, O.; Kimmig, R.; Kasimir-Bauer, S. Longitudinal multi-parametric liquid biopsy approach identifies unique features of circulating tumor cell, extracellular vesicle, and cell-free DNA characterization for disease monitoring in metastatic breast cancer patients. *Cells* **2021**, *10*, 212. [CrossRef]

339. Keup, C.; Suryaprakash, V.; Hauch, S.; Storbeck, M.; Hahn, P.; Sprenger-Haussels, M.; Kolberg, H.-C.; Tewes, M.; Hoffmann, O.; Kimmig, R. Integrative statistical analyses of multiple liquid biopsy analytes in metastatic breast cancer. *Genome Med.* **2021**, *13*, 85. [CrossRef]

340. Keup, C.; Kimmig, R.; Kasimir-Bauer, S. Combinatorial power of cfDNA, CTCs and EVs in oncology. *Diagnostics* **2022**, *12*, 870. [CrossRef]

341. Zhang, W.; Xia, W.; Lv, Z.; Xin, Y.; Ni, C.; Yang, L. Liquid biopsy for cancer: Circulating tumor cells, circulating free DNA or exosomes? *Cell. Physiol. Biochem.* **2017**, *41*, 755–768. [CrossRef]

342. Mitchell, P.S.; Parkin, R.K.; Kroh, E.M.; Fritz, B.R.; Wyman, S.K.; Pogosova-Agadjanyan, E.L.; Peterson, A.; Noteboom, J.; O'Briant, K.C.; Allen, A.; et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 10513–10518. [CrossRef]

343. Zhao, F.; Vesprini, D.; Liu, R.S.; Olkhov-Mitsel, E.; Klotz, L.H.; Loblaw, A.; Liu, S.K.; Bapat, B. Combining urinary DNA methylation and cell-free microRNA biomarkers for improved monitoring of prostate cancer patients on active surveillance. *Urol. Oncol. Semin. Orig. Investig.* **2019**, *37*, 297.e9–297.e17. [CrossRef] [PubMed]

344. Mayers, J.R.; Wu, C.; Clish, C.B.; Kraft, P.; Torrence, M.E.; Fiske, B.P.; Yuan, C.; Bao, Y.; Townsend, M.K.; Tworoger, S.S.; et al. Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. *Nat. Med.* **2014**, *20*, 1193–1198. [CrossRef]

345. Kopreski, M.S.; Benko, F.A.; Kwak, L.W.; Gocke, C.D. Detection of tumor messenger RNA in the serum of patients with malignant melanoma. *Clin. Cancer Res.* **1999**, *5*, 1961–1965. [CrossRef] [PubMed]

346. Wan, N.; Weinberg, D.; Liu, T.-Y.; Niehaus, K.; Ariazi, E.A.; Delubac, D.; Kannan, A.; White, B.; Bailey, M.; Bertin, M. Machine learning enables detection of early-stage colorectal cancer by whole-genome sequencing of plasma cell-free DNA. *BMC Cancer* **2019**, *19*, 832. [CrossRef] [PubMed]

347. Tang, W.; Wan, S.; Yang, Z.; Teschendorff, A.E.; Zou, Q. Tumor origin detection with tissue-specific miRNA and DNA methylation markers. *Bioinformatics* **2017**, *34*, 398–406. [CrossRef] [PubMed]

348. Srivastava, A.; Karpievitch, Y.V.; Eichten, S.R.; Borevitz, J.O.; Lister, R. HOME: A histogram based machine learning approach for effective identification of differentially methylated regions. *BMC Bioinform.* **2019**, *20*, 253. [CrossRef] [PubMed]

349. Soh, K.P.; Szczurek, E.; Sakoparnig, T.; Beerenwinkel, N. Predicting cancer type from tumour DNA signatures. *Genome Medicine* **2017**, *9*, 104. [CrossRef] [PubMed]

350. Siejka-Zielińska, P.; Cheng, J.; Jackson, F.; Liu, Y.; Soonawalla, Z.; Reddy, S.; Silva, M.; Puta, L.; McCain, M.V.; Culver, E.L. Cell-free DNA TAPS provides multimodal information for early cancer detection. *Sci. Adv.* **2021**, *7*, eabh0534. [CrossRef]

351. Sabedot, T.S.; Malta, T.M.; Snyder, J.; Nelson, K.; Wells, M.; DeCarvalho, A.C.; Mukherjee, A.; Chitale, D.A.; Mosella, M.S.; Sokolov, A. A serum-based DNA methylation assay provides accurate detection of glioma. *Neuro-Oncology* **2021**, *23*, 1494–1508. [CrossRef]

352. Razavi, P.; Li, B.T.; Brown, D.N.; Jung, B.; Hubbell, E.; Shen, R.; Abida, W.; Juluru, K.; De Bruijn, I.; Hou, C. High-intensity sequencing reveals the sources of plasma circulating cell-free DNA variants. *Nat. Med.* **2019**, *25*, 1928–1937. [CrossRef]

353. Panagopoulou, M.; Karaglani, M.; Balgkouranidou, I.; Biziota, E.; Koukaki, T.; Karamitrousis, E.; Nena, E.; Tsamardinos, I.; Kolios, G.; Lianidou, E. Circulating cell-free DNA in breast cancer: Size profiling, levels, and methylation patterns lead to prognostic and predictive classifiers. *Oncogene* **2019**, *38*, 3387–3401. [CrossRef]

354. Moran, S.; Martinez-Cardus, A.; Sayols, S.; Musulen, E.; Balana, C.; Estival-Gonzalez, A.; Moutinho, C.; Heyn, H.; Diaz-Lagares, A.; de Moura, M.C.; et al. Epigenetic profiling to classify cancer of unknown primary: A multicentre, retrospective analysis. *Lancet Oncol.* **2016**, *17*, 1386–1395. [CrossRef]

355. Li, B.; Janku, F.; Jung, B.; Hou, C.; Madwani, K.; Alden, R.; Razavi, P.; Reis-Filho, J.; Shen, R.; Isbell, J. Ultra-deep next-generation sequencing of plasma cell-free DNA in patients with advanced lung cancers: Results from the Actionable Genome Consortium. *Ann. Oncol.* **2019**, *30*, 597–603. [CrossRef]

356. Douville, C.; Cohen, J.D.; Ptak, J.; Popoli, M.; Schaefer, J.; Silliman, N.; Dobbyn, L.; Schoen, R.E.; Tie, J.; Gibbs, P. Assessing aneuploidy with repetitive element sequencing. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 4858–4863. [CrossRef]

357. Chabon, J.J.; Hamilton, E.G.; Kurtz, D.M.; Esfahani, M.S.; Moding, E.J.; Stehr, H.; Schroers-Martin, J.; Nabet, B.Y.; Chen, B.; Chaudhuri, A.A. Integrating genomic features for non-invasive early lung cancer detection. *Nature* **2020**, *580*, 245–251. [CrossRef]

358. Doebley, A.-L.; Ko, M.; Liao, H.; Cruikshank, A.E.; Kikawa, C.; Santos, K.; Hiatt, J.; Patton, R.D.; De Sarkar, N.; Hoge, A.C. Griffin: Framework for clinical cancer subtyping from nucleosome profiling of cell-free DNA. *medRxiv* **2021**. [CrossRef]

359. Cortese, R.; Kwan, A.; Lalonde, E.; Bryzgunova, O.; Bondar, A.; Wu, Y.; Gordevicius, J.; Park, M.; Oh, G.; Kaminsky, Z. Epigenetic markers of prostate cancer in plasma circulating DNA. *Hum. Mol. Genet.* **2012**, *21*, 3619–3631. [CrossRef]

360. Budhraja, K.K.; McDonald, B.R.; Stephens, M.D.; Contente-Cuomo, T.; Markus, H.; Farooq, M.; Favaro, P.F.; Connor, S.; Byron, S.A.; Egan, J.B. Analysis of fragment ends in plasma DNA from patients with cancer. *medRxiv* **2021**. [CrossRef]

361. Bendich, A.; Wilczok, T.; Borenfreund, E. Circulating DNA as a possible factor in oncogenesis. *Science* **1965**, *148*, 374–376. [CrossRef]

362. García-Olmo, D.C.; Domínguez, C.; García-Arranz, M.; Anker, P.; Stroun, M.; García-Verdugo, J.M.; García-Olmo, D. Cell-free nucleic acids circulating in the plasma of colorectal cancer patients induce the oncogenic transformation of susceptible cultured cells. *Cancer Res.* **2010**, *70*, 560–567. [CrossRef]

363. Trejo-Becerril, C.; Pérez-Cárdenas, E.; Taja-Chayeb, L.; Anker, P.; Herrera-Goepfert, R.; Medina-Velázquez, L.A.; Hidalgo-Miranda, A.; Pérez-Montiel, D.; Chávez-Blanco, A.; Cruz-Velázquez, J.; et al. Cancer Progression Mediated by Horizontal Gene Transfer in an In Vivo Model. *PLoS ONE* **2012**, *7*, e52754. [CrossRef] [PubMed]

364. Dvořáková, M.; Karafiát, V.; Pajer, P.; Kluzáková, E.; Jarkovská, K.; Peková, S.; Krutílková, L.; Dvořák, M. DNA released by leukemic cells contributes to the disruption of the bone marrow microenvironment. *Oncogene* **2013**, *32*, 5201–5209. [CrossRef]

365. Kostyuk, S.V.; Malinovskaya, E.M.; Ermakov, A.V.; Smirnova, T.D.; Kameneva, L.V.; Chvartatskaya, O.V.; Loseva, P.A.; Ershova, E.S.; Lyubchenko, L.N.; Veiko, N.N. Fragments of cell-free DNA increase transcription in human mesenchymal stem cells, activate TLR-dependent signal pathway, and suppress apoptosis. *Biochem. Suppl. Ser. B Biomed. Chem.* **2012**, *6*, 68–74. [CrossRef]

366. Kostyuk, S.V.; Ermakov, A.V.; Alekseeva, A.Y.; Smirnova, T.D.; Glebova, K.V.; Efremova, L.V.; Baranova, A.; Veiko, N.N. Role of extracellular DNA oxidative modification in radiation induced bystander effects in human endotheliocytes. *Mutat. Res.* **2012**, *729*, 52–60. [CrossRef]

367. Glebova, K.; Veiko, N.; Kostyuk, S.; Izhevskaya, V.; Baranova, A. Oxidized extracellular DNA as a stress signal that may modify response to anticancer therapy. *Cancer Lett.* **2015**, *356*, 22–33. [CrossRef]

368. Ermakov, A.V.; Konkova, M.S.; Kostyuk, S.V.; Smirnova, T.D.; Malinovskaya, E.M.; Efremova, L.V.; Veiko, N.N. An extracellular DNA mediated bystander effect produced from low dose irradiated endothelial cells. *Mutat. Res./Fundam. Mol. Mech. Mutagenesis* **2011**, *712*, 1–10. [CrossRef]

369. Brown, P.C.; Tlsty, T.D.; Schimke, R.T. Enhancement of methotrexate resistance and dihydrofolate reductase gene amplification by treatment of mouse 3T6 cells with hydroxyurea. *Mol. Cell. Biol.* **1983**, *3*, 1097–1107.

370. Carman, M.D.; Schornagel, J.H.; Rivest, R.S.; Srimatkandada, S.; Portlock, C.S.; Duffy, T.; Bertino, J. Resistance to methotrexate due to gene amplification in a patient with acute leukemia. *J. Clin. Oncol.* **1984**, *2*, 16–20. [CrossRef]

371. Anunobi, R.; Boone, B.A.; Cheh, N.; Tang, D.; Kang, R.; Loux, T.; Lotze, M.T.; Zeh, H.J. Extracellular DNA promotes colorectal tumor cell survival after cytotoxic chemotherapy. *J. Surg. Res.* **2018**, *226*, 181–191. [CrossRef]

372. Niu, Z.; Tang, W.; Liu, T.; Xu, P.; Zhu, D.; Ji, M.; Huang, W.; Ren, L.; Wei, Y.; Xu, J. Cell-free DNA derived from cancer cells facilitates tumor malignancy through Toll-like receptor 9 signaling-triggered interleukin-8 secretion in colorectal cancer. *Acta Biochim. Biophys. Sin.* **2018**, *50*, 1007–1017. [CrossRef]

373. Furi, I.; Kalmár, A.; Wichmann, B.; Spisák, S.; Schöller, A.; Barták, B.; Tulassay, Z.; Molnár, B. Cell free DNA of tumor origin induces a 'metastatic' expression profile in HT-29 cancer cell line. *PLoS ONE* **2015**, *10*, e0131699. [CrossRef] [PubMed]

374. Wen, F.; Shen, A.; Choi, A.; Gerner, E.W.; Shi, J. Extracellular DNA in pancreatic cancer promotes cell invasion and metastasis. *Cancer Res.* **2013**, *73*, 4256–4266. [CrossRef] [PubMed]

375. Souza, A.G.d.; Bastos, V.A.F.; Fujimura, P.T.; Ferreira, I.C.C.; Leal, L.F.; da Silva, L.S.; Laus, A.C.; Reis, R.M.; Martins, M.M.; Santos, P.S. Cell-free DNA promotes malignant transformation in non-tumor cells. *Sci. Rep.* **2020**, *10*, 21674. [CrossRef] [PubMed]

376. Lee, T.H.; Chennakrishnaiah, S.; Audemard, E.; Montermini, L.; Meehan, B.; Rak, J. Oncogenic ras-driven cancer cell vesiculation leads to emission of double-stranded DNA capable of interacting with target cells. *Biochem. Biophys. Res. Commun.* **2014**, *451*, 295–301. [CrossRef]

377. Ju, Y.S.; Tubio, J.M.C.; Mifsud, W.; Fu, B.; Davies, H.R.; Ramakrishna, M.; Li, Y.; Yates, L.; Gundem, G.; Tarpey, P.S. Frequent somatic transfer of mitochondrial DNA into the nuclear genome of human cancer cells. *Genome Res.* **2015**, *25*, 814–824. [CrossRef]

378. Bronkhorst, A.J.; Wentzel, J.F.; Ungerer, V.; Peters, D.L.; Aucamp, J.; de Villiers, E.P.; Holdenrieder, S.; Pretorius, P.J. Sequence analysis of cell-free DNA derived from cultured human bone osteosarcoma (143B) cells. *Tumour Biol.* **2018**, *40*, 1010428318801190. [CrossRef]

379. Grabuschnig, S.; Soh, J.; Heidinger, P.; Bachler, T.; Hirschböck, E.; Rodriguez, I.R.; Schwendenwein, D.; Sensen, C.W. Circulating cell-free DNA is predominantly composed of retrotransposable elements and non-telomeric satellite DNA. *J. Biotechnol.* **2020**, *313*, 48–56. [CrossRef]

380. Scott, E.C.; Gardner, E.J.; Masood, A.; Chuang, N.T.; Vertino, P.M.; Devine, S.E. A hot L1 retrotransposon evades somatic repression and initiates human colorectal cancer. *Genome Res.* **2016**, *25*, 745–755. [CrossRef]

381. Burns, K.H.; Boeke, J.D. Human transposon tectonics. *Cell* **2012**, *149*, 740–752. [CrossRef]

382. Garcia-Olmo, D.; Garcia-Arranz, M.; Clemente, L.V.; Gahan, P.B.; Stroun, M. Method for Blocking Tumour Growth. U.S. Patent 9,555,075, 12 March 2015.

383. Garcia-Arranz, M.; Garcia-Olmo, D.; Vega-Clemente, L.; Stroun, M.; Gahan, P.B. *Non-Dividing Cell Virtosomes Affect In Vitro and In Vivo Tumour Cell Replication*; Springer: Berlin/Heidelberg, Germany, 2016; pp. 43–45.

384. Adams, D.; Diaz, N.; Gahan, P. In vitro stimulation by tumour cell media of [3H]-thymidine incorporation by mouse spleen lymphocytes. *Cell Biochem. Funct.* **1997**, *15*, 119–126. [CrossRef]

385. Sawa, C.; Yofu, S.; Kiriyama, K.; Sutoh, K.; Saito, T.; Kishi, S.; Gunji, M.; Inoue, Y.; Sugi, M.; Shioda, S. High concentration of extracellular nucleotides suppresses cell growth via delayed cell cycle progression in cancer and noncancer cell lines. *Heliyon* **2021**, *7*, e08318. [CrossRef]

386. Liang, H.; Peng, B.; Dong, C.; Liu, L.; Mao, J.; Wei, S.; Wang, X.; Xu, H.; Shen, J.; Mao, H.-Q. Cationic nanoparticle as an inhibitor of cell-free DNA-induced inflammation. *Nat. Commun.* **2018**, *9*, 4291. [CrossRef]

387. Burgener, S.S.; Schroder, K. Neutrophil extracellular traps in host defense. *Cold Spring Harb. Perspect. Biol.* **2020**, *12*, a037028. [CrossRef]

388. Branzk, N.; Papayannopoulos, V. Molecular mechanisms regulating NETosis in infection and disease. *Semin. Immunopathol.* **2013**, *35*, 513–530. [CrossRef]

389. Sollberger, G.; Tilley, D.O.; Zychlinsky, A. Neutrophil extracellular traps: The biology of chromatin externalization. *Dev. Cell* **2018**, *44*, 542–553. [CrossRef]

390. Van Avondt, K.; Hartl, D. Mechanisms and disease relevance of neutrophil extracellular trap formation. *Eur. J. Clin. Investig.* **2018**, *48*, e12919. [CrossRef]

391. Bonaventura, A.; Vecchié, A.; Abbate, A.; Montecucco, F. Neutrophil extracellular traps and cardiovascular diseases: An update. *Cells* **2020**, *9*, 231. [CrossRef]

392. Cicco, S.; Cicco, G.; Racanelli, V.; Vacca, A. Neutrophil extracellular traps (NETs) and damage-associated molecular patterns (DAMPs): Two potential targets for COVID-19 treatment. *Mediat. Inflamm.* **2020**, *2020*, 7527953. [CrossRef]

393. Guéant, J.L.; Fromonot, J.; Guéant-Rodriguez, R.M.; Lacolley, P.; Guieu, R.; Regnault, V. Blood myeloperoxidase-DNA, a biomarker of early response to SARS-CoV-2 infection? *Allergy* **2020**, *76*, 892–896. [CrossRef]

394. Pastorek, M.; Dúbrava, M.; Celec, P. On the Origin of Neutrophil Extracellular Traps in COVID-19. *Front. Immunol.* **2022**, *13*, 821007. [CrossRef]

395. Pastor, B.; Abraham, J.-D.; Pisareva, E.; Sanchez, C.; Kudriavstev, A.; Tanos, R.; Mirandola, A.; Mihalovičová, L.; Pezzella, V.; Adenis, A. Association of neutrophil extracellular traps with the production of circulating DNA in patients with colorectal cancer. *iScience* **2022**, *25*, 103826. [CrossRef]

396. Bruschi, M.; Moroni, G.; Sinico, R.A.; Franceschini, F.; Fredi, M.; Vaglio, A.; Cavagna, L.; Petretto, A.; Pratesi, F.; Migliorini, P. Neutrophil extracellular traps in the autoimmunity context. *Front. Med.* **2021**, *8*, 614829. [CrossRef]

397. Filep, J.G. Targeting Neutrophils for Promoting the Resolution of Inflammation. *Front. Immunol.* **2022**, *13*, 866747. [CrossRef]

398. Cao, J.; Su, J.; An, M.; Yang, Y.; Zhang, Y.; Zuo, J.; Zhang, N.; Zhao, Y. Novel DEK-targeting aptamer delivered by a hydrogel microneedle attenuates collagen-induced arthritis. *Mol. Pharm.* **2020**, *18*, 305–316. [CrossRef]

399. Chiang, N.; Sakuma, M.; Rodriguez, A.R.; Spur, B.W.; Irimia, D.; Serhan, C.N. Resolvin T-series reduce neutrophil extracellular traps. *Blood J. Am. Soc. Hematol.* **2022**, *139*, 1222–1233. [CrossRef]

400. Holliday, Z.M.; Earhart, A.P.; Alnijoumi, M.M.; Krvavac, A.; Allen, L.-A.H.; Schrum, A.G. Non-randomized trial of dornase alfa for acute respiratory distress syndrome secondary to COVID-19. *Front. Immunol.* **2021**, *12*, 714833. [CrossRef]

401. Ronquist, G. Prostasomes are mediators of intercellular communication: From basic research to clinical implications. *J. Intern. Med.* **2012**, *271*, 400–413. [CrossRef] [PubMed]

402. Liu, Y. A new perspective on Darwin's Pangenesis. *Biol. Rev.* **2008**, *83*, 141–149. [CrossRef] [PubMed]

403. Gahan, P. Circulating nucleic acids: Possible inherited effects. *Biol. J. Linn. Soc.* **2013**, *110*, 931–948. [CrossRef]

404. Bettegowda, C.; Sausen, M.; Leary, R.J.; Kinde, I.; Wang, Y.; Agrawal, N.; Bartlett, B.R.; Wang, H.; Luber, B.; Alani, R.M.; et al. Detection of circulating tumor DNA in early- and late-stage human malignancies. *Sci. Transl. Med.* **2014**, *6*, 224ra224. [CrossRef]

405. Deichmann, R.; Radsak, M.; Tug, S.; Helmig, S.; Deichmann, E.R.; Schmeier-Jürchott, A.; Wagner, E.; Zimmermann, T.; Radsak, M.; Giacca, M.; et al. Exercise-induced increases in cell free DNA in human plasma originate predominantly from cells of the haematopoietic lineage. *Exerc. Immunol. Rev.* **2015**, *21*, 164–173.

406. Lui, Y.Y.N.; Chik, K.W.; Chiu, R.W.K.; Ho, C.Y.; Lam, C.W.K.; Lo, Y.M.D. Predominant hematopoietic origin of cell-free dna in plasma and serum after sex-mismatched bone marrow transplantation. *Clin. Chem.* **2002**, *48*, 421–427. [CrossRef]

407. Wu, D.C.; Lambowitz, A.M. Facile single-stranded DNA sequencing of human plasma DNA via thermostable group II intron reverse transcriptase template switching. *Sci. Rep.* **2017**, *7*, 8421. [CrossRef]

408. Wong, F.C.; Sun, K.; Jiang, P.; Cheng, Y.K.; Chan, K.C.; Leung, T.Y.; Chiu, R.W.; Lo, Y.M. Cell-free DNA in maternal plasma and serum: A comparison of quantity, quality and tissue origin using genomic and epigenomic approaches. *Clin. Biochem.* **2016**, *49*, 1379–1386. [CrossRef]

409. Neuberger, E.W.; Sontag, S.; Brahmer, A.; Philippi, K.F.; Radsak, M.P.; Wagner, W.; Simon, P. Physical activity specifically evokes release of cell-free DNA from granulocytes thereby affecting liquid biopsy. *Clin. Epigenetics* **2022**, *14*, 29. [CrossRef]

410. Zemmour, H.; Planer, D.; Magenheim, J.; Moss, J.; Neiman, D.; Gilon, D.; Korach, A.; Glaser, B.; Shemer, R.; Landesberg, G.; et al. Non-invasive detection of human cardiomyocyte death using methylation patterns of circulating DNA. *Nat. Commun.* **2018**, *9*, 1443. [CrossRef]

411. Kang, S.; Li, Q.; Chen, Q.; Zhou, Y.; Park, S.; Lee, G.; Grimes, B.; Krysan, K.; Yu, M.; Wang, W.; et al. CancerLocator: Non-invasive cancer diagnosis and tissue-of-origin prediction using methylation profiles of cell-free DNA. *Genome Biol.* **2017**, *18*, 53. [CrossRef]

412. Kundaje, A.; Meuleman, W.; Ernst, J.; Bilenky, M.; Yen, A.; Heravi-Moussavi, A.; Kheradpour, P.; Zhang, Z.; Wang, J.; Ziller, M.J.; et al. Integrative analysis of 111 reference human epigenomes. *Nature* **2015**, *518*, 317–330. [CrossRef]

413. Fernandez, A.F.; Assenov, Y.; Martin-Subero, J.I.; Balint, B.; Siebert, R.; Taniguchi, H.; Yamamoto, H.; Hidalgo, M.; Tan, A.C.; Galm, O.; et al. A DNA methylation fingerprint of 1628 human samples. *Genome Res.* **2012**, *22*, 407–419. [CrossRef] [PubMed]

414. Panagopoulou, M.; Karaglani, M.; Balgkouranidou, I.; Pantazi, C.; Kolios, G.; Kakolyris, S.; Chatzaki, E. Circulating cell-free DNA release in vitro: Kinetics, size profiling, and cancer-related gene methylation. *J. Cell Physiol.* **2019**, *234*, 14079–14089. [CrossRef]

415. Aucamp, J.; Bronkhorst, A.J.; Peters, D.L.; Van Dyk, H.C.; Van der Westhuizen, F.H.; Pretorius, P.J. Kinetic analysis, size profiling, and bioenergetic association of DNA released by selected cell lines in vitro. *Cell Mol. Life Sci.* **2017**, *74*, 2689–2707. [CrossRef] [PubMed]

416. Wang, W.; Kong, P.; Ma, G.; Li, L.; Zhu, J.; Xia, T.; Xie, H.; Zhou, W.; Wang, S. Characterization of the release and biological significance of cell-free DNA from breast cancer cell lines. *Oncotarget* **2017**, *8*, 43180–43191. [CrossRef] [PubMed]

417. Bronkhorst, A.J.; Wentzel, J.F.; Aucamp, J.; van Dyk, E.; du Plessis, L.; Pretorius, P.J. Characterization of the cell-free DNA released by cultured cancer cells. *Biochim. Biophys. Acta* **2016**, *1863*, 157–165. [CrossRef]

418. Stroun, M.; Lyautey, J.; Lederrey, C.; Olson-Sand, A.; Anker, P. About the possible origin and mechanism of circulating DNA Apoptosis and active DNA release. *Clin. Chim. Acta* **2001**, *313*, 139–142. [CrossRef]

419. Stroun, M.; Anker, P.; Beljanski, M.; Henri, J.; Lederrey, C.; Ojha, M.; Maurice, P.A. Presence of RNA in the nucleoprotein complex spontaneously released by human lymphocytes and frog auricles in culture. *Cancer Res.* **1978**, *38*, 3546–3554.

420. Anker, P.; Stroun, M.; Maurice, P.A. Spontaneous release of DNA by human blood lymphocytes as shown in an in vitro system. *Cancer Res.* **1975**, *35*, 2375–2382.

421. Stroun, M.; Anker, P. Nucleic acids spontaneously released by living frog auricles. *Biochem. J.* **1972**, *128*, 100P–101P. [CrossRef]

422. Borenstein, S.; Ephrati-Elizur, E. Spontaneous release of DNA in sequential genetic order by Bacillus subtilis. *J. Mol. Biol.* **1969**, *45*, 137–152. [CrossRef]

423. Holdenrieder, S.; Stieber, P. Apoptotic markers in cancer. *Cinical Biochem.* **2004**, *37*, 605–617. [CrossRef]

424. Nagata, S.; Nagase, H.; Kawane, K.; Mukae, N.; Fukuyama, H. Degradation of chromosomal DNA during apoptosis. *Cell Death Differ.* **2003**, *10*, 108–116. [CrossRef]

425. Delgado, P.O.; Alves, B.C.; Gehrke Fde, S.; Kuniyoshi, R.K.; Wroclavski, M.L.; Del Giglio, A.; Fonseca, F.L. Characterization of cell-free circulating DNA in plasma in patients with prostate cancer. *Tumour Biol.* **2013**, *34*, 983–986. [CrossRef] [PubMed]

426. Jiang, W.W.; Zahurak, M.; Goldenberg, D.; Milman, Y.; Park, H.L.; Westra, W.H.; Koch, W.; Sidransky, D.; Califano, J. Increased plasma DNA integrity index in head and neck cancer patients. *Int. J. Cancer* **2006**, *119*, 2673–2676. [CrossRef] [PubMed]

427. Umetani, N.; Hiramatsu, S.; Hoon, D.S. Higher amount of free circulating DNA in serum than in plasma is not mainly caused by contaminated extraneous DNA during separation. *Ann. N. Y. Acad. Sci.* **2006**, *1075*, 299–307. [CrossRef]

428. Wang, B.G.; Huang, H.Y.; Chen, Y.C.; Bristow, R.E.; Kassauei, K.; Cheng, C.C.; Roden, R.; Sokoll, L.J.; Chan, D.W.; Shih Ie, M. Increased plasma DNA integrity in cancer patients. *Cancer Res.* **2003**, *63*, 3966–3968.

429. Formichi, P.; Radi, E.; Battisti, C.; Tarquini, E.; Leonini, A.; Di Stefano, A.; Federico, A. Human fibroblasts undergo oxidative stress-induced apoptosis without internucleosomal DNA fragmentation. *J. Cell. Physiol.* **2006**, *208*, 289–297. [CrossRef]

430. Ungerer, V.; Bronkhorst, A.J.; Van den Ackerveken, P.; Herzog, M.; Holdenrieder, S. Serial profiling of cell-free DNA and nucleosome histone modifications in cell cultures. *Sci. Rep.* **2021**, *11*, 9460. [CrossRef]

431. Sin, S.T.; Jiang, P.; Deng, J.; Ji, L.; Cheng, S.H.; Dutta, A.; Leung, T.Y.; Chan, K.A.; Chiu, R.W.; Lo, Y.D. Identification and characterization of extrachromosomal circular DNA in maternal plasma. *Proc. Natl. Acad. Sci. USA* **2020**, *117*, 1658–1665. [CrossRef]

432. Zhu, J.; Zhang, F.; Du, M.; Zhang, P.; Fu, S.; Wang, L. Molecular characterization of cell-free eccDNAs in human plasma. *Sci. Rep.* **2017**, *7*, 10968. [CrossRef]

433. Kumar, P.; Dillon, L.W.; Shibata, Y.; Jazaeri, A.A.; Jones, D.R.; Dutta, A. Normal and Cancerous Tissues Release Extrachromosomal Circular DNA (eccDNA) into the Circulation. *Mol. Cancer Res.* **2017**, *15*, 1197–1205. [CrossRef] [PubMed]

434. Sin, S.T.; Ji, L.; Deng, J.; Jiang, P.; Cheng, S.H.; Heung, M.M.; Lau, C.S.; Leung, T.Y.; Chan, K.A.; Chiu, R.W. Characteristics of fetal extrachromosomal circular DNA in maternal plasma: Methylation status and clearance. *Clin. Chem.* **2021**, *67*, 788–796. [CrossRef]

435. Brahimi-Horn, M.C.; Chiche, J.; Pouysségur, J. Hypoxia and cancer. *J. Mol. Med.* **2007**, *85*, 1301–1307. [CrossRef] [PubMed]

436. Sidransky, D.; Tokino, T.; Kinzler, K.W.; Levin, B.; Frost, P.; Vogelstein, B. Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. *Science* **1992**, *256*, 102–105. [CrossRef] [PubMed]

437. Kinde, I.; Bettegowda, C.; Wang, Y.; Wu, J.; Agrawal, N.; Shih Ie, M.; Kurman, R.; Dao, F.; Levine, D.A.; Giuntoli, R.; et al. Evaluation of DNA from the Papanicolaou test to detect ovarian and endometrial cancers. *Sci. Transl. Med.* **2013**, *5*, 167ra164. [CrossRef]

438. Nair, N.; Camacho-Vanegas, O.; Rykunov, D.; Dashkoff, M.; Camacho, S.C.; Schumacher, C.A.; Irish, J.C.; Harkins, T.T.; Freeman, E.; Garcia, I.; et al. Genomic Analysis of Uterine Lavage Fluid Detects Early Endometrial Cancers and Reveals a Prevalent Landscape of Driver Mutations in Women without Histopathologic Evidence of Cancer: A Prospective Cross-Sectional Study. *PLoS Med.* **2016**, *13*, e1002206. [CrossRef]

439. Patel, K.M.; Tsui, D.W.Y. The translational potential of circulating tumour DNA in oncology. *Clin. Biochem.* **2015**, *48*, 957–961. [CrossRef]

440. Birkenkamp-Demtröder, K.; Nordentoft, I.; Christensen, E.; Høyer, S.; Reinert, T.; Vang, S.; Borre, M.; Agerbæk, M.; Jensen, J.B.; Ørntoft, T.F.; et al. Genomic Alterations in Liquid Biopsies from Patients with Bladder Cancer. *Eur. Urol.* **2016**, *70*, 75–82. [CrossRef]

441. Birkenkamp-Demtröder, K.; Christensen, E.; Nordentoft, I.; Knudsen, M.; Taber, A.; Høyer, S.; Lamy, P.; Agerbæk, M.; Jensen, J.B.; Dyrskjøt, L. Monitoring Treatment Response and Metastatic Relapse in Advanced Bladder Cancer by Liquid Biopsy Analysis. *Eur. Urol.* **2018**, *73*, 535–540. [CrossRef]

442. Rentsch, C.A.; Müller, D.C.; Ruiz, C.; Bubendorf, L. Moving Towards Minimally Invasive Genomically Based Diagnosis and Monitoring of Bladder Cancer. *Eur. Urol.* **2016**, *70*, 83–84. [CrossRef]

443. Alekseeva, L.A.; Mironova, N.L.; Brenner, E.V.; Kurilshikov, A.M.; Patutina, O.A.; Zenkova, M.A. Alteration of the exDNA profile in blood serum of LLC-bearing mice under the decrease of tumour invasion potential by bovine pancreatic DNase I treatment. *PLoS ONE* **2017**, *12*, e0171988. [CrossRef]

444. Tamkovich, S.N.; Cherepanova, A.V.; Kolesnikova, E.V.; Rykova, E.Y.; Pyshnyi, D.V.; Vlassov, V.V.; Laktionov, P.P. Circulating DNA and DNase activity in human blood. *Ann. N. Y. Acad. Sci.* **2006**, *1075*, 191–196. [CrossRef]

445. Serpas, L.; Chan, R.W.Y.; Jiang, P.; Ni, M.; Sun, K.; Rashidfarrokhi, A.; Soni, C.; Sisirak, V.; Lee, W.S.; Cheng, S.H.; et al. Dnase1l3 deletion causes aberrations in length and end-motif frequencies in plasma DNA. *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 641–649. [CrossRef]

446. Shi, J.; Zhang, R.; Li, J.; Zhang, R. Size profile of cell-free DNA: A beacon guiding the practice and innovation of clinical testing. *Theranostics* **2020**, *10*, 4737. [CrossRef]

447. Allan, J.; Fraser, R.M.; Owen-Hughes, T.; Keszenman-Pereyra, D. Micrococcal nuclease does not substantially bias nucleosome mapping. *J. Mol. Biol.* **2012**, *417*, 152–164. [CrossRef]

448. Ungerer, V.; Bronkhorst, A.J.; Uhlig, C.; Holdenrieder, S. Cell-Free DNA Fragmentation Patterns in a Cancer Cell Line. *Diagnostics* **2022**, *12*, 1896. [CrossRef]

449. Choy, L.; Peng, W.; Jiang, P.; Cheng, S.H.; Yu, S.C.; Shang, H.; Olivia Tse, O.; Wong, J.; Wong, V.W.S.; Wong, G.L. Single-Molecule Sequencing Enables Long Cell-Free DNA Detection and Direct Methylation Analysis for Cancer Patients. *Clin. Chem.* **2022**, hvac086. [CrossRef]

450. Alcaide, M.; Cheung, M.; Hillman, J.; Rassekh, S.R.; Deyell, R.J.; Batist, G.; Karsan, A.; Wyatt, A.W.; Johnson, N.; Scott, D.W. Evaluating the quantity, quality and size distribution of cell-free DNA by multiplex droplet digital PCR. *Sci. Rep.* **2020**, *10*, 12564. [CrossRef] [PubMed]

451. Heider, K.; Wan, J.C.; Hall, J.; Belic, J.; Boyle, S.; Hudecova, I.; Gale, D.; Cooper, W.N.; Corrie, P.G.; Brenton, J.D. Detection of ctDNA from dried blood spots after DNA size selection. *Clin. Chem.* **2020**, *66*, 697–705. [CrossRef] [PubMed]

452. Lee, E.Y.; Lee, E.-J.; Yoon, H.; Lee, D.H.; Kim, K.H. Comparison of Four Commercial Kits for Isolation of Urinary Cell-Free DNA and Sample Storage Conditions. *Diagnostics* **2020**, *10*, 234. [CrossRef]

453. Augustus, E.; Van Casteren, K.; Sorber, L.; van Dam, P.; Roeyen, G.; Peeters, M.; Vorsters, A.; Wouters, A.; Raskin, J.; Rolfo, C. The art of obtaining a high yield of cell-free DNA from urine. *PLoS ONE* **2020**, *15*, e0231058. [CrossRef] [PubMed]

454. Ulz, P.; Auer, M.; Heitzer, E. Detection of circulating tumor DNA in the blood of cancer patients: An important tool in cancer chemoprevention. In *Cancer Chemoprevention*; Springer: Berlin/Heidelberg, Germany, 2016; pp. 45–68.

455. Shen, N.; Zhang, D.; Yin, L.; Qiu, Y.; Liu, J.; Yu, W.; Fu, X.; Zhu, B.; Xu, X.; Duan, A. Bile cell-free DNA as a novel and powerful liquid biopsy for detecting somatic variants in biliary tract cancer. *Oncol. Rep.* **2019**, *42*, 549–560. [CrossRef]

456. Fernando, M.R.; Jiang, C.; Krzyzanowski, G.D.; Ryan, W.L. Analysis of human blood plasma cell-free DNA fragment size distribution using EvaGreen chemistry based droplet digital PCR assays. *Clin. Chim. Acta* **2018**, *483*, 39–47. [CrossRef]

457. Nakashima, C.; Sato, A.; Abe, T.; Kato, J.; Hirai, M.; Nakamura, T.; Komiya, K.; Sueoka, E.; Kimura, S.; Sueoka-Aragane, N. Automated DNA extraction using cellulose magnetic beads can improve EGFR point mutation detection with liquid biopsy by efficiently recovering short and long DNA fragments. *Oncotarget* **2018**, *9*, 25181–25192. [CrossRef]

458. Aucamp, J.; Calitz, C.; Bronkhorst, A.J.; Wrzesinski, K.; Hamman, S.; Gouws, C.; Pretorius, P.J. Cell-free DNA in a three-dimensional spheroid cell culture model: A preliminary study. *Int. J. Biochem. Cell Biol.* **2017**, *89*, 182–192. [CrossRef] [PubMed]

459. Aucamp, J.; Van Dyk, H.C.; Bronkhorst, A.J.; Pretorius, P.J. Valproic acid alters the content and function of the cell-free DNA released by hepatocellular carcinoma (HepG2) cells in vitro. *Biochimie* **2017**, *140*, 93–105. [CrossRef] [PubMed]

460. Hellwig, S.; Nix, D.A.; Gligorich, K.M.; O'Shea, J.M.; Thomas, A.; Fuertes, C.L.; Bhetariya, P.J.; Marth, G.T.; Bronner, M.P.; Underhill, H.R. Automated size selection for short cell-free DNA fragments enriches for circulating tumor DNA and improves error correction during next generation sequencing. *PLoS ONE* **2018**, *13*, e0197333. [CrossRef] [PubMed]

461. Underhill, H.R.; Kitzman, J.O.; Hellwig, S.; Welker, N.C.; Daza, R.; Baker, D.N.; Gligorich, K.M.; Rostomily, R.C.; Bronner, M.P.; Shendure, J. Fragment Length of Circulating Tumor DNA. *PLoS Genet.* **2016**, *12*, e1006162. [CrossRef]

462. Mouliere, F.; Robert, B.; Arnau Peyrotte, E.; Del Rio, M.; Ychou, M.; Molina, F.; Gongora, C.; Thierry, A.R. High fragmentation characterizes tumour-derived circulating DNA. *PLoS ONE* **2011**, *6*, e23418. [CrossRef]

463. Hudecova, I.; Smith, C.G.; Hänsel-Hertsch, R.; Chilamakuri, C.S.; Morris, J.A.; Vijayaraghavan, A.; Heider, K.; Chandrananda, D.; Cooper, W.N.; Gale, D. Characteristics, origin, and potential for cancer diagnostics of ultrashort plasma cell-free DNA. *Genome Res.* **2022**, *32*, 215–227. [CrossRef]

464. Cheng, J.; Morselli, M.; Huang, W.-L.; Heo, Y.J.; Pinheiro-Ferreira, T.; Li, F.; Wei, F.; Chia, D.; Kim, Y.; He, H.-J. Plasma contains ultrashort single-stranded DNA in addition to nucleosomal cell-free DNA. *iScience* **2022**, *25*, 104554. [CrossRef]

465. Diehl, F.; Li, M.; Dressman, D.; He, Y.; Shen, D.; Szabo, S.; Diaz, L.A., Jr.; Goodman, S.N.; David, K.A.; Juhl, H.; et al. Detection and quantification of mutations in the plasma of patients with colorectal tumors. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 16368–16373. [CrossRef]

466. Chused, T.M.; Steinbergt, A.D.; Talalt, N. The Clearance and Localization of Nucleic Acids by New Zealand and Normal Mice. *Clin. Exp. Immunol.* **1972**, *12*, 465–476.

467. Reckamp, K.L.; Melnikova, V.O.; Karlovich, C.; Sequist, L.V.; Camidge, D.R.; Wakelee, H.; Perol, M.; Oxnard, G.R.; Kosco, K.; Croucher, P.; et al. A Highly Sensitive and Quantitative Test Platform for Detection of NSCLC EGFR Mutations in Urine and Plasma. *J. Thorac. Oncol.* **2016**, *11*, 1690–1700. [CrossRef]

468. Miranda, K.C.; Bond, D.T.; McKee, M.; Skog, J.; Paunescu, T.G.; Da Silva, N.; Brown, D.; Russo, L.M. Nucleic acids within urinary exosomes/microvesicles are potential biomarkers for renal disease. *Kidney Int.* **2010**, *78*, 191–199. [CrossRef]

469. Botezatu, I.; Serdyuk, O.; Potapova, G.; Shelepov, V.; Alechina, R.; Molyaka, Y.; Ananev, V.; Bazin, I.; Garin, A.; Narimanov, M.; et al. Genetic analysis of DNA excreted in urine: A new approach for detecting specific genomic DNA sequences from cells dying in an organism. *Clin. Chem.* **2000**, *46*, 1078–1084. [CrossRef]

470. Tamkovich, S.; Laktionov, P. Cell-surface-bound circulating DNA in the blood: Biology and clinical application. *IUBMB Life* **2019**, *71*, 1201–1210. [CrossRef] [PubMed]

471. Tagawa, M.; Shimbo, G.; Inokuma, H.; Miyahara, K. Quantification of plasma cell-free DNA levels in dogs with various tumors. *J. Vet. Diagn. Investig.* **2019**, *31*, 836–843. [CrossRef]

472. Wilson-Robles, H.; Miller, T.; Jarvis, J.; Terrell, J.; Dewsbury, N.; Kelly, T.; Herzog, M.; Bygott, T.; Hardat, N.; Michel, G. Evaluation of nucleosome concentrations in healthy dogs and dogs with cancer. *PLoS ONE* **2020**, *15*, e0236228. [CrossRef] [PubMed]

473. Wilson-Robles, H.; Bygott, T.; Kelly, T.K.; Miller, T.; Miller, P.; Matsushita, M.; Terrell, J.; Bougoussa, M.; Butera, T. Evaluation of Plasma Nucleosome Concentrations In Healthy Dogs And Dogs With A Variety of Common Cancers. *Res. Artic.* **2021**. [CrossRef]

474. Chibuk, J.; Flory, A.; Kruglyak, K.M.; Leibman, N.; Nahama, A.; Dharajiya, N.; Boom, D.V.D.; Jensen, T.J.; Friedman, J.S.; Shen, M.R.; et al. Horizons in Veterinary Precision Oncology: Fundamentals of Cancer Genomics and Applications of Liquid Biopsy for the Detection, Characterization, and Management of Cancer in Dogs. *Front. Vet. Sci.* **2021**, *8*. [CrossRef]

475. Burnett, D.L.; Cave, N.J.; Gedye, K.R.; Bridges, J.P. Investigation of cell-free DNA in canine plasma and its relation to disease. *Vet. Q.* **2016**, *36*, 122–129. [CrossRef]

476. Beck, J.; Hennecke, S.; Bornemann-Kolatzki, K.; Urnovitz, H.B.; Neumann, S.; Ströbel, P.; Kaup, F.-J.; Brenig, B.; Schütz, E. Genome Aberrations in Canine Mammary Carcinomas and Their Detection in Cell-Free Plasma DNA. *PLoS ONE* **2013**, *8*, e75485. [CrossRef]

477. Beffagna, G.; Sammarco, A.; Bedin, C.; Romualdi, C.; Mainenti, M.; Mollo, A.; Cavicchioli, L.; Ferro, S.; Trez, D.; DE Maria, R.; et al. Circulating Cell-Free DNA in Dogs with Mammary Tumors: Short and Long Fragments and Integrity Index. *PLoS ONE* **2017**, *12*, e0169454. [CrossRef] [PubMed]

478. Troia, R.; Giunti, M.; Calipa, S.; Goggs, R. Cell-Free DNA, high-mobility group box-1, and procalcitonin concentrations in dogs with gastric dilatation–volvulus syndrome. *Front. Vet. Sci.* **2018**, *5*, 67. [CrossRef] [PubMed]

479. Jeffery, U.; Ruterbories, L.; Hanel, R.; LeVine, D. Cell-Free DNA and DN ase Activity in Dogs with Immune-Mediated Hemolytic Anemia. *J. Vet. Intern. Med.* **2017**, *31*, 1441–1450. [CrossRef]

480. Wilson, I.; Burchell, R.; Worth, A.; Burton, S.; Gedye, K.; Clark, K.; Crosse, K.; Jack, M.; Odom, T.; De Grey, S.; et al. Kinetics of Plasma Cell-Free DNA and Creatine Kinase in a Canine Model of Tissue Injury. *J. Vet. Intern. Med.* **2017**, *32*, 157–164. [CrossRef]

481. Letendre, J.-A.; Goggs, R. Determining prognosis in canine sepsis by bedside measurement of cell-free DNA and nucleosomes. *J. Vet. Emerg. Crit. Care* **2018**, *28*, 503–511. [CrossRef]

482. Letendre, J.A.; Goggs, R. Measurement of plasma cell-free DNA concentrations in dogs with sepsis, trauma, and neoplasia. *J. Vet. Emerg. Crit. Care* **2017**, *27*, 307–314. [CrossRef]

483. Li, R.H.L.; Johnson, L.R.; Kohen, C.; Tablin, F. A novel approach to identifying and quantifying neutrophil extracellular trap formation in septic dogs using immunofluorescence microscopy. *BMC Vet. Res.* **2018**, *14*, 210. [CrossRef]

484. Hunt, H.; Cave, N.; Bridges, J.; Gedye, K.; Hill, K. Plasma NT-proBNP and Cell-Free DNA Concentrations after Prolonged Strenuous Exercise in Working Farm Dogs. *J. Vet. Intern. Med.* **2018**, *32*, 135–141. [CrossRef]

485. Rushton, J.G.; Ertl, R.; Klein, D.; Tichy, A.; Nell, B. Circulating cell-free DNA does not harbour a diagnostic benefit in cats with feline diffuse iris melanomas. *J. Feline Med. Surg.* **2019**, *21*, 124–132. [CrossRef]

486. Kowarsky, M.; De Vlaminck, I.; Okamoto, J.; Neff, N.F.; LeBreton, M.; Nwobegabay, J.; Tamoufe, U.; Ledoux, J.D.; Tafon, B.; Kiyang, J. Cell-free DNA Reveals Potential Zoonotic Reservoirs in Non-Human Primates. *bioRxiv* **2018**. [CrossRef]

487. Ohayon, S.; Boyko, M.; Saad, A.; Douvdevani, A.; Gruenbaum, B.F.; Melamed, I.; Shapira, Y.; Teichberg, V.I.; Zlotnik, A. Cell-Free DNA as a Marker for Prediction of Brain Damage in Traumatic Brain Injury in Rats. *J. Neurotrauma* **2012**, *29*, 261–267. [CrossRef] [PubMed]

488. Gruenbaum, B.F.; Boyko, M.; Delgado, B.; Douvdevany, A.; Gruenbaum, S.E.; Melamed, I.; Gideon, M.; Cesnulis, E.; Shapira, Y.; Zlotnik, A. Cell-free DNA as a potential marker to predict carbon tetrachloride-induced acute liver injury in rats. *Hepatol. Int.* **2013**, *7*, 721–727. [CrossRef] [PubMed]

489. Manueldas, S.; Benterud, T.; Rueegg, C.S.; Garberg, H.T.; Huun, M.U.; Pankratov, L.; Åsegg-Atneosen, M.; Solberg, R.; Escobar, J.; Saugstad, O.D.; et al. Temporal patterns of circulating cell-free DNA (cfDNA) in a newborn piglet model of perinatal asphyxia. *PLoS ONE* **2018**, *13*, e0206601. [CrossRef]

490. García-Olmo, D.C.; Samos, J.; Picazo, M.G.; Asensio, A.I.; Toboso, I.; García-Olmo, D. Release of cell-free DNA into the bloodstream leads to high levels of non-tumor plasma DNA during tumor progression in rats. *Cancer Lett.* **2008**, *272*, 133–140. [CrossRef]

491. García-Olmo, D.C.; Picazo, M.G.; Toboso, I.; I Asensio, A.; García-Olmo, D. Quantitation of cell-free DNA and RNA in plasma during tumor progression in rats. *Mol. Cancer* **2013**, *12*, 8. [CrossRef]

492. Phillippe, M.; Adeli, S. Cell-free DNA release by mouse placental explants. *PLoS ONE* **2017**, *12*, e0178845. [CrossRef]

493. Gasparello, J.; Allegretti, M.; Tremante, E.; Fabbri, E.; Amoreo, C.A.; Romania, P.; Melucci, E.; Messana, K.; Borgatti, M.; Giacomini, P.; et al. Liquid biopsy in mice bearing colorectal carcinoma xenografts: Gateways regulating the levels of circulating tumor DNA (ctDNA) and miRNA (ctmiRNA). *J. Exp. Clin. Cancer Res.* **2018**, *37*, 1–11. [CrossRef]

494. Heitzer, E.; Auinger, L.; Speicher, M.R. Cell-Free DNA and Apoptosis: How Dead Cells Inform About the Living. *Trends Mol. Med.* **2020**, *26*, 519–528. [CrossRef]

495. Ibn Sina, A.A.; Lin, T.-Y.; Vaidyanathan, R.; Wang, Z.; Dey, S.; Wang, J.; Behren, A.; Wuethrich, A.; Carrascosa, L.G.; Trau, M. Methylation dependent gold adsorption behaviour identifies cancer derived extracellular vesicular DNA. *Nanoscale Horiz.* **2020**, *5*, 1317–1323. [CrossRef]