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REVIEW

Liquid Biopsy in Neuropsychiatric Disorders: A Step Closer to Precision Medicine

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Abstract

Psychiatric disorders are among the leading causes of disease burden worldwide. Despite their significant impact, their diagnosis remains challenging due to symptom heterogeneity, psychiatric comorbidity, and the lack of objective diagnostic tests and well-defined biomarkers. Leveraging genomic, epigenomic, and fragmentomic technologies, circulating cell-free DNA (ccfDNA)–based liquid biopsies have emerged as a potential non-invasive diagnosis and disease-monitoring tool. ccfDNA is a DNA species released into circulation from all types of cells through passive and active mechanisms and can serve as a biomarker for various diseases, namely, cancer. Despite their potential, the application of ccfDNA in neuropsychiatry remains underdeveloped. In this review, we provide an overview of liquid biopsies and their components, with a particular focus on ccfDNA. With a summary of pre-analytical practices and current ccfDNA technologies, we highlight the current state of research regarding the use of ccfDNA as a biomarker for neuropsychiatric disorders. Finally, we discuss future steps to unlock ccfDNA's potential in clinical practice.

Keywords Liquid Biopsy · Cell-free Nucleic Acids · Cell-free DNA · Cell-free Mitochondrial DNA

Neuropsychiatric disorders affect more than one billion people worldwide [1]. They are among the leading causes of disability, contributing to a large global burden of disease [2]. They account for approximately 20% of all years lived with disability, which measures the impact of an illness on quality of life, and they represent over 400 million disability-adjusted life years globally [1, 3]. Neuropsychiatric disorders are associated with excess mortality, with individuals experiencing these conditions facing a considerably higher risk of premature death compared to the general population [1, 3]. Their economic burden is also substantial, estimated at 5 trillion USD, due to direct medical costs and indirect healthcare costs such as unemployment, absenteeism, and presenteeism [3]. Despite their impact, the diagnosis and treatment of neuropsychiatric disorders remain challenging for several reasons. Their clinical presentations are highly heterogeneous, and the same disorder can present a range of symptoms in different individuals. There is a large symptomatology overlap across different disorders, and comorbidity is the rule rather than the exception [4]. In light of these challenges, there is a pressing need for objective, reliable, and non-invasive measures for diagnosis. Liquid biopsy has emerged as a promising method.

Liquid biopsy, well-known for its non-invasiveness or minimal invasiveness, is a method of analyzing biomolecules in biological fluids. These biomolecules include circulating tumor cells, circulating cell-free DNA (ccfDNA), circulating cell-free RNA, extracellular vesicles and their content (e.g., proteins, DNA, RNA, and lipids), proteins, and metabolites [5]. With the wide availability and accessibility of various genomic, epigenomic, and fragmentomic techniques, ccfDNA-based liquid biopsies have emerged as a promising tool for detection and diagnosis, as well as monitoring of disease progression and treatment response [6].

This review provides an overview of liquid biopsies, with a particular focus on ccfDNA measurement technologies in plasma, serum, and cerebrospinal fluid (CSF). We will also

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summarize the current state of research regarding the use of ccfDNA as a biomarker for neuropsychiatric disorders. Our aim is to offer a perspective on the potential role of ccfDNA in advancing the diagnosis, prognosis, monitoring, and treatment selection for these disorders. Additionally, this paper offers a comprehensive overview of pre-analytical considerations and the broad spectrum of analyses possible with ccfDNA, including its utility in tissue of origin analysis and detection of methylation changes.

Liquid Biopsy

Liquid biopsies have emerged as an alternative to tissue biopsies in cancer. Unlike conventional biopsies, which require invasive procedures (e.g., surgical extractions of a tumor) and are costly and carry the risk of complications, liquid biopsies are minimally invasive, are more cost-effective, and have a faster turnaround time. This allows for serial and real-time monitoring of disease progression and treatment response [7]. This approach involves analyzing various biomarkers present in bodily fluids, most commonly plasma, but also serum, CSF, urine, and saliva.

Circulating Tumor Cells

The concept of liquid biopsy began with the identification of circulating tumor cells, which are cells that detach from the primary tumor or metastatic lesions and enter the bloodstream [8]. These cells are rare in the blood, with 1–10 circulating tumor cells per milliliter of blood, and have a short half-life of up to 2.4 h [9, 10]. The emergence of single-cell omics has enabled the identification of subpopulations of circulating tumor cells, providing a clearer understanding of cancer heterogeneity, progression, and response to treatment [11, 12]. Alongside circulating tumor cells, liquid biopsies encompass a diverse array of components, namely, tumor-educated platelets, extracellular vesicles, and circulating nucleic acids.

Tumor-Educated Platelets

Tumor-educated platelets are circulating anucleate cells that play a role in wound healing, homeostasis, and responses to tumor growth [13]. These cells become "tumor-educated" through the uptake of tumoral RNA and proteins, which alter their transcription, translation, and splicing profiles [14]. This distinct phenotype makes tumor-educated platelets an important biomarker for cancer diagnosis and prognosis [14, 15].

Extracellular Vesicles

Extracellular vesicles are membrane-bound vesicles secreted by all cells and have gained attention recently due to their unique molecular composition, which includes nucleic acids, proteins, and lipids. A key characteristic of extracellular vesicles is that their surface proteins carry the molecular signatures of their parental cells [16, 17]. Extracellular vesicles are classified into three types—exosomes, microvesicles, and apoptotic bodies—based on their biogenesis pathway, size, and cargo [18]. These vesicles may serve as biomarkers for various diseases through their isolation and the analysis of their contents, including the concentration of vesicles in biofluids, sequencing of nucleic acids, and proteomic and lipidomic analyses [19].

Circulating Nucleic Acids

Circulating nucleic acids (ccfDNA, circulating cell-free mitochondrial DNA, circulating tumor DNA, and circulating cell-free RNA) play a pivotal role in liquid biopsies. These nucleic acids are released from cells through apoptosis, necrosis, NETosis, or active secretion [20]. While ccfRNA holds valuable information, this review will specifically focus on ccfDNA.

The advancement of next-generation sequencing techniques allowed for the detection of point mutations, deletions, identification of the fragmentation profile, and epigenetic profiling of ccfDNA. These techniques offer insights into the cellular sources and the mechanisms of cellular release of circulating nucleic acids in multiple physiological and pathological conditions [21]. The distinct characteristics of ccfDNA, such as reflecting genetic alterations and molecular signatures of both healthy and pathological states, make them valuable targets for analysis. To appreciate the significance of ccfDNA as a biomarker, we must first examine its origins and unique characteristics.

Circulating Cell-free DNA: Characteristics and Origins

DNA circulating in the plasma was first described in 1948 [22]. However, its potential was not fully appreciated until decades later when research showed that individuals with systemic lupus erythematosus [23] and cancer [24] exhibited higher levels of ccfDNA compared to healthy individuals. Subsequent studies demonstrated that ccfDNA isolated from cancer patients was in part of tumoral origin

[25] and harbored identical mutations to those found in tumor DNA [26, 27]; thus, the idea of ccfDNA-based liquid biopsy was born.

The ccfDNA is a highly fragmented DNA species present in low concentrations in healthy individuals, usually between 1 and 20 ng/mL [28]. These levels are influenced by factors such as sex, age, exercise, inflammation, pregnancy, and disease states like cancer, systemic inflammatory diseases, and stroke [28–33]. Distinct levels of ccfDNA have also been reported for neuropsychiatric disorders which can vary from the typical concentrations found in healthy individuals [34–37]. While ccfDNA is predominantly of hematopoietic origin, all cell types contribute to the ccfDNA population, including hepatocytes, endothelial cells, and neurons [38]. ccfDNA is also released from tumor cells, fetal cells, and mitochondria (ccf-mtDNA). The mechanisms through which these cells release DNA into circulation remain poorly understood, but it is suggested that release can occur through both passive (apoptosis, necrosis, pyroptosis) and active mechanisms (active secretion, NETosis) [20, 39, 40].

Apoptosis is generally considered the primary mechanism of ccfDNA release, as the fragment size and pattern of ccfDNA suggest DNA degradation by caspase-activated DNase during apoptosis [20]. Massive parallel sequencing and gel electrophoresis show that ccfDNA displays a pattern known as the "apoptotic ladder" with a major peak at ~ 166 bp, representing DNA wrapped around the nucleosomal unit (~ 146 bp) and the linker regions (~ 20 bp), followed by peaks at the dinucleosome (~ 332 bp), and trinucleosome (~ 500 bp) units [41, 42]. The peak at ~ 166 bp is also preceded by smaller peaks at 10-bp periodicities, indicative of enzymatic cleavage [42]. Fragments > 10 Kbp have also been observed in the plasma, suggesting necrotic origins [42, 43].

The ccfDNA can also be actively released by cells. Gahan and Stroun [44] suggested that DNA and RNA fragments are synthesized and complexed with lipoproteins, known as virtosomes, and then actively secreted by cells. NETosis is another active source of ccfDNA and involves the release of neutrophil extracellular traps (NETs), which consist of chromatin and proteins with antimicrobial properties to trap and eliminate microorganisms in the blood [45].

Following its release, the ccfDNA has a short half-life ranging from 4 min up to approximately 2 h [46]. These ccfDNA fragments are cleared from circulation through the action of nucleases in the plasma, with organs such as the liver, spleen, and kidneys also involved in this process [40] (see Fig. 1).

ccfDNA Collection and Processing

There are several challenges in using ccfDNA as a clinical biomarker, including its low concentration and short half-life, contamination with genomic DNA, and the lack of standardization in pre-analytical steps. The National Cancer Institute's Biorepositories and Biospecimen Research Branch [47] has released evidence-based guidelines to standardize the collection and processing of ccfDNA. These standards cover every step of the process, from the choice of biofluid and collection tubes to ccfDNA extraction and quantification. Regarding preferred biofluid choice, serum often contains higher concentrations of ccfDNA than plasma due to clotting during collection, making plasma the preferred choice for accurate ccfDNA composition assessment [48]. The Biorepositories and Biospecimen Research Branch recommends using anticoagulant-coated (EDTA, heparin, citrate) blood collection tubes, with EDTA being preferred, especially when blood processing is delayed ($\geq$ 6 h) and the tubes are stored at room temperature [47]. Tubes with other cell stabilizers may also be used to reduce genomic DNA contamination, particularly when blood processing is delayed for several days [49, 50]. However, for optimal processing, it is recommended to isolate plasma within 4 h of blood draw or up to a day if the blood is stored at 4 °C [51]. There is no standard for the volume of blood collection, as it varies depending on the intended downstream applications [47]. Plasma isolation should be performed in two steps: an initial centrifugation of 10 min at 1200 g–1900 g to isolate the plasma from the blood cells, followed by a second centrifugation at 1600 g for 10 min to minimize cellular contamination [52]. The second centrifugation can be performed immediately after the first one or after storage at – 20 °C or – 80 °C, as no significant changes in the ccfDNA content were observed when the second centrifugation was carried out following storage at these temperatures [53]. It is recommended to store plasma in aliquots of smaller volumes to reduce the number of freeze–thaw cycles and at – 80 °C for long-term storage (up to 10 years) [53]. Several ccfDNA extraction kits are commercially available, including the QIAamp circulating nucleic acid kit and the Maxwell RSC ccfDNA Plasma Kit [54]. The use of these kits is crucial to standardize ccfDNA extraction procedures across labs and ensure reproducibility among research findings. Finally, ccfDNA should be stored at – 20 °C or – 80 °C after extraction, with no more than three freeze–thaw cycles to preserve ccfDNA integrity for downstream analyses [53].

ccfDNA Analysis

The technologies for ccfDNA analyses are versatile, providing extensive information that aids in disease diagnosis, monitoring, and prognosis. Early studies focused on quantitative analysis of ccfDNA by comparing its concentration in individuals with diseases versus healthy controls. Real-time PCR is considered the gold standard for ccfDNA quantification. However, spectrophotometry (NanoDrop)

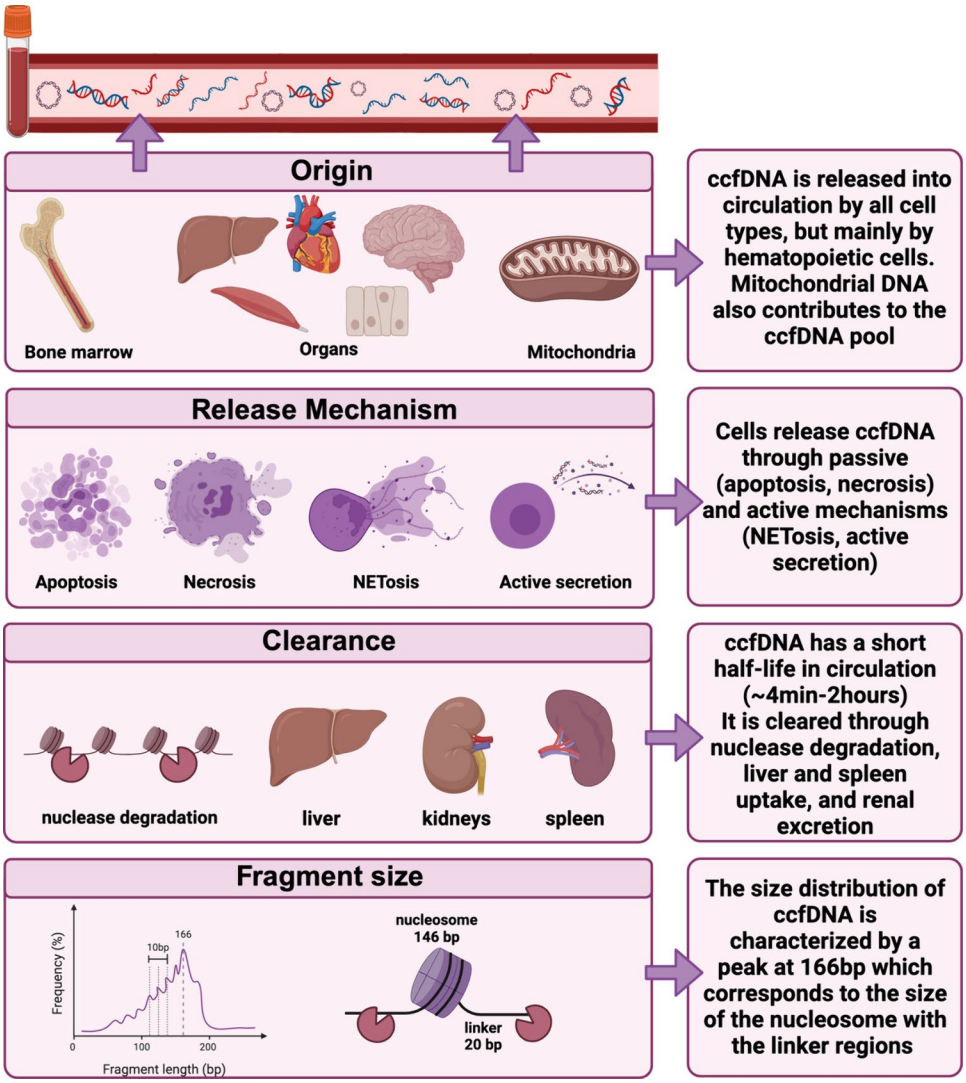

Fig. 1 Characteristics and origin of circulating cell-free DNA (ccfDNA). ccfDNA is released into circulation predominantly by hematopoietic cells as well as cells from other tissues and organs and their mitochondria. These cells release ccfDNA through passive mechanisms (apoptosis and necrosis) and active mechanisms (NETosis and active secretion). ccfDNA is later cleared from circulation through nuclease degradation, but organs such as the liver, kidneys, and spleen aid in its clearance. ccfDNA displays a pattern known as the apoptotic ladder with major peak at ~ 166 bp, which represents DNA wrapped around the nucleosomal unit (~ 146 bp) and the linker regions (~ 20 bp), preceded by smaller peaks at 10-bp periodicities

or fluorometry (PicoGreen, Qubit) methods have also been used to quantify ccfDNA, with results that correlate with qPCR quantification [55, 56].

Detection of mutant ccfDNA is also common in cancer and non-invasive prenatal testing. Using PCR technologies, including real-time PCR, droplet digital PCR, and BEAming, specific known mutations (*EGFR*, *KRAS*, *BRAF* mutations, etc.) can be detected with high sensitivity in plasma [57–59]. Other approaches include next-generation sequencing (NGS)–based techniques, such as whole-genome sequencing or whole-exome sequencing of ccfDNA, which can detect a larger number of mutations [60, 61]. Copy number variations and additional genetic aberrations (insertions, inversions, indels) are also detected using NGS techniques [58]. These methods are more accurate for detecting different types of cancers where mutations directly contribute to the disease but are less useful for studying multifactorial diseases, such as neuropsychiatric disorders, which often lack causal mutations or genomic alterations that can be identified through genetic profiling. This is where fragmentomic and epigenomic techniques have the potential to be more useful.

The size and fragmentation pattern of ccfDNA can also aid in disease detection. For instance, ccfDNA from individuals with cancer exhibits a fragmentation pattern different from the apoptotic ladder observed in healthy individuals; ccfDNA is more fragmented in cancer patients than healthy individuals [62, 63]. Investigating "preferred ends" is another aspect of fragmentomics. ccfDNA is generated by cleavage at selected regions, known as "preferred ends," which can shift in different disease states [64]. For instance, fetal and maternal ccfDNA have different preferred ends, and ccfDNA from cancer patients has different end motifs than healthy individuals [64, 65]. The fragmentation pattern of ccfDNA can also reveal the position of nucleosomes and transcription factors, which can inform the cellular origin of the fragment [66].

The identification of the cell or tissue of origin can also be achieved through epigenetic analysis of ccfDNA. Different tissues exhibit unique DNA methylation patterns that are conserved when DNA is released as ccfDNA [67]. Using methylation sequencing (e.g., bisulfite sequencing or enzymatic methylation sequencing), the methylation signature of ccfDNA can be compared to that of tissues to identify the cellular origin of the ccfDNA fragment [38] (see Fig. 2). For example, Chatterton et al. [68] demonstrated an increase in neuronal and glial ccfDNA following mild head trauma caused by blast wave exposure. This breakthrough is particularly important for neuropsychiatric disorders where direct access to brain tissue in most clinical settings and diseases is not feasible or clinically indicated.

The Potential of ccfDNA in Neuropsychiatric Disorders

The analysis of ccfDNA as a diagnostic or prognostic biomarker remains underdeveloped in neuropsychiatric disorders. Most studies have focused on ccfDNA of mitochondrial origin (ccf-mtDNA), with few exploring the genomic, epigenomic, and fragmentomic ccfDNA technologies for these conditions. While our review emphasizes ccfDNA-based approaches, ccfRNA-based approaches have also been explored elsewhere (see review by Kurtulmuş et al. [69]). A comprehensive summary of all the studies incorporated in this review is presented in Table 1.

Major Depressive Disorder

The results from studies on major depression and ccf-mtDNA are inconsistent. Some studies have suggested that individuals with major depressive disorder (MDD) have lower levels of ccf-mtDNA compared to non-depressed individuals [35, 70]. Conversely, others have found increased levels of ccf-mtDNA, particularly in older adults and those with suicidal behaviors and concurrent physical frailty [34, 37, 71, 72]. Additionally, ccf-mtDNA concentrations were lower in individuals who were acutely depressed compared to those in remission [35].

Lindqvist and colleagues [71] reported a significant increase in ccf-mtDNA in depressed individuals who did not respond to 8 weeks of SSRI treatment. On the other hand, patients who responded to treatment had ccf-mtDNA levels similar to non-depressed individuals. In contrast, Fernström et al. [70] suggest that individuals taking mood stabilizers (lamotrigine, valproic acid, lithium), but not antidepressants, had significantly lower levels of ccf-mtDNA compared to those who were not.

Psychological Stress

The first study to investigate the relationship between psychological stress and ccfDNA examined the effect of stress reduction techniques on ccfDNA levels. They found that cognitive-behavioral intervention and stress reduction techniques reduced cell-free DNA levels in women undergoing in vitro fertilization treatment [73]. Subsequent studies investigated the effect of inducing acute psychological stress on ccfDNA levels. Hummel et al. [75] and Herhaus et al. [74] found that ccfDNA level increased immediately following acute stress induction in the plasma of men, returning to baseline levels 15–30 min later. Additionally, men with higher baseline levels of ccfDNA showed a stronger increase in plasma ccfDNA following stress induction [74]. Acute stress also immediately increased ccf-mtDNA levels

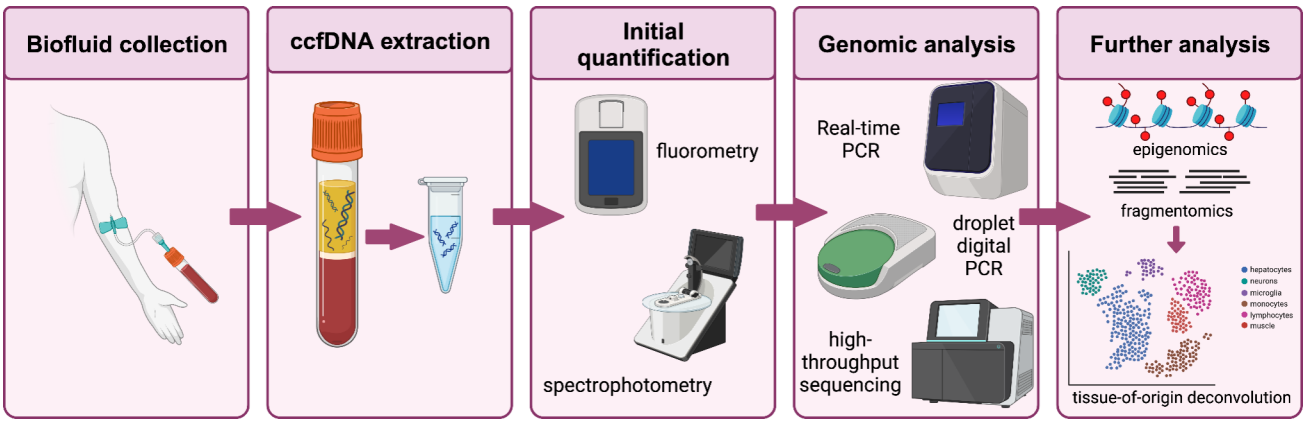

Fig. 2 Workflow of ccfDNA liquid biopsy. ccfDNA liquid biopsies start with biofluid collection then extraction of ccfDNA using commercially available kits. Initial analysis includes ccfDNA quantification via fluorometric or spectrophotometric assays. ccfDNA can then be analyzed by real-time PCR, droplet digital PCR, or high-throughput sequencing. Further epigenomic and fragmentomic analysis are required for tissue deconvolution to identify the tissue of origin of the ccfDNA fragments

Table 1 Summary of studies exploring ccfDNA in neuropsychiatric disorders

Reference	ccfDNA type	Biofluid	Sample	Method	Findings
Major depressive disorder					
Fernström et al. 2021 [70]	ccf-mtDNA	Plasma	281 MDD 49 HC	qPCR	Lower ccf-mtDNA in patients with current and remitted depression Lower ccf-mtDNA in patients taking mood stabilizers ccf-mtDNA not correlated with depression severity
Kageyama et al. 2018 [35]	ccf-mtDNA	Plasma	109 MDD 29 HC	qPCR	Lower ccf-mtDNA in patients with MDD Lower ccf-mtDNA in patients with current depression compared to those in remission
Lindqvist et al. 2018 [71]	ccf-mtDNA	Plasma	50 MDD 55 HC	qPCR	Higher ccf-mtDNA in patients with MDD
Ampo et al. 2022 [34]	ccf-mtDNA	Plasma	32 LLD 21 HC	qPCR	Higher ccf-mtDNA in older adults with LLD ccf-mtDNA correlated with depression severity
Gonçalves et al. 2021 [72]	ccf-mtDNA	Plasma	32 LLD 21 HC	qPCR	Higher ccf-mtDNA in older adults with LLD ccf-mtDNA correlated with depression severity
Psychological stress					
Czamanski-Cohen et al. 2014 [73]	ccfDNA	Plasma	14 women undergoing IVF	Fluorescent quantification (SYBR Gold)	Cognitive behavioral interventions and stress reduction techniques reduce ccfDNA concentration
Herhaus et al. 2023 [74]	ccfDNA	Plasma	29 healthy men	qPCR	Immediate ccfDNA increase following stress induction
Hummel et al. 2018 [75]	ccfDNA and ccf-mtDNA	Plasma	20 healthy men	qPCR	Immediate ccfDNA and ccf-mtDNA increase following stress induction
Trumpff et al. 2019 [76]	ccfDNA and ccf-mtDNA	Serum	30 men 20 women	qPCR	ccf-mtDNA increase 30min following stress induction Men had significantly higher increase in ccf-mtDNA following stress induction compared to women
Bipolar disorder					
Cordeiro et al. 2023 [77]	ccf-mtDNA	Plasma	14 BD 16 HC	qPCR	Higher ccf-mtDNA levels in patients with BD compared to controls
Ho et al. 2021 [78]	ccfDNA	Plasma	47 BD—non-rapid cyclers 46 BD—rapid cyclers	MethylationEPIC BeadChip	Significantly different ccfDNA methylation profiles between rapid cyclers and non-rapid cyclers in pathways related to neuronal and synaptic function

Table 1 (continued)

Reference	ccfDNA type	Biofluid	Sample	Method	Findings
Jeong et al. 2020 [79]	ccf-mtDNA	Serum	64 BD 41 HC	qPCR	No differences in ccf-mtDNA levels between patients with BD and controls ccf-mtDNA negatively correlated with depressive symptoms in patients with BD
Kageyama et al. 2018 [35]	ccf-mtDNA	Plasma	28 BD 29 HC 109 MDD	qPCR	Lower ccf-mtDNA in patients with BD compared to controls Higher ccf-mtDNA in patients with BD compared to MDD
Kageyama et al. 2022 [80]	ccf-mtDNA	Plasma	10 BD 10 HC	qPCR	No differences in ccf-mtDNA levels between patients with BD in remitted and depressed states
Stertz et al. 2015 [81]	ccfDNA and ccf-mtDNA	Serum	20 BD 20 HC	qPCR	Higher levels of ccfDNA in patients with BD compared to controls No difference in ccf-mtDNA in patients with BD compared to controls
Schizophrenia					
Chen et al. 2021 [82]	ccfDNA	Plasma	174 SZ 100 HC	qPCR	Higher levels of ccfDNA in patients with SZ compared to controls
Ershova et al. 2019 [83]	ccfDNA	Plasma	100 SZ 96 HC	Fluorescent quantification (PicoGreen)	Higher levels of ccfDNA in patients with SZ compared to controls Increased endonuclease activity in patients with SZ compared to controls
Ershova et al. 2017 [84]	ccfDNA	Plasma	83 acute psychosis 30 HC	Fluorescent quantification (PicoGreen)	No difference in ccfDNA levels in patients with acute psychosis compared to controls
Jiang et al. 2018 [36]	ccfDNA	Plasma	65 SZ 62 HC	Fluorescence correlation spectroscopy	Higher levels of ccfDNA in patients with SZ compared to controls
Kageyama et al. 2018 [35]	ccf-mtDNA	Plasma	17 SZ 29 HC	qPCR	No difference in ccf-mtDNA levels in patients with SZ compared to controls
Lubotzky et al. 2022 [85]	ccfDNA	Plasma	29 first psychotic episode 31 HC	Fluorometric quantification (Qubit) Bisulfite treatment and sequencing	Higher levels of ccfDNA in patients following a first psychotic episode compared to controls Higher levels of brain-derived ccfDNA in patients following a first psychotic episode compared to controls

Table 1 (continued)

Reference	ccfDNA type	Biofluid	Sample	Method	Findings
Ouyang et al. 2021 [86]	ccf-mtDNA	Plasma	34 SZ 28 HC	qPCR	No difference in ccf-mtDNA levels in patients with SZ following first psychotic episode compared to controls Significant decrease in ccf-mtDNA levels in patients with SZ after the use antipsychotic treatment Correlation between ccf-mtDNA levels and symptom improvement
Psychological trauma					
Morrison et al. 2022 [87]	ccf-mtDNA	Plasma	101 women	qPCR	Higher levels of ccf-mtDNA in women who experienced sexual trauma between 14 and 17 years old compared to women who never experiences psychological trauma, or women who experienced psychological trauma outside of this age range
Anxiety disorders					
Lindqvist et al. 2023 [88]	ccf-mtDNA	Plasma	46 SAD 42 HC	qPCR	Lower ccf-mtDNA levels in patients with social anxiety disorder compared to controls
Autism spectrum disorder					
Shmarina et al. 2020 [89]	ccfDNA	Plasma	133 ASD 27 HC	Fluorescent quantification (PicoGreen)	Higher levels of ccfDNA in patients with ASD compared to controls Higher levels of ccfDNA in patients with severe ASD compared to patients with mild to moderate ASD
Zhang et al. 2010 [90]	ccf-mtDNA	Serum	20 ASD 12 HC	qPCR	Higher levels of ccf-mtDNA in patients with ASD compared to controls
Eating disorders					
Verebi et al. 2024 [91]	ccfDNA and ccf-mtDNA	Plasma	98 ED 12 HC	Droplet digital PCR	Higher levels of long ccfDNA fragments in patients with ED compared to controls
Alzheimer's disease					
Acha et al. 2023 [92]	ccfDNA	Plasma	80 AD 100 HC	Bisulfite pyrosequencing	Significantly differentially methylated CpGs in patients with AD compared to controls
Bahado-Singh et al. 2022 [93]	ccfDNA	Plasma	26 AD 26 HC	MethylationEPIC BeadChip	Significantly differentially methylated CpGs in patients with AD compared to controls

Table 1 (continued)

Reference	ccfDNA type	Biofluid	Sample	Method	Findings
Bahado-Singh et al. 2023 [94]	ccfDNA – CYP genes	Plasma	25 AD 23 HC	MethylationEPIC BeadChip	Significantly differentially methylated CpGs in CYP genes in patients with AD compared to controls
Beadell et al. 2023 [95]	ccfDNA	Serum	20 AD 20 HC	5hmC-Seal assay and high throughput sequencing	Significantly different 5hmC profile between patients with AD and controls, specifically in regions associated with AD related pathways
Chen et al. 2022 [96]	ccfDNA	Plasma	18 AD 24 HC	5hmC-Seal assay and high throughput sequencing	Significantly different 5hmC profile between patients with AD and controls
Ding et al. 2023 [97]	ccf-mtDNA	Plasma	31 AD 26 HC	Sequencing	Significantly different methylation profile of ccf-mtDNA of patients with AD compared to controls
Guemri et al. 2022 [98]	ccfDNA	Plasma	8 AD 14 HC	Bisulfite sequencing	Significantly differentially methylated CpGs in ccfDNA of patients with AD compared to controls
Konki et al. 2019 [99]	ccfDNA	Plasma	395 twins	Bisulfite sequencing, bisulfite pyrosequencing	Significantly differentially methylated sites in ccfDNA in twins discordant for AD Differentially methylated sites are associated with neural function
Maćias et al. 2023 [100]	ccfDNA	Plasma	35 AD 35 HC	Bisulfite pyrosequencing	No significant differences in ccfDNA levels between patients with AD and controls Methylation differences in the promoter region of the *PRLHR* gene in ccfDNA of patients with AD compared to controls
Nidadavolu et al. 2022 [101]	ccfDNA	Serum	134 dementia 135 MCI 362 HC	Digital PCR	Elevated serum ccfDNA linked to a reduction in global cognition and an increased risk of developing dementia Increased ccfDNA levels were associated with a steeper cognitive decline over an 8-year follow-up period
Pollard et al. 2023 [102]	ccfDNA	Plasma	13 AD 12 MCI 25 HC	Bisulfite conversion and sequencing	Increased neuronal ccfDNA in participants with AD compared to controls Increased neuronal ccfDNA in participants with MCI that progressed into AD ($n=6$) compared to participants with MCI who did not later develop AD ($n=6$)
Parkinson's disease					

Table 1 (continued)

Reference	ccfDNA type	Biofluid	Sample	Method	Findings
Lowes et al. 2020 [103]	ccf-mtDNA	CSF	372 PD 159 HC	qPCR	Decreased levels of ccf-mtDNA in patients with PD on treatment compared to controls ccf-mtDNA levels negatively correlated with treatment
Meng et al. 2021 [104]	ccfDNA	CSF	26 PD 10 HC	Bisulfite conversion and sequencing	Significantly differentially methylated genes in ccfDNA of patients with early onset PD
Scalzo et al. 2009 [105]	ccfDNA	Plasma	42 PD 20 HC	qPCR	Decreased levels of ccfDNA in patients with PD compared to controls
Multiple sclerosis					
Beck et al. 2010 [106]	ccfDNA	Serum	28 RMMS 50 HC	High throughput sequencing	Patients with RRMS exhibited distinctive repetitive elements and gene representations compared to controls
Dunaeva et al. 2018 [107]	ccfDNA	Serum	24 RMMS 24 HC	Bisulfite sequencing and methylation specific qPCR	Significantly differentially methylated CpGs in the LINE-1 repeat in the cfDNA of patients with RMMS compared to controls
Lehmann-Wermann et al. 2016 [108]	ccfDNA	Plasma	30 stable MS 19 relapsing MS 47 HC	Bisulfite sequencing	Patients with relapsing MS had higher levels of unmethylated WM1 and MBP3 genes in compared to controls and patients with stable MS
Liggett et al. 2010 [109]	ccfDNA	Plasma	29 RMMS in exacerbation 30 RMMS in remission 30 HC	Fluorescent quantification and methylation hybridization assay	Higher ccfDNA levels in patients with RMMS compared to controls Patients with RMMS have a distinct ccfDNA methylation profile compared to controls Patients with RMMS in remission had a distinct methylation profile compared to patients with RMMS in exacerbation
Olsen et al. 2016 [110]	ccfDNA	Serum	20 RMMS in remission 20 RMMS in relapse 20 HC	Bisulfite conversion and qPCR	Higher signature of *MOG* gene in atients with RMMS in relapse compared to healthy controls and patients with RMMS in remission
Epilepsy					
Alapirtti et al. 2016 [111]	ccfDNA	Serum	51 epilepsy 250 HC	Fluorometric quantification (Qubit)	Lower levels of ccfDNA in patients with extra temporal lobe epilepsy compared to controls Higher levels of ccfDNA in patients with epilepsy for < 18 years Higher levels of ccfDNA in patients with epilepsy who have a BMI ≥ 25

Table 1 (continued)

Reference	ccfDNA type	Biofluid	Sample	Method	Findings
Kim et al. 2021 [112]	ccfDNA	CSF	12 epilepsy	Digital droplets PCR	Known somatic brain mutations were detected in CSF-derived ccfDNA of patients with refractory focal epilepsy
Liimatainen et al. 2013 [113]	ccfDNA	Serum	167 epilepsy 250 HC	Fluorometric quantification (Qubit)	Higher ccfDNA levels in patients with focal epilepsy compared to controls Higher ccfDNA levels in patients with symptomatic epilepsy compared to probably symptomatic patients
Martins-Ferreira et al. 2022 [114]	ccfDNA	Serum	12 epilepsy 11 HC	MethylationEPIC BeadChip	No significant difference in the levels of ccfdna of neuronal origin between patients with mesial temporal lobe epilepsy and controls Significantly differentially methylated regions in the ccfDNA of patients with mesial temporal lobe epilepsy compared to controls

CSF cerebrospinal fluid, *HC* healthy controls, *MDD* major depression disorder, *LLD* late-life depression, *BD* bipolar disorder, *SZ* schizophrenia, *SAD* social anxiety disorder, *ASD* autism spectrum disorder, *ED* eating disorders, *AD* Alzheimer's disease, *MCI* mild cognitive impairment, *PD* Parkinson's disease, *MS* multiple sclerosis, *RMMS* relapsing–remitting multiple sclerosis

[75]. However, Trumpff et al. [76] observed increased serum ccf-mtDNA levels in both men and women 30 min after stress induction, but not immediately. This increase was sex dependent, with men showing a significantly higher ccf-mtDNA increase than women. These mixed results could be attributed to differences in the population studied and the ccfDNA isolation techniques used.

Bipolar Disorder

As with studies of unipolar major depressive disorder, research on bipolar disorder has yielded conflicting results. Some studies showed no significant differences [79, 81], while others found increased ccf-mtDNA in bipolar disorder compared to healthy controls [77]. Interestingly, ccf-mtDNA levels were positively correlated with the severity of depressive symptoms in patients diagnosed with bipolar disorder [79]. Individuals with bipolar disorder also showed higher levels of nuclear ccfDNA compared to healthy individuals [81] and an increase in ccf-mtDNA compared to individuals diagnosed with unipolar major depression [35]. Kageyama et al. [80] reported no changes in ccf-mtDNA levels in patients with bipolar disorder in the remitted state compared to the depressed state.

Ho et al. [78] investigated the ccfDNA methylome in bipolar disorder. They found no significant differences in the tissue of origin of ccfDNA between individuals with rapid cycling bipolar disorder and non-rapid cycling bipolar disorder. However, these two groups had different methylation profiles, and among the differentially methylated CpG sites, there was a significant enrichment in pathways related to synaptic and neuron function.

Schizophrenia

Several studies reported an increase in ccfDNA levels in individuals with schizophrenia and psychotic disorders compared to healthy controls [36, 82, 83, 85]. However, other studies found no significant differences in ccfDNA levels between these groups [35, 84]. Individuals with schizophrenia also exhibited higher endonuclease activity in plasma [83], which is consistent with an increase in shorter ccfDNA fragments compared to healthy controls [36]. Ouyang et al. [86] observed no differences in ccf-mtDNA levels between patients with schizophrenia and healthy controls. However, after 8 weeks of antipsychotic treatment, there was a significant decrease in ccf-mtDNA levels in patients with schizophrenia, and the ccf-mtDNA copy number correlated with symptom improvement. Lubotzky and colleagues [85] performed methylation analysis to identify differences in brain-derived ccfDNA levels. They reported a significant increase in total ccfDNA levels in individuals following their first psychotic episode compared to healthy controls, as well

as a significant increase in total brain-derived ccfDNA and ccfDNA of neural, oligodendrocytic, and astrocytic origin after a first psychosis episode.

Psychological Trauma

One study has investigated the link between ccf-mtDNA and psychological trauma. A group of women were examined for lifetime trauma history and post-traumatic stress disorder (PTSD) symptoms [87]. Interestingly, women who experienced trauma between the ages 14 and 17 had significantly higher ccf-mtDNA levels in plasma compared to women with no trauma history and those who experienced trauma outside of this age range. Women in this group also exhibited a significantly increased startle response to a fear conditioning paradigm. However, the ccf-mtDNA levels did not correlate with PTSD symptoms.

Anxiety Disorders

Individuals with social anxiety disorder had significantly lower ccf-mtDNA levels compared to healthy individuals at two assessments separated by 11 weeks. However, ccf-mtDNA levels did not correlate with the severity of anxiety or depressive symptoms. Interestingly, cognitive behavioral therapy improved anxiety symptoms but had no significant effect on ccf-mtDNA levels, which remained significantly lower in anxious individuals compared to healthy controls [88].

Autism Spectrum Disorder (ASD)

Shmarina and colleagues [89] found that individuals with ASD had significantly higher concentrations of ccfDNA compared to healthy controls, with individuals with severe autism showing the highest levels of ccfDNA. They also reported no changes in blood endonuclease activity between individuals with ASD and healthy controls, suggesting ineffective clearance of ccfDNA. Elevated levels of serum ccf-mtDNA were also reported in young individuals with autism [90].

Eating Disorders

One paper has explored the association between ccfDNA and eating disorders. Verebi et al. [91] reported that patients with eating disorders, particularly bulimia nervosa, had significantly higher levels of long ccfDNA fragments compared to healthy controls. Notably, this increase was observed specifically in long ccfDNA fragments rather than total ccfDNA levels. As previously mentioned, long ccfDNA fragments are likely released through necrosis [42, 43]. This finding is consistent with previous research indicating dysregulation of immune function in individuals with eating disorders [115, 116].

Alzheimer's Disease and Dementia

Macías and colleagues [100] reported no significant differences in total plasma ccfDNA levels between patients with Alzheimer's disease and healthy individuals. However, another study showed that, over an 8-year follow-up period, elevated levels of total serum ccfDNA were linked to worse cognitive performance, faster cognitive decline, and increased risk of developing dementia in older adults [101]. Individuals with Alzheimer's disease also had significantly elevated levels of ccfDNA of neuronal origin compared to healthy individuals [102]. Interestingly, patients with mild cognitive impairment who later developed Alzheimer's disease had higher levels of neuronal ccfDNA when compared to those who did not develop Alzheimer's disease [102].

Previous studies have explored the methylation profile of ccfDNA in patients with Alzheimer's disease, detecting significantly differentially methylated CpGs and regions [92–94, 98–100]. Some of these differentially methylated sites are associated with neural function [99] and include genes such as the *HOXA3*, *LHX2*, and *ADARB2* [92, 98, 99]. Combining the methylation data with artificial intelligence analysis accurately predicted the diagnosis of Alzheimer's disease [93]. Additionally, ccfDNA from older adults with Alzheimer's disease had significantly different 5-hydroxymethylcytosine (5hmC) profiles compared to healthy controls [95, 96].

Parkinson's Disease

Scalzo et al. [105] found that older adults with Parkinson's disease had significantly lower levels of plasma ccfDNA compared to healthy controls. To our knowledge, this is the only study evaluating blood ccfDNA in individuals diagnosed with Parkinson's disease. Patients with Parkinson's disease who were on treatment had significantly lower CSF ccf-mtDNA levels compared to controls, with treatment showing a negative correlation with ccf-mtDNA levels [103]. Additionally, Parkinson's disease patients also diagnosed with depression and anxiety initially had significantly reduced levels of ccf-mtDNA compared to Parkinson's disease patients with no comorbid depression and anxiety; however, this trend was reversed after 36 months [103]. Meng et al. [104] reported that the patients with early-onset Parkinson's disease have significantly differentially methylated genes in ccfDNA compared to controls, and these genes were associated with both neural and immune function.

Multiple Sclerosis

Patients with relapsing–remitting multiple sclerosis had higher plasma ccfDNA concentrations than age-matched controls [109]. The serum ccfDNA of these patients exhibited distinctive representations of repetitive elements and genes, particularly those predominantly expressed in the central nervous system, compared to controls [106]. They also displayed distinct methylation profiles [107, 109], including higher levels of unmethylated *MOG*, *WM1*, and *MBP3* genes [108, 110] compared to healthy controls and patients with inactive or stable multiple sclerosis.

Epilepsy

Liimatainen et al. [113] showed that patients with focal epilepsy had significantly higher ccfDNA concentrations compared to healthy controls, and patients with symptomatic epilepsy had significantly elevated levels of ccfDNA compared to patients diagnosed with cryptogenic epilepsy (a form of epilepsy where the cause cannot be identified). However, Alapirtti et al. [111] found significantly lower levels of ccfDNA in patients with temporal lobe epilepsy compared to healthy controls. Additionally, patients with epilepsy of less than 18 years in duration showed higher ccfDNA concentrations than those with epilepsy greater than 18 years in duration; likewise, patients with a BMI greater than 25 had higher ccfDNA concentrations than those with a BMI of less than 25 [111]. Patients with mesial temporal lobe epilepsy also had significantly differentially methylated regions in promoters, gene bodies, and CpG islands associated with central nervous system structure and function, including synaptic assembly, neurotransmission, and GABAergic pathways [114]. Kim et al. [112] identified somatic brain mutation in the CSF-derived ccfDNA of patients with refractory focal epilepsy, further highlighting ccfDNA's diagnostic potential.

Conclusion and Future Perspectives

The lack of non-invasive, objective, and reliable diagnostic biomarkers has been a major challenge in psychiatry. The heterogeneous and comorbid nature of psychiatric disorders, the overlap of symptoms across different disorders, and variability in response to treatment further complicate diagnosis and long-term prognosis for these conditions. In the face of these challenges, ccfDNA-based liquid biopsies offer a promising avenue for developing more precise diagnostic and prognostic models. This dynamic and non-invasive approach has the potential to not only support the diagnosis of various psychiatric disorders but also monitor disease progression and treatment response. In this context, our review offers a new and more comprehensive perspective on the utility of ccfDNA in a wider range of neuropsychiatric disorders. By examining both mitochondrial and genomic ccfDNA, addressing pre-analytical considerations, and exploring diverse analyses such as tissue of origin deconvolution and methylation changes of ccfDNA, our review stands out for its unique approach.

Mitochondrial ccfDNA has been extensively studied in the context of neuropsychiatric disorders and was the subject of a recent meta-analysis [117, 118], which showed a significant decrease in CSF, but not peripheral, ccf-mtDNA levels between patients with neuropsychiatric disorders and healthy controls. While there has been an ongoing discussion about the use of ccf-mtDNA as a diagnostic or screening tool for different neuropsychiatric disorders, current methodological limitations, low specificity of the ccf-mtDNA to specific neuropsychiatric disorders, and an insufficient number of well-powered studies preclude its routine clinical use for diagnosing or screening of these conditions.

Despite the genetic and epigenetic advancements in the analysis of ccfDNA, its utility in psychiatry remains largely underexplored and far from being integrated into clinical workflows due to specific challenges and limitations. Sensitivity is a critical issue, especially considering the low levels of ccfDNA in circulation and the potential variability among individuals with different neuropsychiatric conditions, supporting the notion that these conditions are biologically highly heterogeneous. Standardizing pre-analytical processing is crucial for exploring ccfDNA's potential as a biomarker in neuropsychiatry. Variability in the choice of biofluid, sample handling, ccfDNA extraction, and storage can impact the reliability and reproducibility of the results. Adhering to the Biorepositories and Biospecimen Research Branch's guidelines for ccfDNA processing ensures reproducibility across studies.

Moving forward, studies in psychiatry should take full advantage of ccfDNA technologies. Continued advancements in high-throughput sequencing, epigenetic profiling, and fragmentomics will enhance ccfDNA's power to serve as an accurate biomarker for psychiatric disorders. ccfDNA has the unique ability to offer a glimpse into the brain. Through epigenetic profiling, we can detect ccfDNA of neural and glial origin, opening a window into the brain's molecular landscape. This makes ccfDNA a powerful biomarker for elucidating the biological processes and mechanisms underlying psychiatric disorders. Studies should also investigate changes in ccfDNA released from other cells and tissues to provide a deeper and more comprehensive understanding of these disorders.

ccfDNA holds significant promise in uncovering the intricate biological mechanisms underpinning neuropsychiatric disorders. By examining how changes in ccfDNA profiles correspond to specific symptoms, treatment responses, and

disease progressions, we can gain deeper insights into the pathophysiology of these disorders. For instance, the size of ccfDNA fragments could reveal the mechanism underlying ccfDNA release, thereby providing valuable insights into the disease pathophysiology.

In the future, ccfDNA data could be integrated with clinical and neuroimaging biomarkers through machine learning algorithms to improve the detection, classification, and diagnosis of psychiatric disorders. Additionally, collaboration among researchers, clinicians, and stakeholders is imperative for translating ccfDNA research findings into clinically actionable tools and interventions. Such collaboration ensures that research is conducted efficiently and effectively, with the ultimate goal of improving patient outcomes. This accelerates the translation of ccfDNA research into tangible benefits for patients, such as improved diagnostic accuracy, personalized treatment strategies, and better monitoring of disease progression. This will be a step closer to precision psychiatry, where insights derived from ccfDNA could revolutionize diagnosis, treatment approaches, and disease monitoring, tailoring interventions based on personalized disease profiles.

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Data Availability No datasets were generated or analyzed during the current study.

Declarations

Ethics Approval Not applicable to this work.

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