Circadian Rhythm and Circulating Cell-Free DNA Release on Healthy Subjects

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Abstract

Background

In the last decade, clinical studies have investigated the clinical relevance of circulating cell-free-DNA (ccfDNA) as a diagnostic and prognosis tool in various diseases including cancers. However, limited knowledge on ccfDNA biology restrains its full development in the clinical practice. To improve our understanding, we evaluated the impact of the circadian rhythm on ccfDNA release in healthy subjects over a 24-hour period.

Methods

20 healthy male subjects underwent serial blood sampling (8:00 AM, 9:00 AM, 12:00 AM, 4:00 PM, 8:00 PM, 12:00 PM, 4 AM (+1 Day) and 8 AM (+1 Day)). We performed digital droplet-based PCR (ddPCR) assays to target 2 DNA fragments (69 & 243 bp) located in the *KRAS* gene to determine the ccfDNA concentration and fragmentation profile. For control, half of the samples were re-analyzed by capillary miniaturized electrophoresis (BIAbooster system).

Results & discussion

Overall, we did not detect any influence of the circadian rhythm on ccfDNA release. Instead, we observed a decrease in the ccfDNA concentration after meal ingestion, suggesting either a post-prandial effect or a technical detection bias due to a higher plasma load in lipids and triglycerides. We also noticed a potential effect of gender, weight and creatinine levels on the ccfDNA concentration.

Introduction

The presence of fragmented circulating cell-free DNA (ccfDNA) within peripheral blood was first described by Mandel & Metais in 1948 ¹. The medical community has grown great interests in ccfDNA analyses over various disciplines, including infections, obstetrics, transplantation, surgery and oncology. In 1989, Stroun *et al.* ² opened the door for ccfDNA to numerous applications when they demonstrated that a fraction of ccfDNA corresponded to cell-free tumor DNA (ctDNA) in cancer patients. Since then, the utility of ccfDNA has been largely explored for cancer screening, diagnosis and prognosis ³⁻⁵ through the analysis of somatic mutations ^{5,6}, chromosomal alterations ⁷ and epigenetic changes ⁸. More recently, several teams demonstrated the pertinence of ctDNA analyses to detect post-operative minimal residual disease ⁹ and early cancer recurrence ¹⁰. The gains in sensibility and specificity, acquired through analytical methods like optimized Next Generation Sequencing (NGS), droplet-based digital PCR (ddPCR) or optimized qPCR, have allowed for the detection of rare events in particular the presence of low concentrations of ctDNA molecules in the bloodstream ^{11,12}.

Despite the great potential of ctDNA as a cancer biomarker, its application in clinical practice remains limited. Currently, the routine use of ctDNA is restricted to the Cobas® EGFR Mutation Test v2 (Roche) approved by the FDA for patients with non-small-cell-lung-carcinoma (NSCLC) eligible to TKi-EGFR therapy and the Natera Test (granted as “Breakthrough Device” by FDA) to identify minimal residual disease after surgery. Amongst parameters that may limit the use of ctDNA in the clinical practice, it is important to underline that the biological variables likely to modify the release of ccfDNA are not clearly comprehended. The origin of ccfDNA remains poorly understood and may imply several cell death processes¹³. Apoptosis has been proposed as a primary mechanism by which ccfDNA is released into circulation due to its distinctive fragment length of 140 – 180 bp, which mirror the size of nucleosome-bound DNA fragments¹⁴. Necrosis and active release have also been described as a source of ccfDNA release¹³. Overall, the ccfDNA fragmentation profile has been studied in different clinical situations although results remain inconclusive^{15–17}. Moreover, ccfDNA could be combined with diverse biological structures, including nucleosomes, extracellular vesicles (EVs; e.g., exosomes and virosomes) and neutrophil extracellular DNA trap (NETs) that could modify its integrity and physiological role¹⁸. For example, Moss *et al.* used a deconvolution methylation model in healthy subjects to show that ccfDNA mostly comes from white blood cells and erythrocyte progenitors¹⁹.

Increased ccfDNA levels have been observed in various pathology such as cancers²⁰, acute and chronic inflammatory diseases²¹, traumas²², and cardiovascular diseases²³. The increase in ccfDNA concentration can also be observed in physiological processes such as physical activities²⁴, pregnancy²⁵ or obesity²⁶. The analysis of ccfDNA concentration alone is therefore insufficient for the proper exploration of oncological processes. Analytical tests specifically targeting ctDNA by using cancer specific genetic and epigenetic alteration, have thus been developed to meet such demands^{27–29}.

Although considerable efforts have been devoted to understanding the origin and the evolution of ccfDNA under various biological conditions, less attention has been paid to the daily dynamics of ccfDNA. The estimated half-life of ccfDNA is comprised between 15 mins and 2.5 hours, depending on the biological context³⁰. Looking for potential daily variations of ccfDNA, linked to the circadian rhythm or digestion, could improve the reliability of ctDNA monitoring within liquid biopsy. Indeed, the circadian rhythmicity is a molecularly driven, individual clock. It generates cyclic changes through the activity of various compounds, with a periodicity of about a day³¹. This observation has led to chrono-pharmaceutical approaches to optimize regimen dosing and administration schedules³². Such insights may allow defining the optimal conditions for plasma sampling including time, resting and/or fasting.

The present study was designed to evaluate the influence of circadian rhythm on ccfDNA release in healthy subjects by focusing on its absolute quantification and fragmentation profile. Such work could provide clues to determine the optimal realization of liquid biopsy sampling for the introduction of these analyses into clinical practice. To reach this goal, we performed our analyses using ddPCR assays as well as BIABooster system technology (miniaturized capillary electrophoresis) that allow for precise quantification of the total ccfDNA¹⁷. We first validated repeatability and precision of the quantification

method on commercial plasma samples from healthy subjects. We then analyzed ccfDNA from a cohort of patients who underwent longitudinal plasma sampling over a 24-hour period. The impact of different variables on ccfDNA release such as weight, gender, creatinine concentration levels and ethnic origin was also evaluated.

Materials & Methods

Patients & procedures

For the healthy cohort, we used biological samples that had been collected for a previous study carried on by the clinical investigation center of Hospital Europeen Georges Pompidou (Plasma Soluble (Pro)Renin Receptor)³³. The study received the ethical approval from CPP Ile de France III (N°EUDRACT: 2010-A00365-34) ethics committee and all subjects provided with written informed consent. All methods were performed in accordance with the relevant guidelines and regulations. This healthy prospective cohort was composed of 20 healthy males and 10 healthy females for a total of 170 samples (figure 1). Blood samples were collected in fasting conditions after 1-hour rest in a semi recumbent position. To limit biases, patients were asked to maintain a resting state throughout the study.

Healthy male underwent serial blood sampling over a period of 24 hours. Each male underwent 8 blood sampling at 8 AM, 9 AM, 12 AM, 4 PM, 8 PM, 12 PM, 4 AM (+1 Day) and 8 AM (+1 Day) using K2-EDTA tubes (Greiner Bio-One International, Cat. N°: 456043). Their meals were provided after blood sampling at 9:00 AM, 12:00 AM and 8:00 PM.

Healthy female underwent only one blood sampling at 8 AM.

The clinical characteristics of this healthy cohort are presented in the Supplementary Table 1. For one male, the weight and creatinine level data were not reported. The Cortisol level was measured. Secreted in a pulsatile fashion, it is known to display a circadian rhythm in adults and older children. Cortisol levels are highest in the morning and subsequently decrease to a nadir in the evening³⁴.

To evaluate the repeatability of the method, we applied commercial plasma from healthy individuals (n = 13) (BIOPREDICT International). The analysis was carried out in triplicate for plasma preparation (n = 39), DNA extraction and ddPCR analysis. These ccfDNA samples were quantified by 69 bp ddPCR assay. The workflow of this analysis is summarized in the Supplementary Figure 1.

Plasma ccfDNA preparation

The collection tubes (K2-EDTA) were processed within 4 hours to prevent the destruction of white-blood cells using two-centrifugation steps (1200g for 15 min at 4°C; 8000g for 10 min at 4°C). For each sample, 1 mL of plasma was aliquoted and stored at – 80°C. The DNA was extracted using the Maxwell RSC ccfDNA Plasma Kit (Promega, Cat No./ID: AS1480) following the manufacturer's instruction and was eluted in 62 µL of elution buffer.

ccfDNA analysis

✓ Droplet-based digital PCR assays

Since the half-life of circulating DNA molecules has been shown not to exceed 90 min^{13,30,35}, we assumed that each sample content along the day corresponding to new circulating DNA molecules. Thus, we normalized the DNA released for each time-point of sampling and calculated the total of DNA concentration released along the day.

We designed droplet-based digital PCR assays to target the wild-type sequence of *KRAS* gene exon 2, codon 13. These assays allowed amplifying two sequences with lengths of 69 and 243 bp, which reflected the quantification of DNA fragments > 69 bp and > 243 bp, respectively. In both cases, a probe bearing a VIC fluorophore (λ_{ex} 538 nm / λ_{em} 554 nm) detected the wild-type sequence of the *KRAS* gene.

✓ ddPCR assays and DNA integrity index (DII) calculation

A total of 11 μL of ddPCR Supermix for Probes (2X) (No dUTP) (Ref: 1863024, BIO-RAD laboratories) was mixed with 1.1 μL of assay Mix solution (20X) containing 8 μM of forward (69 or 243 bp) and reverse primers, and 4 μM VIC Taqman® labeled-probes. The extracted plasma DNA was added to a final reaction volume of 22 μL . Probes and primers are provided in Supplementary Table 2.

The PCR assay mixes were prepared in a pre-PCR room to limit the risks of contamination. The probe bearing VIC-fluorophore was designed to be specific to the *KRAS* exon 2 WT allele. Emulsifications of DNA samples were generated according to manufacturer protocol (Ref: 1864002, QX200™ Droplet Generator, BIO-RAD laboratories). The emulsions were thermal cycled following different PCR programs described in the Supplementary Table 3. After completion, the emulsions were either stored at 4°C or processed immediately to measure the end-point fluorescence signal from each droplet.

Since the number of positive droplets reveals the quantity of amplifiable target DNA, the fraction of amplifiable DNA in each patient samples could be determined. The number of DNA copies was determined using the QuantaSoft™ Analysis Pro Software (BIO-RAD laboratories). DII was defined as a ratio between the number of amplified copies of “long” (243 pb) and “short” (69 pb) DNA fragments. DII is calculated as below (Equation 1):

$$DII_{WT} = \frac{\text{Quantity of fluorescent droplets corresponding to 243 bps fragment (WT)}}{\text{Quantity of fluorescent droplets corresponding to 69 bps fragment (WT)}} \quad \text{Equation 1}$$

BIAboster analysis

The fragmentation profiles and concentration analyses of ccfDNA from 10 males (80 DNA samples) were also performed using BIAboster System (ADELIS) as previously described³⁶. The system is based on the principle of DNA fragment migration by capillary electrophoresis coupled to LED induced fluorescence (LEDIF) detectors. It allows performing size and concentration analyses of double stranded DNA with a

sensitivity of 10 fg/μL in an operating time of 20 min. Based on the quantity of the different fragments obtained, a ratio highlighting the size distribution of the tested sample is calculated by BIAbooster technology as below (Equation 2):

$$ratio = \frac{\text{Detected Quantity of fragments with a size ranging between 240–1650 bps}}{\text{Detected Quantity of fragments with a size ranging between 75–1650 bps}} \quad \text{Equation 2}$$

Statistical analyses

Statistical analyses were performed using R software (version 4.0.3). To compare the DNA concentrations and integrity index between the different blood collection times, unpaired nonparametric Mann-Whitney U-test & Wilcoxon-tests were performed.

Results

Evaluation of the repeatability of the method

The preliminary evaluation of the repeatability by ddPCR - 69 bp assay was carried out. Figure 2 shows a representation of the variation of ccfdNA concentrations within replicates. The coefficient of variation was 11%. The supplementary Figure 2 confirms that there were no significant differences between the DNA triplicates (Kruskal-Wallis test, p-value = 0.93). Both results suggest a repeatability of the pre-analytical and analytical methods.

Implication of the circadian rhythm on circulating DNA concentration

CcfDNA quantification by ddPCR

Using the ddPCR - 69 bp assay in simplex format, the number of *KRAS* exon 2 codon 13 copies could be precisely quantified (Supplementary Figure 3 & 4: Qubit versus ddPCR linear regression model and bland-Altman representation).

Using that method, significant differences were observed between each blood collection times (Figure 3 Panel A, Kruskal-Wallis test, p-value = 0.0012). Yet, we observed no differences in concentrations between the collection points distant from meal ingestion (Kruskal-Wallis test, p-value = 0,76; Figure 3, Panel b). The ccfdNA concentration was significantly decreased 3 to 4 hours after meal ingestion (Time 12:00 AM, 4:00 PM & 12:00 PM) (Figure 3, Panel c1 & c2). More specifically, ccfdNA concentration decreased between 9:00 AM and 12: 00 AM and between 8:00 PM and 12:00 PM. It increased between 4:00 PM and 8:00 PM and between 12:00 PM and Day 1 + 4:00 AM.

The variation in cortisol concentration during the 24-hour inclusion period was taken as a control of the circadian rhythm (Supplementary Figure 5). The daily variation of ccfdNA and cortisol concentration were not correlated (Pearson R coefficient = 0.23). Between the healthy individual's entrance in hospital

(blood sampling at 8:00 AM) and the last sampling (the next day at the same time), ccfDNA concentration and proportion remained stable (respectively 7.97 (14.7%) and 6.56 (12.8%) ng/mL of plasma for 8:00 AM & Day 1 + 8:00 AM) (Supplementary Figure 6).

CcfDNA quantification by BIAbooster system

Using the BIAbooster system, the ccfDNA concentration at each time-point was determined and compared. As observed with ddPCR determination, we showed significant differences in ccfDNA concentrations between time-points of blood collection over 24 hours period, related with meal ingestion (Kruskal-Wallis test, p-value = $1e-04$) (Figure 4, Panel a). Apart from the samples collected after meal ingestion (12:00 AM, 4:00 PM & 12:00 PM), we noticed no differences between other blood collection points (Kruskal-Wallis test, p-value = 0,068; Figure 4, Panel b).

Evaluation of the DNA integrity index over 24 hours

Using the ddPCR -243 bp assay, we quantified the DNA fragments. The DII index was determined for each DNA sampling-point following Equation 2. Overall, no significant differences in ccfDNA integrity were observed between the different times-points (Kruskal-Wallis test, p-value = 1; Figure 5, Panel a).

The ratio calculated using the BIAbooster System showed similar results (Figure 5, Panel b; Kruskal-Wallis, p-value = 0.44)

Influence of clinical parameters on ccfDNA concentration in healthy subjects

Gender (n = 20 men & n = 10 women)

Within the whole group, the ccfDNA concentration was higher in men (Wilcox-test, p-value = 8.9×10^{-5} , Figure 6, Panel a).

Ethnic status (n = 10 non-Caucasian men & n = 10 Caucasian men)

In male patients, we showed non-significant difference in regards to ccfDNA concentrations according to the ethnical status (Wilcox-test, p-value = 0.2) Figure 6, Panel b. The 2 groups were identical in terms of weight average (respectively 72.2 kg & 74.84 kg for non-Caucasian and Caucasian men).

Weight (n = 15 with a weight < 67.5 kg, n = 14 with a weight > 67.5 kg)

The median weight of the whole cohort (women and men) was 67.5kg. We therefore defined two groups, one with a weight < 67.5 kg and one with a weight > 67.5 kg. The higher weight group was associated with higher ccfDNA concentrations (Wilcox-test, p-value = 0.022. Figure 6, Panel c).

Creatinine (n= 15 with a creatinine concentration < 75 $\mu\text{mol /L}$, n = 14 with a creatinine concentration > 75 $\mu\text{mol /L}$)

Assuming the absence of renal failure in this population, the creatine level could be considered as a biomarker reflecting the muscular mass³⁷. The median creatinine concentration level in this cohort (women and men) was 75 mol/L. We defined two groups, one with a creatinine concentration < 75 mol/L and one with a creatinine concentration > 75 mol/L. The high creatinine concentration group showed higher levels of ccfDNA concentration (Wilcox-test, p-value = 0.014), suggesting a potential effect of muscular mass on circulating DNA release (Figure 6, Panel d).

Discussion

Defined as a critical tool to identify minimal residual disease or cancer progression^{38–40}, the clinical value of ctDNA detection is under evaluation in several randomized control trials^{41–43}. The interpretation of those clinical studies requires careful attention to the pre-analytical and analytical processes, as well as confounding factors related to physiologic conditions.

In the first part of this study, we confirmed the repeatability of our method. These data validated the absence of technical variability linked to the pre-analytical condition, which ensured the pertinence of our biological data. Our process benefited from a reproducible pre-analytical process starting from plasma preparation up to the DNA extraction^{44,45}.

Overall, the ccfDNA concentration and fragmentation profile remained stable during the day at each blood collection time (Figure 3, Panel C; Figure 4, Panel B; Figure 5), except for samples drawn after meal ingestion. This variation could be related to postprandial effect on ccfDNA released or to a technical bias. Indeed, difficulties to extract circulating DNA fragments in plasma enriched in lipids and triglycerides (4 hours after a meal absorption) have already been described⁴⁶. In 2019, Meddeb *et al.* performed a similar study but the sampling was limited to 4 blood collection times (9:00 AM; 12:00 AM, 3:00 PM, 6:00 PM). They also observed a decreasing amount of circulating DNA between 9:00 and 12:00 AM after meal ingestion⁴⁷. In 2020, Lois Gardner *et al.* demonstrated that biomolecule corona of lipid nanoparticles (lipid structure) contains circulating cell-free DNA, suggesting a potential interaction between lipids and circulating DNA⁴⁸.

We also noticed a significant difference in ccfDNA concentrations between women and men. However, the average weight between these groups was also different, which appears as a major confounding factor. It was not possible to conclude between gender or weight contribution on ccfDNA release on a small cohort. This difference was not observed for ctDNA in the ALGECOLS cohort⁴⁹.

The significant difference observed between the low and high weight groups (Figure 6 Panel C) in the present study is in line with the literature^{26,50,51}. Previous studies have suggested a relationship between the adipocyte inflammatory process and the ccfDNA concentration in overweighted individuals²⁶.

We found that the creatinine level⁵² could play an important role in ccfDNA release, as shown by the relation between creatinine and ccfDNA concentration level. Further analyses including the measurement

of the muscular mass could answer these questions.

This work presents several limitations. Firstly, the circadian rhythm study included a small cohort of only 20 male healthy subjects. Another drawback is that the age of healthy individuals (18-35 years old) does not reflect the age of patients developing a cancer disease (> 60 years old). Since aging, inflammatory processes and cell senescence may increase the circulating DNA concentration, a dedicated study should be performed on a larger cohort of healthy volunteers aged over 60 years old and including women, in order to statistically confirm our results. Further investigations on the potential postprandial effect on circulating DNA variation are also required.

In conclusion, circadian rhythm in healthy individuals did not clearly contribute to the variation in ccfDNA concentrations. However these finding also suggest that blood should be drawn ideally before meal ingestion to optimize the ccfDNA concentration. Yet further analysis are required to confirm those observations.

Declarations

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Author contributions:

The individual contributions for this work are:

GP: Experimental work and analysis; Data acquisition; Writing - Original Draft; Writing - Review & Editing

JSH: Data acquisition; Formal analysis; Investigation; Methodology; Resources; Data Curation; Writing - Original Draft; Writing - Review & Editing; Funding acquisition

AB: Formal analysis; Investigation; Methodology; Resources; Data Curation; Writing - Review & Editing

DB: Data acquisition; Investigation; Methodology; Resources; Data Curation; Writing - Review & Editing

WX: Data interpretation; Writing - Review & Editing

FG: Data acquisition; Experimental work and analysis; Writing - Review & Editing

AB: Data acquisition; Experimental work and analysis; Writing - Review & Editing

GB: Experimental work and analysis; Writing - Original Draft; Writing - Review & Editing

VG: Supervision; Writing - Review & Editing

LP: Supervision; Writing - Review & Editing

MA : Investigation ; Resources ; Supervision; Funding acquisition; Writing - Review & Editing;

PLP : Conceptualization ; Methodology ; Investigation ; Resources ; Supervision;

LB : Formal analysis; Data Curation ; Writing - Original Draft; Writing - Review & Editing

VT : Conceptualization; Methodology; Resources; Data Curation ; Writing - Original Draft; Writing - Review & Editing; Visualization; Supervision; Funding acquisition

All authors reviewed the manuscript.

Data availability statement

The data that support the findings of this study are available from the corresponding authors VT or LB, upon reasonable request.

Competing interests' statements:

VT: Honoraria from Raindance Technologies and Boehringer Ingelheim; cofounder emulseo; board emulseo

PLP: Honoraria and board: Amgen, Merck-Serono, Boehringer Ingelheim, Sanofi, Roche, Lilly

AB: Honoraria and board: Amgen

JSH : speaker, advisory board or consultancy fees from Amgen, Astra Zeneca, Bayer, Bristol-Myers Squibb, Novartis, Novo Nordisk, Vifor Pharma, all unrelated to the present work

GP: Eurofins-Biomnis employee

PL: Eurofins-Biomnis employee

VG: Eurofins-Biomnis employee

AB (Dr Audrey Boutonnet): ADELIS employee

FG: ADELIS employee

The other authors declare no potential conflicts of interest.

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Figures

Figure 1

Workflow of the circadian study

In total, 20 healthy males and 10 healthy females were recruited. For each male, 8 blood samples were drawn. For each female, only one blood collection was drawn.

Figure 2

Repeatability of the pre-analytical and analytical process (n = 39 plasmas from n = 13 healthy subjects)

Variation within repeated analysis of identical samples is represented for each DNA specimen.

Figure 3

Impact of the circadian rhythm on ccfdDNA release measured by ddPCR

Panel a: provides the circulating ccfdDNA proportion for each blood collection point over 24 hours.

Panel b: ccfdDNA proportion for blood collection after exclusion of the post-prandial periods

Panel c; part 1: ccfdDNA proportion for blood collection drawn before the meal ingestion (9:00 AM and 8:00 PM) and 4 hours later (digestion period 12:00 AM and 12:00 PM).

Panel c; part 2: ccfdDNA proportion for blood collection during digestion period (12:00 AM and 12:00 PM) and after 8 hours of fasting (8:00 PM and Day 1 + 4:00 AM).

Figure 4

Impact of the circadian rhythm on ccfDNA release measured by BIAboster system

Panel a: ccfDNA proportion at each timepoint over a 24-hour period

Panel b: ccfDNA proportion for blood collection after exclusion of the post-prandial

Figure 5

Influence of the circadian rhythm on ccfDNA integrity

Panel a: ccfDNA integrity index (DII) for each blood collection point over 24 hour period.

Panel b: ccfDNA ratio (equation 2)

Figure 6
Influence of others clinical and biological parameters on ccfDNA release.

Panel a: Gender. The ccfDNA concentration was higher for male. However, the 2 groups did not have the same weight average (59.6 kg for females and 73.5 kg for males).

Panel b: Ethnicity.

Panel c: Weight. The median weight of the cohort (female and male) was 67.5 kg. We defined two groups.

Panel d: Creatinine concentration. The median creatinine concentration of the cohort (female and male) 75 mol/L. We defined two groups.

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