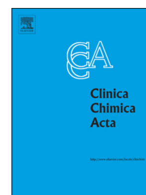
Clinica Chimica Acta xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim



Invited critical review

Circulating cell free DNA: Preanalytical considerations☆

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ARTICLE INFO

Article history:
Received 12 March 2013
Received in revised form 21 May 2013
Accepted 23 May 2013
Available online xxxx

Keywords:
Circulating cell-free DNA
Standard operating procedure
Preanalytical handling
Diagnosis
Stability

ABSTRACT

Despite the growing interest in circulating cell-free DNA (ccfDNA) analysis in various clinical fields, especially oncology and prenatal diagnosis, few studies on sample handling have been reported and no analytical consensus is available. The lack of consistency between the various protocols for sample handling and the techniques used for ccfDNA analysis is one of the major obstacles in translating ccfDNA analysis to clinical practice. Although this point is highlighted regularly in the published reviews on ccfDNA analysis, no standard operating procedure currently exists despite several ongoing clinical studies on ccfDNA analysis.

This review examines the pre-analytical parameters potentially affecting ccfDNA concentration and fragmentation at each pre-analytical step from blood drawing to the storage of ccfDNA extracts.

Analysis of data in the literature and our own observations revealed the influence of preanalytical factors on ccfDNA analysis. Based on these data, we determined the optimal preanalytical protocols for ccfDNA analysis and ultimately, a guideline for the translation of ccfDNA analysis in routine clinical practice.

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Abbreviations: ccfDNA, circulating cell-free DNA; EDTA, ethylenediaminetetraacetic acid; Q-PCR, quantitative polymerase chain reaction; DII, DNA integrity index; RT, room temperature; *KRAS*, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog [GenBank: NG_007524.1]; *BRAF*, v-raf murine sarcoma viral oncogene homolog B1 [GenBank: NG_007873.2].

☆ F. Mouliere is supported by a grant from the Centre National de la Recherche Scientifique (CNRS) and the Region of Languedoc-Roussillon (CNRS044406). The study was granted by the GEFLUC (DCMLP10-184, France). A.R. Thierry is supported by the Institut National de la Santé et de la Recherche Médicale (INSERM) (France).

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0009-8981/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.cca.2013.05.022

Please cite this article as: El Messaoudi S, et al, Circulating cell free DNA: Preanalytical considerations, Clin Chim Acta (2013), http://dx.doi.org/10.1016/j.cca.2013.05.022

UNCORRECTED PROOF

1. Introduction

The discovery of circulating cell-free DNA (ccfDNA) in the human circulatory system has led to intensive research on its use in various clinical fields. CcfDNA was discovered in 1948 by Mandel and Metais [1] although at the time, it did not attract much curiosity. Thirty to 40 years later, however, the interest of ccfDNA was demonstrated by several groups: Leon et al. [2] found that ccfDNA concentration was significantly increased in cancer patients and Stroun et al. [3,4] described a proportion of ccfDNA that was tumor derived and carried its molecular characteristics, thus leading to the concept of a "liquid biopsy". Additionally, ccfDNA fragmentation has grown in interest in terms of diagnosis since the revelation of significant differences between cancer patients and healthy subjects [1–8]. Therefore, ccfDNA analysis could provide diagnostic, pronostic, and theranostic information. Several researchers are intensively developing techniques that allow detection and characterization of genetic and epigenetic alterations of tumor cells using ccfDNA analysis in the plasma/serum of cancer patients. Such techniques could revolutionize the management care of cancer patients through the detection of mutations leading to resistance to targeted therapies, personalized therapeutic monitoring and non-invasive follow-up of the disease. Several works and reviews have been published on this topic over the last decade [9–15]. In the field of prenatal diagnosis, ccfDNA was used to develop a risk-free, non-invasive method to analyze fetal molecular genetics in pregnant women to avoid the risks associated with certain practices, such as amniocentesis [16]. In 1997, Lo et al. [17] showed fetal DNA in the plasma of pregnant women, which stimulated vigorous research in this field and the implementation of current clinical tests, such as fetal sex assessment, fetal rhesus D genotyping, or detection of fetal chromosomal aneuploidy [18]. Other groups have demonstrated the interest of ccfDNA in other clinical fields, such as autoimmune diseases [9,19–21], trauma, sepsis [22], or myocardial infarction [23].

Despite intensive research, few ccfDNA-based tests have been translated to clinical practice. Currently, some tests are available for specific prenatal diagnosis [2] and only one technique exists for oncology, namely BEAMing [24], a sophisticated technique allowing detection of mutations in various genes, particularly in colorectal cancer (CRC) patients. Several techniques are under development to detect and characterize ccfDNA in cancer patients including restriction fragment length polymorphism, direct sequencing, high-resolution melting analysis, digital PCR, cold PCR, and other techniques usually used for tumor-tissue analysis [14]. Nevertheless, ccfDNA concentration has not yet been validated as a cancer biomarker as the literature reveals conflicting data: plasma ccfDNA concentrations in cancer patients range from a few ng/ml to several thousand ng/ml, which overlaps with the concentration range for healthy individuals [10,11,15,25]. Furthermore, the estimation of ccfDNA fragmentation in cancer patients has been found to be lower, equivalent, or higher than in control subjects [1–8]. Our group's work on ccfDNA analysis in CRC cancer patients found that ccfDNA fragmentation was higher in cancer patients than in healthy subjects [26,27]. Such differences may be due not only to bias when selecting patients, but also to variation in the technical procedures for extracting and quantifying ccfDNA used by each laboratory, since no standard operating procedure (SOP) for ccfDNA analysis is available.

Over the last decade, ccfDNA reviews have regularly highlighted the lack of a SOP. Thus, a SOP for ccfDNA analysis is necessary to translate ccfDNA analysis to clinical practice [9–15,28,29]. The lack of pre-analytical and analytical consensus for ccfDNA analysis, such as type of matrix, storage conditions, or particular handling of blood sampling, constitutes pre-analytical factors affecting ccfDNA concentration and fragmentation, thus presenting major obstacles to clinical application.

This is the first review that examines the main pre-analytical factors affecting ccfDNA analysis, from blood drawing to the storage of ccfDNA extracts, and provides a summary of the optimal conditions for pre-analytical handling of samples for ccfDNA analysis.

Data from the literature presented in this review are supported by our own observations on the impact of different handling protocols on ccfDNA concentration. Particular attention was given to the study of ccfDNA fragmentation considering that it is an indicator of ccfDNA stability during handling and the storage of samples. Our robust and precise ccfDNA quantification method enabled us to precisely study the pre-analytical handling and portability of ccfDNA analysis.

2. Optimal blood sampling

2.1. Serum or plasma

The matrix of choice, i.e. serum or plasma, is the first question to ask when standardizing ccfDNA analysis. Several works comparing ccfDNA concentrations in paired plasma and serum samples [5–7] have revealed significantly higher ccfDNA concentrations in serum than in plasma. Table 1 summarizes some of the results published in various clinical fields. Nevertheless, several publications have demonstrated that the increased level of ccfDNA in serum is due to the clotting process of white blood cells in the collection tube leading to their lysis [31,34–37]. As a consequence, ccfDNA in serum is at least slightly contaminated by genomic DNA released from white blood cells and specific ccfDNA is diluted by high concentrations of non-specific genomic DNA. Even though it has been established for a few years that plasma is better than serum, studies in the field are still based on serum samples, certainly due to the propensity of clinical laboratories to prepare sera conventionally and to carry out retrospective studies.

In the field of oncology, our group has shown unequivocally that plasma is a better source of specific tumor-derived ccfDNA [38]. We used xenografted mice (n = 8) with a human CRC cell line to show that total ccfDNA concentration determined with a murine wild-type *KRAS* primer set (i.e. targeting non tumor-derived ccfDNA) was higher in serum samples than in plasma samples. On the other hand, when studying ccfDNA concentration using a human wild-type *KRAS* primer set (i.e. targeting specific tumor-derived ccfDNA), ccfDNA concentrations were higher in plasma samples. This observation confirms that the increase of ccfDNA in serum samples is due to the release of DNA from blood cells.

As plasma appears to be the matrix of choice, this review focuses on the pre-analytical factors potentially affecting ccfDNA in the plasma fraction.

Table 1
Non-exhaustive data focusing on differences in ccfDNA concentrations in paired serum and plasma samples.

Article	Clinical field	n	Plasma cfDNA concentration	Serum cfDNA concentration	p-value
Quantification of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma [31]	Healthy subjects	18	Approximately 40 copies/mL	Median: 8000 copies/mL	ND
Higher amount of free circulating DNA in serum is not mainly caused by contaminated extraneous DNA during separation [33]	Cancer	24	Mean ± sd: 180 ± 150 pg/μL	Mean ± sd: 970 ± 730 pg/μL	p = 0.0002
Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone narrow transplantation [34]	Transplantation	22	Median: 1195,1 copies/mL	Median: 16,344,8 copies/mL	p < 0.0001
Effects of preanalytical factors on the molecular size of cell free DNA in blood [35]	Fetal cfDNA	27	Median: 600 copies/mL	Median: 975 copies/mL	p < 0.05

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