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EDTA-mediated inhibition of DNases protects circulating cell-free DNA from *ex vivo* degradation in blood samples

Gustavo Barcelos Barra [a,b,*], Ticiane Henriques Santa Rita [a,b], Júlia de Almeida Vasques [a,b], Camilla Figueiredo Chianca [b], Lídia Freire Abdalla Nery [a], Sandra Santana Soares Costa [a]

[a] Sabin Laboratory of Clinical Analysis, Brasilia, Federal District, Brazil
[b] Post-Graduation in Health Sciences, University of Brasilia, Brasilia, Federal District, Brazil

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ABSTRACT

Objetives: The extracellular DNA occurring in plasma-EDTA and serum is a biomarker of growing interest, especially in prenatal diagnosis and oncology. The objectives of the present study were to compare the DNase activity in these specimens and to investigate its *ex-vivo* impact over the circulating cell-free DNA yield (ccfDNA), using the circulating cell-free fetal DNA (ccffDNA) as a tool.

Design and methods: EDTA-plasma and serum from women bearing male fetus were submitted to an endogenous DNase activity assay based on qPCR hydrolysis probe degradation, they were treated with DNAse I to investigate the action of an exogenous nuclease and also submitted to different temperature conditions to investigate the temperature-dependent degradation of the ccffDNA. In all instances, all male ccffDNA were quantified by qPCR targeting the Y chromosome-specific sequence DYS-14. Moreover, a serial dilution of EDTA was added to nonanticoagulated plasma and serum before the endogenous DNAse activity assay, to investigate the EDTA-mediated inhibition of the blood's DNase.

Results: The endogenous nuclease activity was 14.9-fold higher in serum compared to EDTA-plasma. The DNAse I treatment did not alter the ccffDNA yields in EDTA-plasma, but completely degraded it in serum. The addition of increasing doses of EDTA to nonanticoagulated plasma and serum resulted in a stepwise inhibition of their nucleases activity. Finally, we observed a much more pronounced temperature-mediated decrease on the ccffDNA amount in serum compared to EDTA-plasma.

Conclusion: The exogenous and endogenous DNases are more active in serum, the anticoagulant EDTA indirectly inhibits blood DNases, and consequently ccfDNA is protected from the blood's DNase preanalytical impact in EDTA-plasma.

1. Introduction

Circulating cell-free DNA (ccfDNA) is defined as an extracellular DNA occurring in the blood [1]. It is a biomarker of growing interest in various clinical fields, especially in prenatal diagnosis and oncology. The analysis of the circulating cell-free fetal DNA (ccffDNA) in the mother's blood circulation is the method of choice for non-invasive investigation of the fetus genetic traits. Moreover, the analysis of the tumor derived from ccfDNA could provide diagnostic and prognostic information for cancer patients [2].

However, the existence of a DNA degrading activity in the blood is well established [3] and some authors reported an inverse correlation between the circulating DNA yield and blood's DNase activity [4,5]. The DNAse I is the major nuclease present in blood and its products (e.g., plasma and serum) [3,6,7]. It hydrolyses phosphodiester bonds of single-strand or double-strand DNA to generate oligonucleotides and nucleotides with 5′-phospho and 3′-hydroxy termini [8–10].

Despite of these knowledge, blood's DNase impact as a preanalytical variable for ccfDNA analysis is neither clear nor well studied [11]. To our knowledge, no study has specifically links on both subjects. Then we speculated if the ccfDNA could be susceptible to degradation by DNase activity present in the biological samples, which would be a factor affecting *ex vivo* ccfDNA yields. If DNases are active in the blood specimens it could be a source of variation (e.g. decrease the sensitivity of molecular assays).

The ccffDNA in maternal blood would be an invaluable tool to investigate this issue because placenta cells (trophoblasts) are its primary source [12], and the supply is lost after the maternal blood draw. In other words, it means that the ccffDNA quantity cannot increase in the

* Corresponding author at: Laboratório Sabin de Análises Clínicas, SHIGN 710/711 Norte loja 64 bloco A Sobreloja, Asa norte, Brasília, Distrito Federal, ZIP 70750-531. Fax: +55 61 8184 2213.
E-mail address: gbbarra@gmail.com (G.B. Barra).

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ex vivo sample itself as it can occur with the genomic ccfDNA [13]. Indeed, the ccffDNA is present in similar concentrations in maternal EDTA-plasma and serum [14].

In addition, EDTA-plasma and serum paired samples would be the second invaluable tool to investigate the questions mentioned before because the anticoagulant EDTA is an indirect DNase I inhibitor [7,15,16], since it chelates divalent ions (Ca^{+2}, Mg^{+2} and Mn^{+2}), which are essential for the enzyme structure [17] and full enzymatic activity [18].

Thus, the objectives of the present study were to compare the DNase activity in EDTA-plasma and serum paired samples and to investigate its *ex vivo* impact over the ccffDNA yield.

2. Materials and methods

2.1. Ethics statement

The health science faculty ethical committee of the University of Brasilia approved this study (IRB protocol no. 188/12), and written informed consent was obtained from each participant.

2.2. Subjects, blood collection, processing and transporting

This experimental study enrolled 34 women with male fetus (mean gestational age, 12 ± 4 weeks). Blood samples were collected simultaneously in two 4 mL tubes with gel barrier: Vacuette K2 EDTA Sep and Vacuette Z serum sep with clot activator (both from Greiner Bio-one). After the venipuncture, the tubes were both centrifuged for 2,000*g* for 10 min at room temperature (to form a gel barrier), transported to processing center at 22 ± 4 °C and stored at −20 °C until further use. After thawing, 1000 μL of the supernatants was transferred into polypropylene tubes, centrifuged at 14,000*g* for 10 min at room temperature, and each subsequent experiment was performed with sets of 9–11 paired supernatants.

2.3. Endogenous DNase activity assay

We used a fluorescence assay to investigate the samples endogenous DNase activity. Briefly, the assay consisted of 20 μL of the crude EDTA-plasma or serum, 250 nM of a hydrolysis probe (5′-6-FAM-CTCCAGCTC/ZEN/CACCTGAACGGCC-IABFQ-3′), 10 μl of 2X Maxima Probe qPCR master mix (Fermentas) and nuclease-free water (Fermentas) to a final volume of 35 μL. The reactions were incubated isothermally at 37 °C for 24 h on the Step-One qPCR System, and the fluorescence was measured at the beginning of the reaction and every 30 min thereafter. The maxima Probe qPCR master mix was used as a source of a passive reference dye (ROX), and the data were shown as deltaRn, which is the FAM fluorescence divided by the ROX fluorescence and them subtracted by the reaction baseline. The baseline for this assay was set as the first FAM normalized by the ROX fluorescence value (Fig. S1). To summarize, this assay investigated the action of sample's DNase over a single-stranded DNA molecule. Nuclease-free water and one unit of DNase I (Fermentas) were used as negative and positive controls, respectively.

2.4. EDTA-mediated inhibition of DNases

A serial dilution of EDTA (Disodium Salt, Dihydrate, USB) was added to six paired nonanticoagulated plasma and serum samples before the endogenous DNase activity assay in order to investigate the EDTA-mediated inhibition of hydrolysis probe degradation. The nonanticoagulated plasma was prepared through blood draw in plain tube (Vacuette serum tubes 4 ml, Greiner Bio-one), followed by centrifugation at 2,000*g* for 5 min at room temperature immediately after venipuncture, the supernatants were transferred into polypropylene tube and then centrifuged at 14,000*g* for 10 min at room temperature. The serum sample was prepared as described in the *blood collection, processing and transporting*. Thereafter, a 10-fold serial dilution of EDTA from 5×10^{-9} to 5×10^{-2} M was added to the samples and the endogenous nuclease activity assay was performed as described before. The experiments were repeated twice, and the dose–response data were analyzed using the log-dose versus response function of the software Prism 6.0 (GraphPad).

2.5. Plasma and serum treatments

The specimens were submitted to 37 °C for 24 h and 48 h and to −20 °C, 4 °C and 22 °C for 24 h in order to investigate the effect of the temperature over ccffDNA. Furthermore, the samples were treated with 25 U of DNase I for 1 h at 37 °C in order to assess the action of an exogenous nucleases on the fetal DNA sequences. All treatments were performed on EDTA-plasma and serum before the DNA extraction.

2.6. DNA extraction

The DNA was extracted from 500 μL of each sample by using the generic protocol 2.0.1 of the NucliSens easyMAG system (bioMerieux), 50 μL of magnetic silica particle suspension and elution in 25 μL.

2.7. Male ccffDNA quantification

Male cffDNA were quantified by amplifying a well-described Y-chromosome-specific sequence (DYS-14) [11] in an Step-One qPCR System (Life Technologies) using Maxima Probe qPCR master mix (Fermentas) and PrimeTime qPCR assay (Integrated DNA Technologies) consisting of the primers DYS14-F (5′-CCATGACCCCAGAGTCTGC-3′) and DYS14-R (5′-CTTCCTGGCTTGGGCATTAAC-3′) and hydrolysis probe (5′-6-FAM-CTCCAGCTC/ZEN/CACCTGAACGGCC-IABFQ-3′) as recommended by the manufactures. The cycle conditions were as follows: pre-incubation for 10 min at 95 °C, followed by 60 cycles of 15 s at 95 °C and 60s at 60 °C. The reaction volume was 24 μL, including 10 μL of the eluate. The equation describing the DYS-14 calibration curve was $Y = -3.26X + 29.75$ (efficiency = 102.45% and $R^2 = 0.998$). All samples were run in duplicate and each run included two negative controls. The quantity of male DNA in genomic equivalents (GE) per PCR reaction was determined by comparison with a 5-fold dilution series of the 007 control (life technologies), 151, 30.2, 6.02, 1.21 and 0.24 GE. The conversion factor of 6.6 pg of DNA per genome was used and the DYS-14 concentration in GE/mL was calculated as described before [14]. The qPCR experiments and description followed the minimum information for qPCR experiments guideline (MIQE) [19].

2.8. Statistical analysis

The statistical method used was Wilcoxon's matched-pairs signed rank test. All graphs and statistical analysis were performed using the software Prism 6.0.

3. Results

3.1. Endogenous and exogenous DNase activities in EDTA-plasma and in serum

In the endogenous DNase activity assay, we observed a sustained hydrolysis probe degradation in serum over time, which was not observed in EDTA-plasma. In EDTA-plasma, the probe degradation reached a plateau at 1–3 h of reaction (Fig. 1). Considering the reaction's last reading point, the median deltaRn was 14.9-fold higher in serum compared to the paired EDTA-plasma (median 1.64 deltaRn *versus* 0.11 deltaRn, $p = 0.0156$) (Fig. S2).

The treatment with DNase I showed no change of the median ccffDNA amount in EDTA-plasma (untreated 17.86 GE/mL *versus* treated 19.68 GE/mL, $p = 0.35$) (Fig. 2A) but degraded it to undetectable

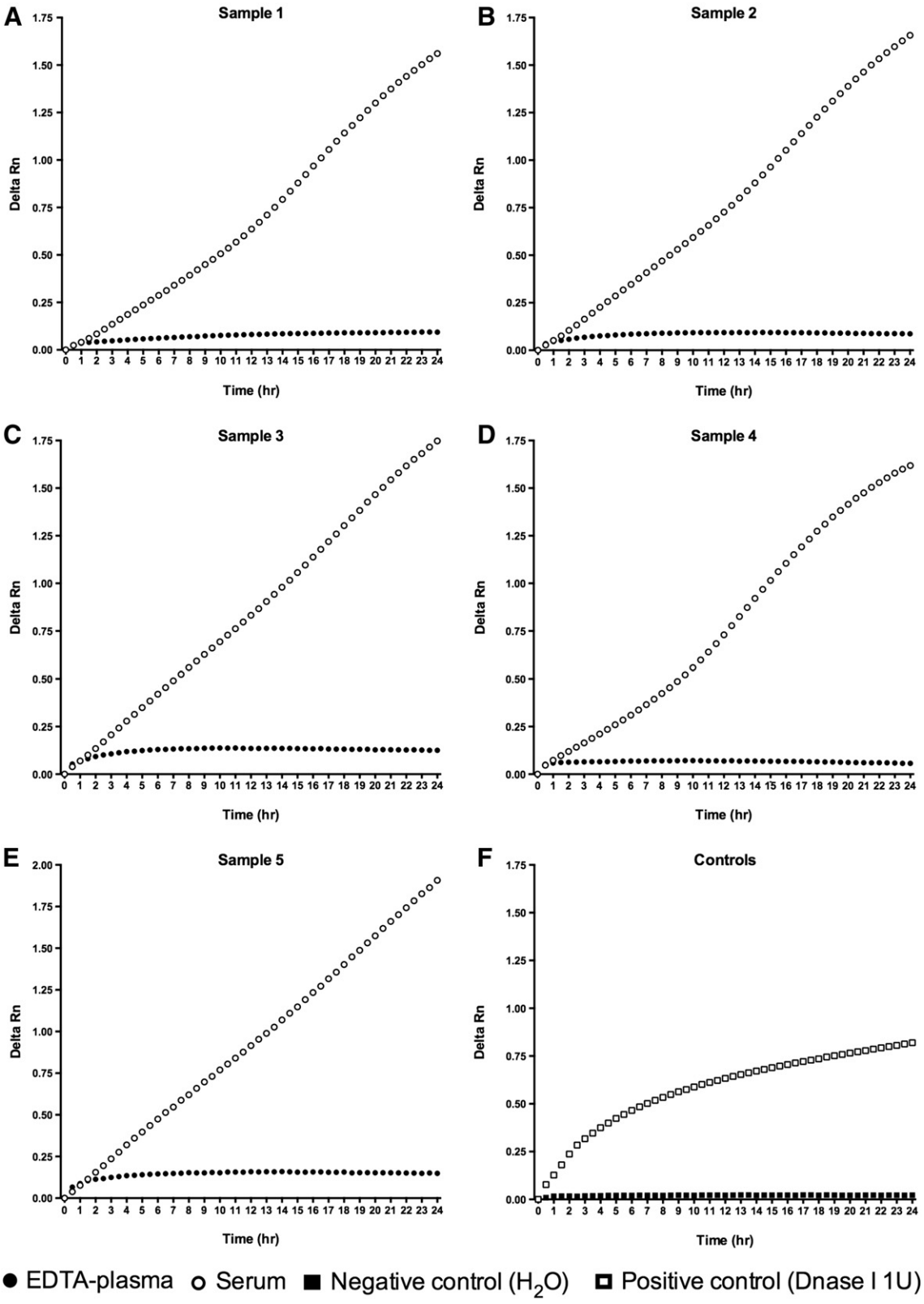

Fig. 1. Representative endogenous DNase activity assay in EDTA-plasma and serum paired samples of 5 subjects. In 24 h, the hydrolysis probe degradation was higher in serum (open circles) compared to the matched EDTA-plasma (closed circles), suggesting that the blood DNA degrading activity was inhibited in EDTA-plasma. The negative control (closed square) showed negligible levels of hydrolysis probe degradation. The positive control (open square) showed hydrolysis probe degradation kinetic that was not as continuous as the serum samples. We cannot distinguish if this effect was secondary to a higher DNases amount in the serum together with presence of inhibitors or if it was a complex composition of DNA degrading enzymes.

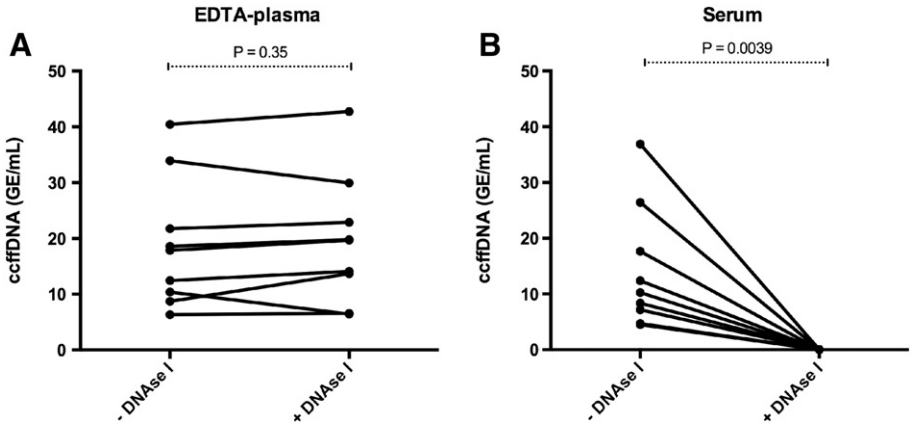

Fig. 2. Before-and-after plot showing the effect of DNAse I treatment on ccffDNA yields in EDTA-plasma and serum. (A and B) The specimens were treated (+) or not (−) with 25U of DNAse I for one hour at 37 °C before the cffDNA measurement. Each line represents one-studied sample ($n = 10$). The ccffDNA was completely degraded in the serum sample, but not in plasma-EDTA meaning that this exogenous nuclease was inhibited in the latter specimen.

levels in serum (untreated 10.23 *versus* treated 0.00 GE/mL, $p = 0.0039$) (Fig. 2B).

3.2. EDTA dose-dependent inhibition of nucleases in fresh plasma and in serum

The additions of a 10-fold serial dilution of EDTA to nonanticoagulated plasma and to serum (Fig. S3) before the endogenous nuclease activity assay showed a stepwise inhibition of the hydrolysis probe degradation in both specimens. The IC_{50} ranged from 1.46×10^{-4} M to 5.70×10^{-5} M in nonanticoagulated plasma and from 1.09×10^{-4} M to 6.66×10^{-5} M in serum.

3.3. Temperature effect over ccffDNA in EDTA-plasma and serum samples

The initial ccffDNA concentration was 1.4-fold higher in EDTA-plasma than in the matched serum (27.40 *versus* 18.57 GE/mL) (Fig. 3A *versus* 3B at 0 h). In the first 24 h, the exposure at 37 °C resulted in a decrease on the ccffDNA concentration of 1.07-fold in EDTA-plasma (median 27.40 GE/mL *versus* 25.55 GE/mL, $p = 0.07$) and 3.52-fold in serum (median 18.57 GE/mL *versus* 5.26 GE/mL, $p = 0.001$). In the next 24 h, the reduction in the ccffDNA yields was 1.02-fold (median 25.55 GE/mL *versus* 24.83 GE/mL, $p = 0.24$) and 1.68-fold (median 5.26 GE/mL *versus* 3.13 GE/mL, $p = 0.001$) for EDTA-plasma and serum, respectively. Considering the total experiment time (48 h), the ccffDNA degradation was 1.10-fold (median 27.40 GE/mL *versus* 24.83 GE/mL, $p = 0.001$) in EDTA-plasma and 5.9-fold (median 18.57 GE/mL *versus* 3.13 GE/mL, $p = 0.001$) in serum (Fig. 3A and B).

Next, samples were subjected to the pragmatic transportation and storage temperatures for 24 h. The median ccffDNA concentration in EDTA-plasma was similar during the experiment duration, 40.27 GE/mL for −20 °C, 42.25 GE/mL for 4 °C and 41.45 GE/mL for 22 °C (Fig. 3C). In serum, the same observation was made for −20 °C and 4 °C (20.72 *versus* 19.89 GE/mL, $p = 0.91$), on the other hand, a 1.6-fold and 1.54-fold decreases were observed at 22 °C (12.88 GE/mL) compared to −20 °C and 4 °C, respectively ($p = 0.0039$ for both) (Fig. 3D).

4. Discussion

In this study, we compared the DNase activity in EDTA-plasma and serum samples and investigated its *ex vivo* impact over the ccffDNA yield. Our first finding, that endogenous and exogenous DNases were more active in serum, is supported by the observation of sustained hydrolysis probe degradation over the reaction time in this specimen. Conversely, we observed a deltaRn increase in EDTA-plasma, but it reached a plateau in the beginning of the reaction and no further fluorescence change was observed in the remaining 21–23 h. In addition, the treatment with a high amount of DNase I degraded the ccffDNA in serum, but not on ccffDNA in EDTA-plasma. Taken together, these results suggested that sample's DNase activity is a preanalytical variable which could affect the ccffDNA yields in serum more extensively than in plasma-EDTA.

Furthermore, in the endogenous DNase assay the probe degradation kinetics of the reaction positive control (1 U of DNase I) was different from the serum samples. This observation should be secondary to the fact that total DNase activity in the serum sample is dependent on the complex combination of the concentration and composition of the DNA degrading enzymes and unknown factors influencing their activity [6].

Our second finding was the evidence that EDTA indirectly inhibits the sample's DNase activity, probably by chelation of divalent ions. Adding increasing doses of EDTA to nonanticoagulated plasma and serum samples resulted in a stepwise inhibition of the enzymatic activity. The K_2EDTA concentration in the gel barrier tubes used in this study was 1.8 mg per mL of blood (4.5×10^{-3} M), and the anticoagulant amount contained in the regular EDTA-plasma tubes ranges from 1.5 to 2.0 mg per mL of blood [20]. We observed a very important, but not complete, inhibition of the DNase activity in the dose–response curves at this concentration (5×10^{-3} M). Similar conclusion can be draw from the small but still detectable nuclease activity found in EDTA-plasma samples. Taken together, these results suggested that the molecular tests based on the ccfDNA analysis would benefit from a 10-fold increase of EDTA concentration in the collection tube since no or negligible DNAse activity was observed at 5×10^{-2} M.

Furthermore, the EDTA IC50 for nonanticoagulated plasma and serum were similar, suggesting that equal concentration of this anticoagulant is able to elicit the same inhibitory effects in both specimens [21]. We observed similar DNA degrading activity in nonanticoagulated plasma compared to the paired serum, although they were different between the individuals.

Our third finding, that ccffDNA is protected from a temperature-triggered degradation in EDTA-plasma, was supported by our exposition experiments. At 37 °C, the ccffDNA concentration showed a greater decrease in serum compared to EDTA-plasma probably because EDTA indirectly inhibited DNase activity in the latter specimen. These observations confirmed that blood DNase activity could exerts its effects on the sample *ex vivo* and it can lower the ccffDNA yields in the biological samples. However, despite the EDTA inhibition, the small but still detectable nuclease activity found in EDTA-plasma samples lead to an almost significant reduction of the ccffDNA yield in the first 24 h and significant reduction after 48 h. The elimination of this residual enzyme activity in the EDTA-plasma should increase ccfDNA stability.

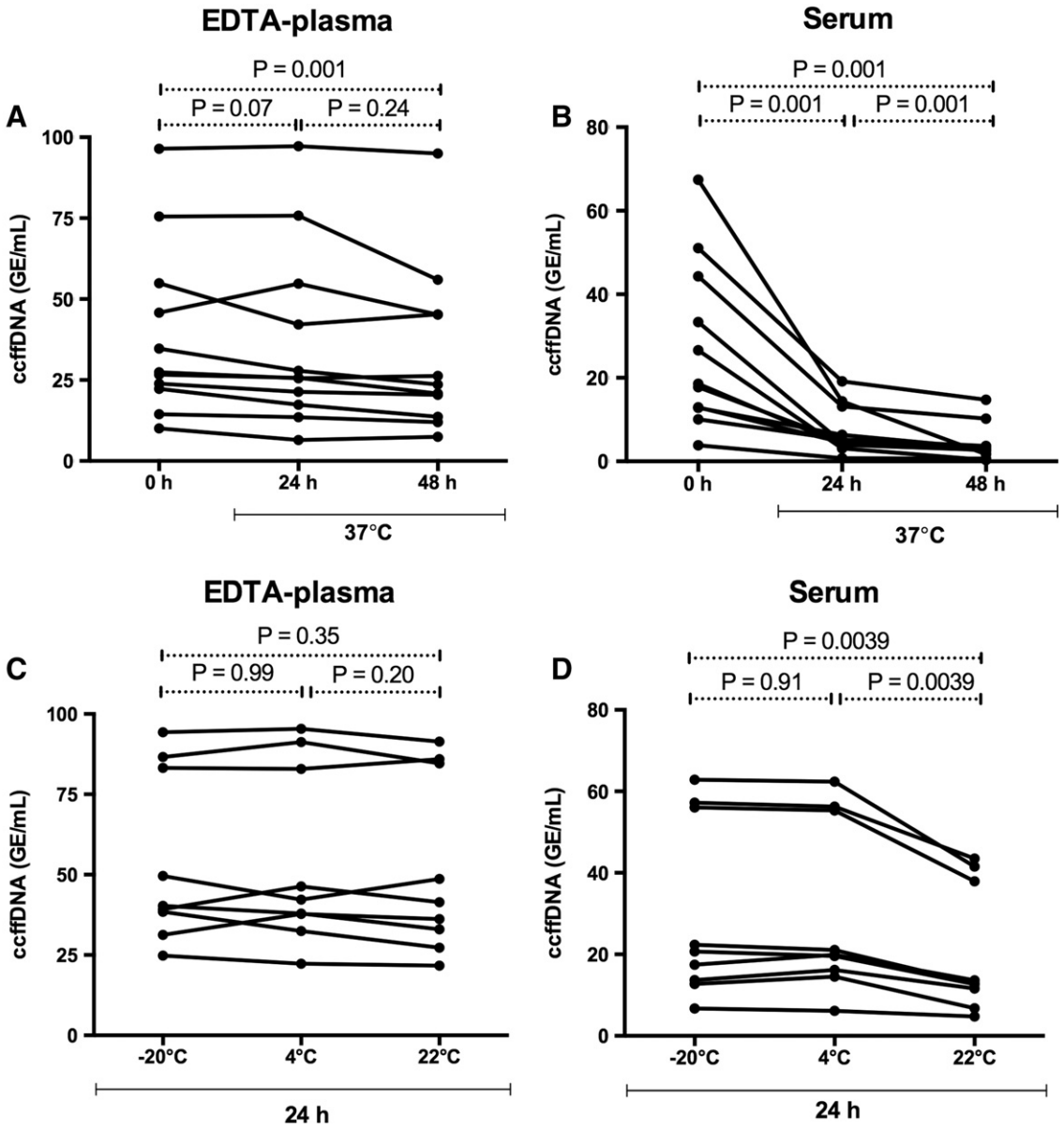

Fig. 3. Before-and-after plot showing the effect of temperature over ccffDNA in EDTA-plasma and serum. (A and B) Basal (0 h) and after exposure to 37 °C for 24 h and 48 h ($n = 11$). (C and D) Samples exposed to −20 °C, 4 °C and 22 °C for 24 h. Each line represents one-studied sample ($n = 9$). The results showed that ccffDNA was protected from sample's DNAse activity in plasma-EDTA and the DNA degrading activity in serum was a temperature-triggered mechanism.

Considering the pragmatic transport and storage temperatures of clinical laboratories, no differences were observed in ccffDNA at −20 °C and 4 °C in both specimens, otherwise, a decrease occurs at 22 °C in serum but not in plasma-EDTA. These evidences suggested that sample's DNase activity is a temperature-triggered mechanism and a preanalytical factor that is more important to serum samples. Therefore, the consistently lower initial ccffDNA yield in serum compared to the EDTA-plasma found in our paired samples could be secondary to the transportation at 22 ± 4 °C to processing center, we cannot exclude the potentially contribution of the higher maternal DNA background in serum for this observation. Thus, in the ccfDNA field strictly low-temperature condition control should be applied to serum samples. On the other hand, it can be more flexible for plasma-EDTA.

Actually, several preanalytical considerations for ccfDNA and ccffDNA analysis have been studied, and EDTA-plasma is the matrix of choice because it ensured the stability of the analyte and also because of its low contamination with leukocyte's genomic DNA [2]. Many studies have already demonstrated that the ccffDNA remained stable for 24 h after phlebotomy in EDTA-plasma and concluded that it can be measured in referral laboratories after the sample has been shipped overnight [11,22]. Others have described better results using plasma-EDTA compared to serum [23,24]. All these evidences supported our conclusion that EDTA inhibits the sample's DNase activity and protects ccffDNA *ex vivo* in biological samples. Indeed, the EDTA provides the higher ccffDNA stability observed in EDTA-plasma.

Finally, the protection conferred by EDTA can be extrapolated for other DNA targets, and the use of this anticoagulant should mitigate preanalytical effects of blood nucleases. Furthermore, our group conceived independently the endogenous DNase activity assay used in this study; however, previous similar protocols were identified after literature research [25,26]. In conclusion, these evidences showed that the blood DNases are active in serum and the widely used anticoagulant EDTA also works as an indirect blood's DNase inhibitor that avoids *ex vivo* degradation of the ccfDNA.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.clinbiochem.2015.02.014.

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