LabMed

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Impact of Tube Additives on Baseline Cell-Free DNA, Blood Nuclease Activity, and Cell-Free DNA Degradation in Serum and Plasma Samples: A Comparative Study

Gustavo Barcelos Barra *, Ticiane Henriques Santa Rita, Rafael Henriques Jácomo and Lídia Freire Abdalla Nery

Genomics Section, Sabin Diagnóstico e Saúde, Brasília 70632-340, Brazil; ticihenriques@gmail.com (T.H.S.R.); rafaeljacomo@sabin.com.br (R.H.J.); lidia@sabin.com.br (L.F.A.N.)

* Correspondence: gustavo@sabin.com.br; Tel.: +55-61-3012-8371

Abstract: Cell-free DNA (cfDNA) analysis is a pivotal tool in non-invasive diagnostics, including cancer monitoring and prenatal testing. However, the preanalytical phase, particularly the choice of anticoagulant, significantly impacts cfDNA integrity and yield. This study aims to compare cfDNA yield, stability, and DNase activity in plasma-citrate and plasma-heparin, using plasma-EDTA and serum as established controls, to explore more deeply the impact of blood DNAse activity on cfDNA in these specimens. Blood samples from 15 healthy volunteers were collected in four types of tubes (citrate, heparin, EDTA, and serum). cfDNA was extracted and quantified using qPCR, and endogenous DNase activity was assessed through hydrolysis probe assays. Samples were incubated at 37 °C for 24 h to evaluate cfDNA degradation rates. Heparin-plasma exhibited the highest DNase activity, with baseline cfDNA levels intermediate—higher than EDTA but lower than serum—leading to substantial cfDNA degradation (85.3%). Combined with its known PCR inhibition, this renders heparin-plasma unsuitable for cfDNA analysis. Citrate-plasma, with baseline cfDNA levels similar to EDTA, showed partial DNase inhibition, resulting in intermediate cfDNA degradation (13.3%), a limitation that diminishes its viability compared to EDTA-plasma. Serum, with the highest baseline cfDNA levels, exhibited high DNase activity and significant cfDNA degradation (55.6%), making it unsuitable for cfDNA preservation. EDTA-plasma demonstrated complete DNase inhibition and minimal cfDNA degradation (8%), confirming it as the most suitable specimen for cfDNA analysis. These findings emphasize the importance of anticoagulant selection, highlighting critical limitations of heparin-plasma and citrate-plasma while reinforcing EDTA-plasma as the gold standard for preserving cfDNA integrity in diagnostic applications.

Keywords: cell-free DNA; serum; EDTA plasma; heparin plasma; citrate plasma; nuclease activity; DNase activity

Academic Editors: Weiyong Liu and Emmanouil Magiorkinis

Received: 11 January 2025
Revised: 30 January 2025
Accepted: 12 February 2025
Published: 3 March 2025

Citation: Barra, G.B.; Santa Rita, T.H.; Jácomo, R.H.; Nery, L.F.A. Impact of Tube Additives on Baseline Cell-Free DNA, Blood Nuclease Activity, and Cell-Free DNA Degradation in Serum and Plasma Samples: A Comparative Study. *LabMed* **2025**, 2, 4. https://doi.org/10.3390/labmed2010004

1. Introduction

Cell-free DNA (cfDNA) analysis has revolutionized non-invasive diagnostics, offering genetic insights from blood samples without the need for invasive tissue biopsies [1]. In oncology, circulating tumor DNA (ctDNA) allows for the detection of tumor-specific mutations, aiding in early cancer diagnosis, monitoring treatment response, and identifying minimal residual disease [2,3]. This method is particularly beneficial as it captures tumor heterogeneity and allows for repeat sampling to track disease progression [1].

In prenatal care, cfDNA analysis has enabled non-invasive prenatal testing (NIPT) for fetal chromosomal abnormalities like trisomy 21, 18, and 13, with high accuracy. This

technology has reduced the need for invasive procedures like amniocentesis, offering a safer option for both mother and fetus [4,5].

The preanalytical phase is critical in cfDNA analysis as it affects cfDNA yield, quality, and integrity [6]. Key factors include the blood collection tube type and time to processing [7]. Additionally, DNase activity and the interaction of tube additives can significantly impact cfDNA stability [8,9], making proper management of these variables essential for reliable analysis.

Specialized tubes like Streck and PAXgene effectively stabilize leukocytes and prevent genomic DNA (gDNA) contamination by preserving cell integrity [8,10,11]. Streck tubes, with their formaldehyde-releasing agent, better prevent cell lysis and maintain cfDNA stability for up to 7 days at room temperature, while inhibiting DNase activity [8]. Although PAXgene tubes offer similar DNase inhibition, they are less effective in long-term prevention of cell lysis [10,11]. Both tubes, however, are specialized and may not always be available for cfDNA studies.

The American Society of Clinical Oncology and the College of American Pathologists recommend using plasma-EDTA for cfDNA collection, with processing within 6 h to minimize leukocyte lysis and gDNA contamination [12]. Plasma-EDTA benefits from EDTA-mediated inhibition of blood DNases, preserving cfDNA during the preanalytical phase when immediate processing is not feasible [9]. When specialized tubes like Streck or PAXgene are unavailable, plasma-EDTA, with strict time management, serves as a viable alternative.

Serum, on the other hand, presents challenges for cfDNA analysis. While it may appear to yield higher cfDNA levels, this is primarily due to contamination with gDNA from lysed leukocytes during clotting, which compromises the sensitivity and specificity of the analysis [4,13]. Additionally, DNase activity in serum accelerates cfDNA degradation [9], making it less suitable for cfDNA analysis compared to plasma collected in EDTA or specialized tubes designed to preserve cfDNA integrity.

Other common anticoagulants, such as heparin and citrate, are less well-characterized for cfDNA analysis. Although these anticoagulants are typically chosen based on specific test requirements [14], their impact on cfDNA yield, gDNA contamination, and DNase activity remains insufficiently understood, highlighting a gap in their evaluation for cfDNA studies [15].

This study aims to compare cfDNA yield, stability, and DNase activity across plasma-citrate, plasma-heparin, plasma-EDTA, and serum specimens. We will evaluate the initial cfDNA amount, blood DNase activity and cfDNA degradation in plasma-citrate and plasma-heparin, using plasma-EDTA and serum as positive and negative controls, respectively. Plasma-EDTA is expected to show low gDNA contamination, effective DNase inhibition and minimal cfDNA degradation, while serum is anticipated to exhibit high gDNA contamination, high DNase activity and substantial cfDNA degradation. For plasma-citrate and plasma-heparin, these variables remain less explored, particularly their DNase activity and its cfDNA ex vivo effect on cfDNA stability. The goal is to highlight the drawbacks of plasma-citrate and plasma-heparin, focusing on the underexplored variable of blood DNase activity, and to determine whether there is a second commonly available anticoagulant as effective as EDTA for cfDNA analysis, which could expand the options for this application.

2. Materials and Methods

2.1. Subjects, Blood Collection, and Processing

This experimental study enrolled 15 healthy volunteers (7 men and 8 women). Blood samples were simultaneously collected in four 4 mL tubes: Vacuette K3EDTA (coated with

1.8 mg of K3EDTA per mL of blood), Vacuette Sodium Citrate (containing 0.109 mol/L (3.2%) sodium citrate), Vacuette Sodium Heparin (14 IU of heparin/mL of blood), and Vacuette Z Serum with Clot Activator (plain tubes coated with micronized silica particles), all from Greiner Bio-One, Kremsmünster, Austria. Within 10–15 min after venipuncture, the tubes were centrifuged at 2000× *g* for 10 min at room temperature. Subsequently, 1000 µL of the supernatants were transferred into polypropylene tubes and stored at −20 °C until further use. After thawing, the samples were centrifuged again at 14,000× *g* for 10 min at room temperature, and paired supernatants were used for subsequent experiments.

2.2. Endogenous DNase Activity Assay

Endogenous DNase activity was assessed as previously described, and detailed description of the method can be found elsewhere [9]. Briefly, the assay included 20 µL of the crude samples, 250 nM of a hydrolysis probe (5′-6-FAM-CTCCAGCTC/ZEN/CACCTGAA CGGCC-IABFQ-3′) (Integrated DNA Technologies, Coralville, IA, USA), 10 µL of 2X Maxima Probe qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), and nuclease-free water (Thermo Fisher Scientific, Waltham, MA, USA), to a final volume of 35 µL. The reactions were incubated at a constant temperature of 37 °C for 24 h using the Step-One qPCR System (Thermo Fisher Scientific, Waltham, MA, USA). Fluorescence was measured at the beginning of the reaction and then at 30-minute intervals throughout the incubation period.

The Maxima Probe qPCR Master Mix was utilized as a source of the passive reference dye (ROX) to normalize the fluorescence readings. The data were presented as delta Rn (ΔRn), calculated by dividing the FAM fluorescence by the ROX fluorescence, followed by subtraction of the baseline value, which was established using the initial fluorescence measurement. This approach allowed us to monitor the degradation of the single-stranded DNA probe by endogenous DNase activity. Nuclease-free water and DNase I (Thermo Fisher Scientific, Waltham, MA, USA) were employed as negative and positive controls, respectively.

2.3. Specimen Treatment

Serum and plasma samples were incubated at 37 °C for 24 h to investigate the effect of endogenous nucleases on cfDNA. Treatments were conducted prior to DNA extraction, and cfDNA levels were measured as described below.

2.4. DNA Extraction

DNA was extracted from 500 µL of each sample using the generic protocol 2.0.1 of the NucliSens EasyMAG system (bioMérieux, Marcy-l'Étoile, France). The extraction involved 50 µL of magnetic silica particle suspension, and the DNA was eluted in 25 µL.

2.5. cfDNA Quantification

cfDNA was quantified using qPCR, targeting a 60 base pair sequence from the RNAse P gene. The reactions were performed on the Step-One qPCR System (Thermo Fisher Scientific, Waltham, MA, USA) under the following conditions: 12.5 µL of 2X Maxima Probe qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 2.5 µL of 10X PrimeTime qPCR Assay (Integrated DNA Technologies, Coralville, IA, USA), consisting of the primers RNAse P-F (5′-AGATTTGGACCTGCGAGCG-3′), RNAse P-R (5′-GAGCGGCTGTCTCCACAAGT-3′), and the RNAse P probe (5′-HEX-TTCTGACCT/ZEN/GAAGGCTCTGCGCG-IABFQ-3′). A total of 5 µL of extracted DNA was used in a final reaction volume of 25 µL. The thermal cycling conditions were as follows: an initial pre-incubation at 95 °C for 10 min, followed by 40 cycles of 15 s at 95 °C and 15 s at 60 °C. The RNAse P calibration curve was described by the equation Y = −3.306X + 36.613

(efficiency = 100.68% and R^2 = 0.998). All samples were run in duplicate, and each run included a no-template control.

The quantity of cfDNA in genomic equivalents (GE) per reaction was determined by comparison with a 5-fold dilution series of a well-characterized DNA sample (ranging from 150,000 to 5 GE per reaction) extracted from whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). A conversion factor of 6.6 pg of DNA per cell (GE) was applied. The RNAse P concentration in GE/mL was calculated as previously described [16]. This measurement in GE/mL was chosen because it offers a direct estimation of the number of cells. To convert GE/mL to ng/mL, multiply the GE/mL value by 6.6×10^{-3}. The qPCR experiments and their description followed the Minimum Information for qPCR Experiments (MIQE) guidelines [17].

2.6. Statistical Analysis

The statistical methods employed included the D'Agostino and Pearson omnibus normality test to assess data distribution, followed by repeated-measures one-way ANOVA for comparing multiple conditions. Sidak's multiple comparisons test was used for post hoc pairwise comparisons to control for type I error. The significance level was set at $p < 0.05$ for all analyses. Data that passed the normality test were further analyzed using the repeated measures ANOVA. All graphs and statistical analyses were performed using Prism 6.0h software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Basal cfDNA Yields in Serum, Heparin-Plasma, Citrate-Plasma, and EDTA-Plasma

First, we measured the basal cfDNA levels (15 min after blood draw) in serum, heparin-plasma, citrate-plasma, and EDTA-plasma. The basal cfDNA yields (mean ± SD) were highest in serum, at 1473 ± 797 GE/mL, significantly exceeding those in the other specimens ($p < 0.0001$ compared to heparin, citrate, and EDTA). Heparin-plasma presented intermediate cfDNA levels of 472 ± 190 GE/mL, which were distinct from those in serum ($p < 0.0001$), citrate-plasma ($p < 0.0001$), and EDTA-plasma ($p < 0.0001$). Citrate-plasma and EDTA-plasma had the lowest cfDNA quantities, 147.4 ± 41.6 GE/mL and 154.8 ± 37.3 GE/mL, respectively, and these quantities were not significantly different from each other ($p > 0.99$) (Figure 1).

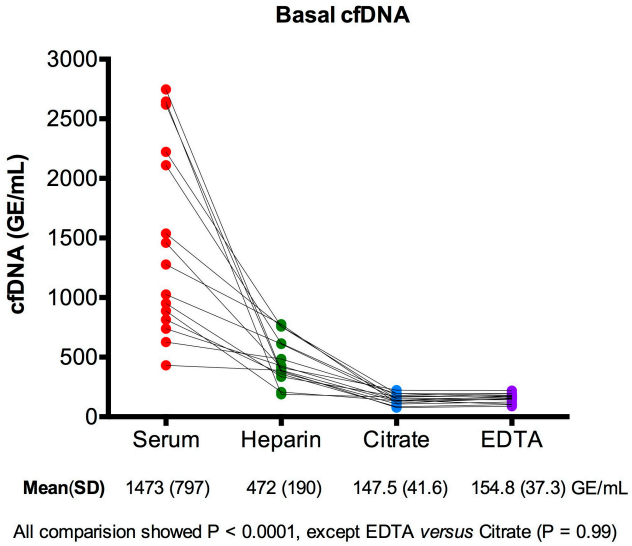

Figure 1. Basal cfDNA levels in genomic equivalents per mL (GE/mL) in serum, heparin-plasma, citrate-plasma, and EDTA-plasma samples, measured 15 min after blood draw. The cfDNA yield [mean

(SD)] was highest in serum, followed by heparin-plasma, citrate-plasma, and EDTA-plasma (n = 15). Statistical analysis revealed that all comparisons were highly significant ($p < 0.0001$), except for the comparison between EDTA-plasma and citrate-plasma ($p = 0.99$).

3.2. Direct Measurement of the Endogenous DNase Activity in Serum, Heparin-Plasma, Citrate-Plasma, and EDTA-Plasma

Next, we measured the endogenous DNase activity in serum, heparin-plasma, citrate-plasma, and EDTA-plasma using a fluorescent DNase activity assay based on hydrolysis probe degradation to evaluate the effect of each tube additive on blood DNase activity. Sustained hydrolysis probe degradation (indicating DNase activity) was observed over time in serum, heparin-plasma, and most of citrate-plasma samples. The kinetics of probe degradation in serum and heparin-plasma were similar, indicating active DNase presence; however, serum consistently exhibited lower delta Rn levels at each time point compared to heparin-plasma.

In contrast, the kinetics of probe degradation in citrate-plasma differed from those in serum and heparin-plasma, suggesting a partial inhibitory effect on DNase activity. On the other hand, in EDTA-plasma, the probe degradation plateaued at the beginning of the reaction, with no further increase observed, confirming an extensive inhibitory effect of EDTA on blood DNase activity (a representative depiction of endogenous DNase activity in a paired set of tested plasmas and serum can be found in Figure 2A).

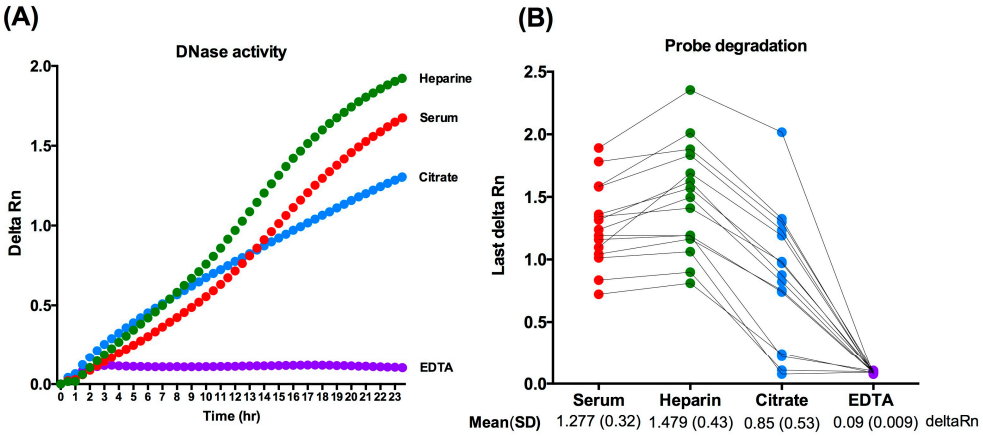

Figure 2. (**A**) Representative curves showing the endogenous DNase activity over time in serum, heparin-plasma, citrate-plasma, and EDTA-plasma. The curves illustrate the hydrolysis of a DNA probe, with activity represented as delta Rn (ΔRn) values over a 24-hour period. DNase activity was evident in serum, heparin-plasma, and citrate-plasma, with serum and heparin-plasma showing the highest activity. EDTA-plasma exhibited minimal activity, indicating strong inhibition of DNase. (**B**) Final delta Rn (ΔRn) values from the DNase activity assay for individual samples (n = 15). Each line connects measurements from the same sample across different plasma and serum types. The mean (SD) delta Rn values are provided for each sample type, showing that DNase activity was highest in heparin-plasma and serum, partially inhibited in citrate-plasma, and fully inhibited in EDTA-plasma. Statistical analysis showed all comparisons to be significant ($p < 0.0001$) except for the comparison between serum and heparin-plasma ($p = 0.17$).

Considering the last reading point of the DNase activity assay, the mean (SD) delta Rn observed in serum and heparin-plasma was [1.277 ± 0.32 delta Rn] and [1.479 ± 0.43 delta Rn], respectively, with no significant difference between them ($p = 0.17$). Citrate-plasma exhibited an intermediate DNase activity level [0.85 ± 0.53 delta Rn], which was significantly different from heparin-plasma ($p < 0.0001$), serum ($p = 0.0002$), and EDTA-plasma ($p < 0.0001$). DNase activity in EDTA-plasma was highly inhibited

[0.09 ± 0.009 delta Rn], and significantly different from heparin-plasma, serum, and citrate-plasma ($p < 0.0001$ for all) (Figure 2B).

3.3. Effect of Endogenous DNase Activity on cfDNA Levels in Plasma and Serum

Next, to observe the effect of blood DNase activity on cfDNA ex vivo, we measured the degradation of cfDNA by quantifying its levels after incubation at 37 °C for 24 h. In serum, cfDNA levels decreased by a mean of 55.6% [from 1473 ± 797 GE/mL to 653 ± 522 GE/mL, $p < 0.0001$] (Figure 3A). Heparin-plasma showed a mean reduction of 85.3% [from 472 ± 190 GE/mL to 69 ± 60 GE/mL, $p < 0.0001$] (Figure 3B). Conversely, in citrate-plasma, cfDNA levels declined by 13.3% [from 147.4 ± 41.6 GE/mL to 127.8 ± 40.1 GE/mL, $p = 0.99$] (Figure 3C), and in EDTA-plasma, there was an 8.0% reduction [from 154.8 ± 37.3 GE/mL to 142.4 ± 38.7 GE/mL, $p > 0.99$] (Figure 3D).

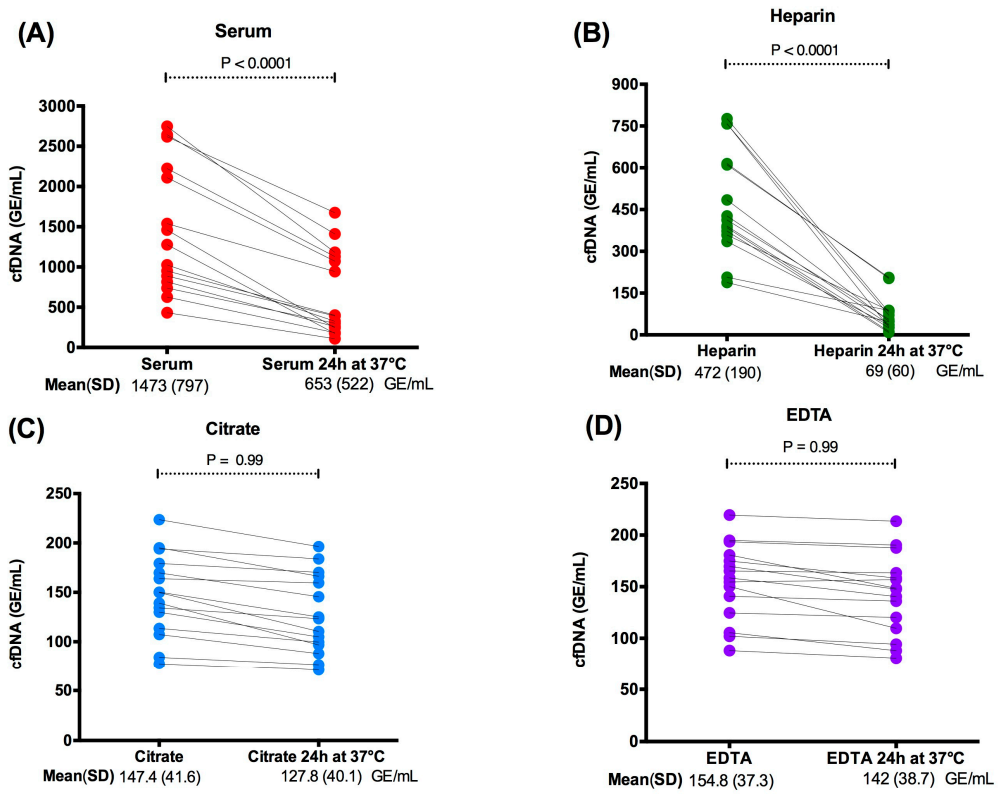

Figure 3. Changes in cfDNA levels (in genomic equivalents per mL, GE/mL) after 24 h of incubation at 37 °C across different sample types: (**A**) serum cfDNA levels decreased significantly by 55.6%; (**B**) heparin-plasma showed a significant reduction of 85.3%; (**C**) EDTA-plasma exhibited an 8.0% reduction, with no significant difference; (**D**) citrate-plasma levels decreased by 13.3%, with no significant difference. In conclusion, heparin-plasma and serum samples showed the most substantial cfDNA degradation, likely due to active blood DNase activity, whereas EDTA-plasma and citrate-plasma demonstrated greater cfDNA stability, indicating inhibited blood DNase activity.

4. Discussion

In this study, we comprehensively examined the baseline levels of cfDNA, endogenous DNase activity, and their impact on cfDNA stability, with a particular focus on the less-explored sample types, heparin-plasma and citrate-plasma, compared to the established controls, i.e., serum and EDTA-plasma. Our findings reveal three key points:

(a) Citrate-plasma and EDTA-plasma exhibited significantly lower initial cfDNA concentrations compared to serum and heparin-plasma;

(b) DNase activity was almost completely inhibited in EDTA-plasma, partially inhibited in citrate-plasma, and highly active in both heparin-plasma and serum;
(c) DNase-driven cfDNA degradation was substantial in heparin-plasma and serum, moderate in citrate-plasma and minimal EDTA-plasma.

Our quantitative analysis, conducted within 15 min of blood draw, showed that citrate-plasma and EDTA-plasma had lower baseline cfDNA levels compared to heparin-plasma and serum. These findings indicate that citrate-plasma and EDTA-plasma introduce minimal ex vivo cfDNA, with values (~75–225 GE/mL or ~0.5–1.485 ng/mL) comparable to those reported in the circulation of healthy individuals (0.06 to 22.5 ng/mL) [18].

The elevated cfDNA levels observed in serum, as expected, were attributed to leukocyte lysis during coagulation [6]. Unexpectedly, heparin-plasma showed higher cfDNA levels than EDTA-plasma and citrate-plasma, despite the assumption that anticoagulation would prevent the release of additional gDNA into cfDNA.

These results suggest that different anticoagulants have distinct pre-analytical effects on baseline cfDNA levels. Heparin, citrate, and EDTA anticoagulate through different mechanisms.

Heparin works by binding to antithrombin, a naturally occurring inhibitor of blood clotting. Once bound, heparin significantly enhances antithrombin's ability to inhibit key clotting factors, such as thrombin (factor IIa), factor Xa, and factor IXa. This prevents the formation of fibrin, the protein that forms the structural framework of a blood clot [19].

On the other hand, EDTA and citrate inhibit coagulation by acting as divalent ion chelators. They bind to calcium ions (Ca^{2+}), which are essential cofactors in the coagulation cascade. Calcium ions facilitate the activation of several clotting factors, including prothrombin (factor II) and factor X, which are crucial for converting fibrinogen to fibrin. By sequestering calcium, EDTA and citrate effectively disrupt this cascade, preventing blood clot formation [19].

The observed cfDNA levels in paired citrate-plasma, EDTA-plasma, and heparin-plasma indicate that different anticoagulants have distinct pre-analytical effects on baseline cfDNA levels due to their varied influences on blood coagulation and cell stability.

Corroborating this observed effect, in vitro research has shown that heparin can induce leukocyte lysis in a dose-dependent manner, with concentrations of 10 IU/mL causing 10–30% cell lysis [20]. Another in vitro study demonstrated that heparin at 10 IU/mL can also promote the formation of neutrophil extracellular traps (NETs) within 15 min, which are often associated with cell lysis, death, and the release of extracellular DNA [21]. We hypothesize that the slightly higher ex vivo cfDNA levels observed in heparin-plasma shortly after blood draw (15 min) could be linked to this in vitro phenomenon, as the amount of heparin in collection tubes typically ranges from 14 to 18 IU/mL [19].

These observations suggest that divalent ion chelators, such as EDTA and citrate, are preferable for cfDNA analysis as they minimize unwanted gDNA contamination from leukocytes. This prevention reduces pre-analytical interference from non-tumor or non-fetal DNA sequences, preserving assay sensitivity in applications like liquid biopsies and fetal DNA analysis.

Our second major finding, that DNase activity is highly inhibited in EDTA-plasma, partially inhibited in citrate-plasma, and active in both heparin-plasma and serum, is supported by two key observations: (1) the direct measurement of DNase activity using a hydrolysis probe assay; and (2) cfDNA levels assessed after incubation at 37 °C for 24 h to promote DNA degradation [22].

DNase activity was highest in heparin-plasma and serum, intermediate in citrate-plasma, and lowest in EDTA-plasma. After incubation at 37 °C for 24 h, cfDNA degradation

was most pronounced in heparin-plasma (85.3%) and serum (55.6%), compared to citrate (13.3%) and EDTA (8%).

These results can be explained by the fact that EDTA and citrate chelate divalent ions (Mg^{2+} and Ca^{2+}), rendering them unavailable for DNase activity, as these ions are essential for most blood DNases [23,24]. This dual effect—preventing both coagulation and DNase activity—makes EDTA the preferred anticoagulant for cfDNA analysis as it minimizes gDNA contamination and reduces DNase activity.

Citrate-plasma showed intermediate DNase inhibition, with cfDNA degradation rates similar to EDTA-plasma after 24 h at 37 °C (13% versus 8%). While citrate interferes with DNase activity, the concentration tested (3.2%) may be insufficient to achieve the higher inhibition seen with EDTA. Despite this intermediate DNase activity, the baseline cfDNA amount did not significantly change after 24 h at 37 °C, making citrate-plasma the second-best option for cfDNA analysis among the specimens tested when plasma separation is performed soon after blood draw. The main drawback remains the intermediate DNase activity. Indeed, we found that increasing the citrate concentration to 8% in collection tubes improves its effectiveness, making it comparable to EDTA for cfDNA analyses (preliminary results) [25].

A previous study evaluated the cfDNA quality of citrate-plasma and EDTA-plasma in terms of gDNA contamination after 72 h of storage at 4 °C. It demonstrated that citrate-plasma (3.2%) provided superior cfDNA quality, with minimal gDNA contamination, compared to EDTA-plasma, which exhibited larger cfDNA fragments due to gDNA contamination. cfDNA quality and quantity in citrate tubes were maintained throughout this period, making the results comparable to those obtained with cell-stabilizing tubes like Streck [26].

In the above-cited study, better results from citrate-plasma were observed only after 72 h of contact with leukocytes, and they were comparable to EDTA before this time. Storage at 4 °C is less favorable for blood DNase activity and could lead to its full inhibition. While the pre-analytical conditions in our study and the previously mentioned one were different and not directly comparable, taken together, these observations suggest that citrate-plasma is a viable option for cfDNA analysis. Storing samples at 4 °C may compensate for the intermediate DNase activity observed, thereby helping preserve cfDNA quality.

Heparin-plasma and serum, with fully active DNases, present significant drawbacks due to gDNA contamination and cfDNA degradation, making these specimens unsuitable for cfDNA analysis. Despite this, several studies on cfDNA have used heparin-plasma. The results from these prior cfDNA studies should be interpreted cautiously, considering the preanalytical effects observed in our study.

Although DNase activity in heparin-plasma was not significantly different from serum, cfDNA degradation was higher in heparin-plasma (85.3% vs. 55.6%). This could be attributed to serum DNase inhibition by G-actin released from lysed cells [27], which is more pronounced due to higher gDNA contamination. On the other hand, heparin at 10 IU/mL has been shown to form polymeric complexes with cell-free DNA in whole blood [28] and facilitates the DNase I-mediated digestion of cfDNA, independent of its anticoagulant properties [29].

Since heparin-plasma exhibited higher cfDNA levels compared to citrate-plasma and EDTA-plasma, which seem to represent the basal levels of cfDNA in circulation, our observations support the hypothesis that heparin, at least to some extent, directly induces ex vivo cell death and the subsequent release of gDNA into plasma. The simultaneous occurrence of G-actin inhibition in serum and heparin stimulation of DNase I activity could have contributed to the observed cfDNA degradation levels after 24 h of incubation at 37 °C in our experiments.

Indeed, heparin is not the sample of choice for cfDNA studies due to its inhibitory effects on polymerase chain reaction (PCR) assays. Heparin is known to inhibit DNA polymerase activity, which can significantly interfere with the amplification of cfDNA, leading to unreliable results [30,31]. However, the addition of heparinase, an enzyme that degrades heparin, has been shown to enable reliable quantification of circulating tumor DNA (ctDNA) from heparinized plasma samples using droplet digital PCR [31].

We cannot exclude the possibility that heparin inhibited the qPCR reactions observed in this study. However, it is unlikely that modern DNA extraction methods, which result in highly purified DNA templates, are significantly affected by this issue. Although we did not observe any qPCR inhibition, the experiments were not specifically designed to test for this effect. We speculate that the observed inhibitory effect of heparin on cfDNA could be due to its high DNase activity and cfDNA degradation, potentially being confounded with PCR inhibition.

Additionally, several studies have consistently shown that heparin use in pregnant women poses a preanalytical problem for non-invasive prenatal testing (NIPT) due to increased degradation of cell-free fetal DNA to the production of shorter cfDNA fragments and lower fetal fractions [32,33]. These factors lead to more frequent retests and a significantly higher likelihood of test failure, with an odds ratio of 21.87 for nonreportable results in heparin-treated patients [34].

This underscores the need for careful consideration of heparin use in NIPT testing. We hypothesize that the increased degradation of cell-free fetal DNA and non-reportable NIPT results in women using heparin could be secondary to increased blood DNase activity in vivo. This hypothesis should be tested in specific experiments evaluating DNase activity in individuals using heparin.

Despite the strengths of this study in providing a comparative analysis of cfDNA stability and DNase activity across commonly used anticoagulants, some limitations should be acknowledged. First, our study was conducted using blood samples from healthy volunteers, which may not fully reflect cfDNA dynamics in patients with pathological conditions such as cancer, inflammatory diseases, or pregnancy-related cfDNA alterations. Additionally, our sample size was limited to 15 individuals, which, while sufficient to detect statistically significant differences, does not fully capture potential inter-individual variability in cfDNA metabolism and DNase activity. A larger cohort, including patients with various medical conditions, would provide a more comprehensive understanding of how different anticoagulants impact cfDNA integrity in diverse populations.

Second, we focused on cfDNA quantification but did not assess its quality in terms of purity (A260/280 ratio) or fragmentation profiles. However, it is important to note that in our routine clinical practice and previous studies using the same extraction platform (Easymag), we have never observed significant issues related to DNA purity or PCR inhibition. This semi-automated system has consistently provided high-quality nucleic acid extractions for molecular diagnostics, including HPV detection, fetal sex determination, and viral RNA quantification (HIV, dengue, Zika, chikungunya, and SARS-CoV-2). While this supports the reliability of our extractions, future studies should include a more detailed evaluation of cfDNA purity and fragmentation, particularly to assess the potential presence of high molecular weight DNA contamination, especially in heparin-plasma.

Third, this study did not include specialized blood collection tubes such as Streck and PAXgene, which are specifically designed to stabilize cfDNA by preventing leukocyte lysis and inhibiting DNase activity. While EDTA-plasma remains the gold standard when specialized tubes are unavailable, a direct comparison with these tubes would provide a more comprehensive understanding of cfDNA preservation across different collection methods. Future research will focus on evaluating these specialized tubes to determine

their advantages and limitations in comparison to EDTA, particularly in settings where EDTA may not be ideal.

Despite these limitations, our study provides critical insights into the impact of citrate and heparin on cfDNA integrity and DNase activity, reinforcing the importance of anticoagulant selection in cfDNA-based applications. These findings should guide future studies aiming to refine preanalytical protocols for liquid biopsies, non-invasive prenatal testing, and other cfDNA-based diagnostic approaches.

5. Conclusions

In conclusion, citrate-plasma and heparin-plasma presented distinct challenges and insights for cfDNA analysis. Citrate-plasma exhibited intermediate DNase inhibition and low gDNA contamination, suggesting it could be a potential alternative when EDTA is unavailable. Heparin-plasma, on the other hand, displayed the highest DNase activity, leading to substantial cfDNA degradation and, combined with its known PCR inhibition, makes it highly unsuitable for cfDNA analysis. Serum, while yielding the highest baseline cfDNA levels, suffered from significant DNase activity and gDNA contamination, compromising its utility in cfDNA-based applications. With minimal DNase activity and low gDNA contamination, EDTA-plasma remains the gold standard for cfDNA analysis among common anticoagulants. These findings emphasize the importance of anticoagulant selection, particularly for critical applications such as liquid biopsies and prenatal testing, and call for caution when interpreting results from previous studies using serum and heparin-plasma due to their significant pre-analytical limitations.

Author Contributions: Conceptualization, G.B.B. and T.H.S.R.; methodology, G.B.B. and R.H.J.; formal analysis, G.B.B., T.H.S.R. and R.H.J.; resources, L.F.A.N.; data curation, G.B.B.; writing—original draft preparation, T.H.S.R.; writing—review and editing, G.B.B. and R.H.J.; supervision, R.H.J.; project administration, G.B.B.; funding acquisition, R.H.J. and L.F.A.N. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: This study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Federal District Faculty of Health Sciences (FEPECS) under protocol code CAAE 35684414.5.0000.5553 (Approved on 24 November 2014).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data supporting the results reported in this study are included within the article.

Acknowledgments: We thank Janete Ana Ribeiro Vaz and Sandra Santana Soares Costa for their support in this study.

Conflicts of Interest: The authors declare no conflicts of interest.

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