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# Heat treatment of samples improve the performance of the Nijmegen–Bethesda assay in hemophilia A patients undergoing immune tolerance induction

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ABSTRACT

Nijmegen–Bethesda assay is the gold standard to assess inhibitory antibodies against factor (F) VIII. This method has some limitations, including high coefficient of variation and possible interference of residual endogenous or exogenous factor VIII. Heat-treatment of samples at 56 °C for 30 min could be a strategy to improve the sensitivity of this test. The aim of this study was to compare inhibitor quantification in hemophilia patients with and without inhibitor performed in previously heated and non-heated samples. A total of 109 analyses from 46 patients with severe hemophilia A were performed. Patients were divided into three groups: 20 patients with no history of inhibitor, recently and not recently exposed to FVIII (group I), 21 patients with history of inhibitor not exposed to FVIII (group II), and 5 patients (68 samples) undergoing an immune tolerance induction (ITI) protocol (group III). For patients with no history of inhibitor, heat-treatment did not modify the results ($p = 0.24$). However, differences in inhibitor levels between heated and non-heated samples were observed in patients with history of inhibitor (group II, $p < 0.05$) and in patients in ITI (group III, $p < 0.001$). In 11 samples, inhibitor quantification shifted from negative to positive. Additionally, a longitudinal evaluation of each ITI patient showed similar trend line for the results of heated and non-heated samples. In this study, we demonstrated that heating samples increase sensitivity of Nijmegen–Bethesda assay, with no shift from negative to positive results in patients with no history of inhibitor. Furthermore, this procedure has an important role to patients undergoing an ITI protocol.

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## 1. Introduction

The assessment of inhibitory antibodies against factor (F) VIII or IX is critical for hemophilia care in the clinical setting. The first method for inhibitor evaluation, Bethesda assay, was described by Kasper in 1975 [1], and later Verbruggen proposed the Nijmegen modification [2]. Currently, Nijmegen–Bethesda assay is the gold standard method for inhibitor assessment. The method evaluates FVIII or FIX residual coagulant activity from normal pooled plasma (NPP) after 2 h of incubation with patient plasma under inhibitor investigation. The percentage of residual factor activity is converted into Bethesda units (BU). One BU is defined as the amount of inhibitor producing a residual activity of 50% [1]. In spite of the sensitivity improvement provided by the Nijmegen modification, Nijmegen–Bethesda assay retains a lack of reproducibility evidenced by high coefficient of variation (CV), in different external quality assessment programs, including, External quality Control of Assays and Tests (ECAT), and UK National External Quality Assurance Scheme (NEQAS) [3,4].

Immune tolerance induction (ITI) treatment, which is based on regular infusions of FVIII concentrates, is the main strategy for inhibitor eradication in hemophilia A patients. The management of ITI is highly dependent on the accuracy of inhibitor quantification using the Nijmegen–Bethesda assay. However, the presence of exogenous FVIII administrated within an ITI protocol affects the principle of the Bethesda assay, where patient and control samples should be equivalent before incubation, leading to underestimation of the inhibitor titers. This interference has been shown to be minimized when the samples are heated before the assay, to eliminate the residual factor [5]. However, the impact of this procedure in the context of ITI has not yet been evaluated. The objective of this study was to evaluate the effect of plasma heating in split plasma samples from

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patients with hemophilia A with or without inhibitor, and in patients submitted to ITI.

## 2. Patients and methods

### 2.1. Ethical issues

This study was part of a multicenter study, (EMBIH — Estudo Multicêntrico Brasileiro de Inibidores em Hemofilia) involving six Hemophilia Treatment Centers (HTCs) from Brazil and coordinated by the Hemophilia Unit from Hemocentro Unicamp in Campinas, São Paulo, Brazil. The Institutional Review Board of the participating centers approved data collection. All procedures were in accordance with the ethical standards and with the Helsinki Declaration.

### 2.2. Human plasma samples

Plasma samples from hemophilia A patients were collected in 0.109 M citrated tubes in accordance with the Clinical and Laboratory Standards Institute (CLSI) recommendation, into evacuated siliconized glass tubes (Becton Dickinson, Franklin Lakes, NJ, USA). The plasma samples were separated by 2500 *g* for 10 min and stored at −80 °C until the assay was performed. Blood processing was completed within 2 h. Two different aliquots were separated from the same plasma sample of each patient. One of the aliquots was heated at 56 °C for 30 min and then centrifuged at 2500 *g* for 5 min whereas the second aliquot was not heat-treated.

### 2.3. Nijmegen–Bethesda assay

Both plasma aliquots underwent the same procedure for Nijmegen–Bethesda assay, as previously described [2]. Briefly, all patient samples were initially screened undiluted, and when the result was positive, dilutions from 1:2 until 1:320 with 4% bovine serum albumin (BSA) (Sigma, St. Louis, MO, USA) buffered in 0.1 M imidazole buffer, pH 7.4 (Instrumentation Laboratory Bedford, Massachusetts USA) were performed. Undiluted and diluted samples were incubated volume to volume with normal pool plasma buffered (NPPB) with 0.1 M imidazole to pH 7.4, prepared in house by the addition of solid imidazole (Sigma, St. Louis, MO, USA), for 2 h at 37 °C. FVIII coagulant activity (FVIII:C) in each sample was measured based on the one stage by activated partial thromboplastin time (aPTT) method, using PTT-A reagent (Instrumentation Laboratory Bedford, Massachusetts USA), FVIII-deficient plasma (Instrumentation Laboratory Bedford, Massachusetts USA) as substrate on a TOP 500 coagulation analyzer (Instrumentation Laboratory Bedford, Massachusetts USA). Calibration curves were prepared using standard reference plasma (Instrumentation Laboratory Bedford, Massachusetts USA) diluted in 0.1 M imidazole buffer, pH 7.4 (Instrumentation Laboratory Bedford, Massachusetts USA). The detection limit for FVIII calibration curve was 0.1 IU/dL. The residual FVIII:C in the patients' mixture from each sample was divided by the remaining FVIII:C in the control mixture (FVIII-deficient plasma from Instrumentation Laboratory Bedford, Massachusetts USA and NPPB) expressed as percentage of residual activity. The results were calculated by linear regression of a curve containing 1 BU as the 50% residual activity and 0 BU as 100% residual activity. When the residual activity was ≥100% for undiluted samples, the final result was reported as 0 BU. When the residual factor of undiluted sample was <100%, additional dilutions were evaluated until a residual activity between 25% and 75% was achieved. In this case, at least three dilutions were plotted on the line curve to check the antibody kinetic. The parallelism between the three dilutions was evaluated and a coefficient of variation <10% was considered acceptable. The dilution of approximately 50% of residual activity was selected as the final result. For all diluted samples the dilution factor applied was considered as the final result. Positive inhibitor was defined as >0.6 BU.

### 2.4. Factor VIII antigen

FVIII antigen was determined by enzyme-linked immunosorbent assay (ELISA) with VisuLize™ FVIII Antigen Kit (Affinity Biological, Ancaster, Ontario, Canada) according to the manufacturer's instructions. Briefly, strip wells were pre-coated with sheep polyclonal antibody to human FVIII. Plasma samples and calibrator plasma were diluted and applied to the wells. FVIII antigen when present, binds to the coated antibody. After washing away unbound material, peroxidase-labeled sheep detecting antibody was applied and allowed to bind to the captured FVIII. The wells were again washed and a solution of peroxidase substrate tetramethylbenzidine (TMB) was applied and allowed to react for a fixed period of 15 min. A blue color develops which changes to yellow and the color formed was measured spectrophotometrically in a microplate reader at 450 nm. The absorbance at 450 nm was directly proportional to the quantity of FVIII antigen captured.

### 2.5. Statistical analyses

Mann–Whitney test and Spearman correlation coefficient, with a significance level at $p < 0.05$, were calculated using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

## 3. Results

We performed 109 analyses on 46 severe hemophilia A patients (FVIII < 1.0 IU/dL). For evaluation, patients were divided into three distinct groups: 20 patients with no history of inhibitor, recently and not recently exposed to FVIII (group I); 21 patients with history of inhibitor not exposed to FVIII (group II); and 5 patients (68 analyses) undergoing an ITI protocol (group III).

In patients from group I, no statistical significance was observed between heated and non-heated samples submitted to Nijmegen–Bethesda assay, in that heat treatment of plasma samples did not modify the results (Fig. 1A). In group II, a statistically significant increase in the inhibitor titer was observed when samples were heat-treated ($p < 0.05$) (Fig. 1B). In this group, samples from 6 out of 21 patients, which were negative with no heat-treatment before the assay, became positive after heat-treatment. We also evaluated FVIII activity and FVIII antigen (FVIII:Ag) levels in each plasma sample from these patients, as these could interfere in the performance of the inhibitor detection assay. As shown in Table 1, all these samples contained FVIII:Ag before heat-treatment (Table 1). In the group of patients undergoing ITI (group III) an even more evident difference was observed between the mean results obtained in heated and non-heated samples ($p < 0.001$) (Fig. 1C). This group consisted of 5 patients, aged between 1 yr 11 months and 35 yr 6 months when ITI started. Mean time from first inhibitor detection until the beginning of ITI was 53.2 m (SD 41 m; ranging from 1 m to 111 m). When samples were heat-treated, the mean titer of inhibitor was 3 fold higher when compared to samples not submitted to heat treatment. In order to confirm these data, samples from ITI patients were longitudinally evaluated. Interestingly, the results obtained with heated and non-heated samples from each ITI patient showed the same trend line (Fig. 2). In 5 of these samples, ITI-1, 2 and 4 previously negative results became positive (>0.6 BU) after heat treatment (Table 1).

## 4. Discussion

ITI represents the most important therapeutic strategy for inhibitor treatment in hemophilia A. However, ITI is a complex and often long-lasting therapy, involving a fair amount of human and material resources. Inhibitor titers measured by the Nijmegen–Bethesda assay are the most important parameter for ITI management, thus, the accuracy of this assay can be regarded as a critical determinant of ITI success

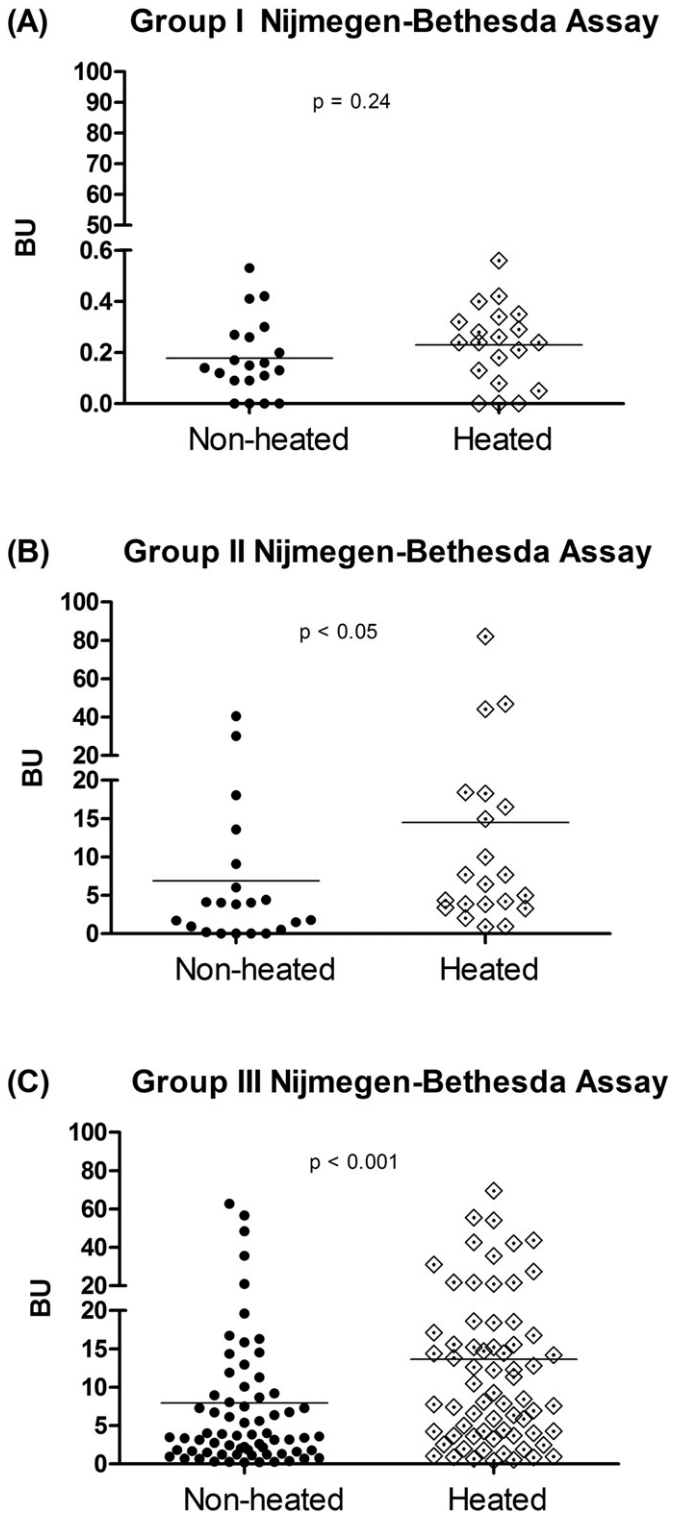


**Fig. 1.** Comparison of non-heated and heated samples for Nijmegen–Bethesda assay. A. 20 patients without history of inhibitor, recently and not recently exposed to FVIII (group I). B. 21 patients with history of inhibitor not exposed to FVIII (group II). C. 5 patients (68 samples) undergoing an ITI protocol (group III).

[6]. In this study, we demonstrated that heat treatment of plasma samples improved the sensitivity of the Nijmegen–Bethesda method, particularly in patients who were recently exposed to FVIII concentrates, as during ITI treatment. This improvement was achieved with no apparent increase of false positive results in patients with no history of inhibitor.

**Table 1**
FVIII coagulation activity (FVIII:C) and FVIII antigen (FVIII:Ag) in samples from group II and group III patients that became positive in Nijmegen–Bethesda assay after heat treatment.

| Patient | | FVIII:C (IU/dL) | FVIII:Ag (ng/dL) | Inhibitor titer (BU) | |
|---|---|---|---|---|---|
| | Reference range | 60–120 | 64–189[a] | 0.6 | |
| | Detection limit | 0.2 | 0.8 | 0 | |
| | | | | Non-heated sample | Heated sample |
| *Group II patients with history of inhibitor not recently exposed to FVIII* | | | | | |
| 1 | | 0.8 | 3.25 | 0 | 0.95 |
| 2 | | 0.1 | 4.46 | 0 | 0.9 |
| 3 | | 0.1 | 1.42 | 0 | 3.31 |
| 4 | | 0.1 | 1.43 | 0 | 3.41 |
| 5 | | 0.3 | 1.50 | 0.2 | 2.05 |
| 6 | | 0.3 | 1.53 | 0.54 | 6.46 |
| *Group III patients undergoing ITI treatment* | | | | | |
| ITI-2 | | 1.6 | 2.11 | 0.19 | 2.47 |
| ITI-1 | | 1.8 | 20.55 | 0.24 | 3.62 |
| ITI-4 | | 0.2 | 7.42 | 0.28 | 0.87 |
| ITI-4 | | 0.2 | 6.00 | 0.29 | 1.8 |
| ITI-2 | | 0.5 | 1.64 | 0.39 | 3.69 |

[a] Reference range according to the manufacture, assuming FVIII:Ag concentration of 100–200 ng/mL per 1 IU/mL of FVIII activity.

In our study, heat treatment of plasma samples did not result in any significant change in inhibitor levels in 20 patients with no history nor clinical suspicion of inhibitor, in whom inhibitor was not expected. These results indicate that heating of plasma samples does not lead to an increase in false positive results, preserving the specificity of the assay. Next, we evaluated the effect of heat-treatment in samples from patients with a history of inhibitor, with no recent exposure to FVIII. This represents a population in whom false negative results could result in inadequate therapeutic choices. We demonstrated a significant increase in inhibitor levels, with the higher fold from 4.14 to 14.98 BU. More importantly, in 6 of 21 patients, positive results were obtained in heat-treated samples, which were otherwise reported as negative in non-heated samples. The maximum increase in inhibitor levels was from 0 to 6.46 BU. Interestingly, FVIII antigen levels could be detected in all these samples, suggesting that heat-inactivation of residual protein was indeed the mechanisms behind the improvement of test performance. An even more striking difference between heated and non-heated samples was observed in samples from patients on ITI, in whom higher FVIII antigen levels were present in non-heated samples. ITI patients are treated with high amounts of FVIII protein; therefore, any deleterious effect of residual protein on assay performance becomes even more critical. Accordingly, the presence of higher levels of FVIII antigen, and the higher difference of inhibitor titers between these samples corroborate that heat-treatment improves assay performance by minimizing the influence of residual FVIII levels. The longitudinal follow-up of our patients demonstrate that despite the fact that the inhibitor titers obtained by the two methods presented a similar pattern, heating resulted in significantly different levels of inhibitor titer at critical time points, which could potentially influence the management of these patients and ultimately, the success of ITI. Therefore, our data suggest that heat treatment could be an important step for patients undergoing ITI, since even low levels of FVIII antigen in the test sample could potentially hamper the good performance of the Nijmegen–Bethesda assay. In fact, one of the most basic principles of this assay is that patient and control mixtures should be comparable before incubation [2]. Thus, denaturation of FVIII protein by heat treatment could guarantee this comparability.

Definition of ITI success is based on adequate pharmacokinetics parameters of FVIII, including negative results of Nijmegen–Bethesda assay, which recovered more than 66% of the expected coagulation activity after FVIII had been administered, with a FVIII half-life of more

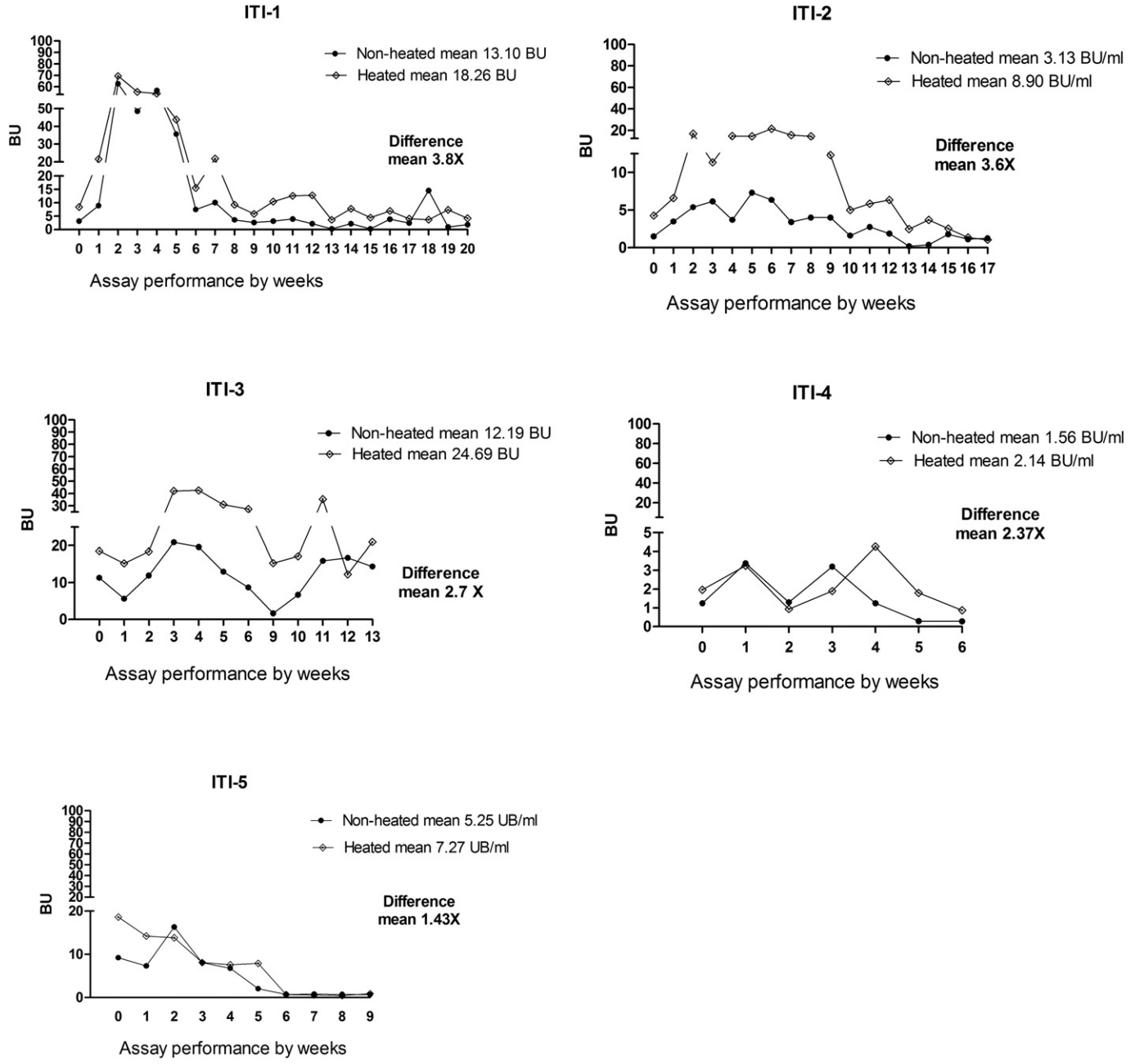


**Fig. 2.** Longitudinal evaluation of inhibitor by Nijmegen–Bethesda assay from heated and non-heated samples from five hemophilia A patients (ITI 1–5) submitted to ITI treatment.

than 6 h [7]. In some cases, a discrepancy of results is found with a negative Nijmegen–Bethesda assay, and an inadequate pharmacokinetics parameter. This observation can be correlated with the presence of non-neutralizing antibodies or also due to false negative results from samples without heat treatment.

Previously, Verbruggen and colleagues reported that there were no significant changes in the results with heat treatment in inhibitor positive specimens [8]. However, in this study, samples were spiked with FVIII for *in vitro* evaluation, and this could explain the different results. More recently, Miller et al. demonstrated that plasma heating could indeed improve the sensitivity of the Nijmegen–Bethesda assay, by minimizing the effect of residual FVIII protein in non-heated samples [5]. Recently this application was further evaluated in acquired hemostatic disorder resulting from autoantibodies developed against FVIII and in this context, heat treatment of the sample also appeared to improve the sensitivity of the Nijmegen–Bethesda assay [9]. Despite these demonstrations, heat-treatment of plasma samples is not yet widely used by hemostasis laboratories worldwide [5]. Another important scenario is related to high coefficient of variation (CV %) of Bethesda assay and of the Nijmegen–Bethesda assay. The poor reproducibility of the test is even more pronounced in inhibitor titers below 20 BU [10]. The lack of sensitivity mainly in low titer inhibitor range can interfere in different clinical settings, such as the choices of the treatment product and in management of ITI therapy, resulting in less effective replacement therapy and increasing bleeding complications [10]. In other words, no inhibitor detection by Nijmegen–Bethesda assay due to high variability results inter-laboratory can also affect treatment choices. In this context, standardization of methods and reagents, use of controls and heat treatment may overcome this circumstance.

Until now, none of the studies that addressed the effect of heat-treatment of plasma during inhibitor surveillance correlated their results with clinical variables such as bleeding response to clotting factor concentrates, in the case of patients off ITI, or of ITI success, which occurred in our case. This limitation of our study should be

taken into consideration when analyzing our results. However, the fact that in group II, inhibitors were later detected in 6 patients in whom false negative results had been obtained without heat-treatment is highly suggestive of the clinical relevance of our findings.

## 5. Conclusion

Residual factor VIII in plasma samples of hemophilia A patients which can interfere with the Bethesda or Nijmegen–Bethesda assays, can be a particularly relevant challenge in ITI context. In our study, we demonstrated the benefits of the heat treatment in improving the sensitivity of the Nijmegen–Bethesda assay in inhibitor positive patients, and in the ITI context. Future studies are required to evaluate the clinical impact of this simple laboratory step in the laboratory surveillance of inhibitor levels.

## Authorship

*Contribution*: S.A.L.M. designed the study, performed the assays, collected and analyzed the data, and wrote the manuscript; A.C.T. performed the assays, A.L.A.S. collected the data; E.V.D.P and S.S.M. analyzed the data, and reviewed the manuscript; and M.C.O. designed the study, collected the data, and critically revised and approved the final version of the manuscript.

*Conflict-of-interest disclosure*: The authors declare no competing financial interests.

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