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IgG subclasses of FVIII inhibitors in an argentine cohort of severe hemophilia A patients: Analysis by flow cytometry

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Abstract

Introduction: FVIII inhibitors consist of a polyclonal population of antibodies. Previous studies have demonstrated different distribution of IgG subclasses. IgG4 was associated to high level of FVIII inhibitors and failure of immune tolerance induction (ITI) treatment. This study monitored the relative distribution of IgG subclasses of anti-FVIII in patients with severe hemophilia A (SHA).

Methods: Anti-FVIII antibodies were measured employing an immunomethod, developed in our laboratory, that combines flow cytometry (FC) with microspheres coupled (FVIII-m) or not (Control-m) to FVIII. Seventy-five patients with SHA were studied, 17 without inhibitors (Group I); 58 with inhibitor history, 13 low responders: (LR: Group II), and 45 high responders (HR: Group III). Eight patients undergoing ITI were also included.

Results: We found anti-FVIII antibodies in 11 of 27 patients (40%) without inhibitors and in 45 of 48 with inhibitors at the moment of the study. IgG4 was predominant only in the Group III: P=0.02 in patients with low level of inhibitors and P=0.0001 with high titer of inhibitors. Longitudinal analysis performed on patients undergoing ITI showed a gradual decrease of IgG4 values that was associated to improvement of clinical parameters during treatment.

Conclusion: We suggest the use of the FC method to supplement functional traditional assays and to help to improve the management of patients with SHA.

KEYWORDS

flow cytometry, FVIII, hemophilia, immunoglobulins, inhibitors

1 | INTRODUCTION

FVIII, glycoprotein that circulates bound noncovalently to VWF, participates in the coagulation cascade. Genetic defects of FVIII result in Hemophilia A, a coagulation disorder which is treated with FVIII products. About 25% of patients with severe hemophilia A (SHA) develop antibodies against the FVIII molecule, a fact that represents a serious therapeutic problem.[1] If the antibodies are directed to functional sites of the molecules they inhibit FVIII function (inhibitor antibodies: I-Ab), neutralizing the procoagulant activity of the factor. Antibody responses against FVIII are routinely identified using the Bethesda or Nijmegen-modified Bethesda assays.[2] These methods detect only antibodies that inhibit the FVIII function, has a limited sensitivity for low titer inhibitors, a high coefficient of interlaboratory variations,[3,4] with high number of false-positive and false-negative results, and the results are affected by the presence of thrombin inhibitors, heparin, and Lupus Anticoagulants (LA).[5]

The International Society of Thrombosis and Haemostasis (ISTH) classification of I-Ab is accepted worldwide.[6] Low responder patients (LR) (whose historical inhibitor peak never exceeded 5 Bethesda Unit

[BU] mL^{-1}) do not develop an increase in inhibitor levels after further exposure to FVIII. In contrast, high responder patients (HR) (whose historical inhibitor peak exceeded 5 BU mL^{-1} at least once) increased I-Ab upon re-exposure to FVIII. This group of patients needs to be treated with bypassing agents like Factor VII and activated prothrombin complex concentrates.[7]

The humoral response to FVIII may also include antibodies, which do not inhibit FVIII function (NI-Ab) but may affect the biological half-life of infused FVIII and be clinically relevant increasing the clearance of the Factor.[8-10] NI-Ab escape detection by the functional neutralization assays. In about 20% of normal healthy donors, FVIII inhibitors also have been identified.[11,12]

Immune tolerance induction (ITI) is the strategy of choice for eradication of FVIII inhibitors. This treatment includes frequent administration of high or intermediate doses of FVIII, which results in a gradual decline of the inhibitor titer in 75% of treated patients.[13,14] ITI may take 1-3 years to achieve tolerance.[7] Little is known about the immunological mechanisms that cause the down modulation of the humoral anti-FVIII immune response.

FVIII elicits a polyclonal IgG response.[15] In humans, four IgG subclasses with distinct structural and functional properties, IgG1, IgG2, IgG3, and IgG4 constitute approximately 65%, 25%, 6%, and 4% of total IgG, respectively.[16-18] IgG antibody responses to different types of antigens leads to marked skewing toward one of the subclasses. IgG1 and IgG3 antibodies are generally induced in response to T-dependent protein antigens, whereas IgG2 antibodies are associated with polysaccharide antigens.[16] Chronic antigen stimulation elicits IgG4 antibodies.[17] Previous reports have shown different contribution of each subclass in FVIII immune response depending on the method used for evaluation and/or the group of patients included in the study.[19-24] Although all subclasses have been found, IgG4 is the major component of the anti-FVIII response, specially in cases with high inhibitor level and when ITI fails.[25]

The Bethesda assay is not useful to determine the relative contribution of the different IgG subclasses to the total amount of anti-FVIII IgG in patient samples.

We have developed a sensitive immunomethod to evaluate anti-FVIII antibodies using a combination of FVIII-coated microspheres and flow cytometry (FC). This semiquantitative assay detects both I-Ab and NI-Ab and has been useful to evaluate total anti-FVIII IgG.[26]

In this study, we investigated the relative contribution of IgG subclasses to the anti-FVIII response of SHA patients of our institution, employing the FC assay, with particular emphasis in the evaluation of the results in relation to the response of patients to ITI.

2 | MATERIALS AND METHODS

2.1 | Patients

Plasma samples were collected at least 7 days after the last infusion of FVIII concentrate from patients with SHA referred to our center. None of the samples were hemolyzed, lipemic, or heat inactivated. Seventy-five patients were stratified according to Bethesda assay: Group I: 17 patients without history of I-Ab, Group II: 13 LR patients, five without current inhibitors, and eight showing a low but detectable I-Ab (≤1 BU mL^{-1}), Group III: 45 HR patients: five without current inhibitors, 17 with low I-Ab titers (≤5 BU mL^{-1}), and 23 with high I-Ab levels (>5 BU mL^{-1}). Eight patients during ITI treatment were also included. Patients under ITI received 200 IU/kg of anti-hemophilic Factor VIII once a day during 18 months.

All hemophiliacs were negative for HIV infection. The study was approved by the Ethics Committee of Academia Nacional de Medicina of Buenos Aires.

2.2 | Adsorbing rFVIII to microspheres

The procedure was detailed in a previous report.[26] Briefly, rFVIII (Baxter Healthcare Corporation, Glendale, CA, USA) was attached to microspheres (Polysciences, Inc, Warrington, PA, USA) following manufacturer' instructions (FVIII-m). Microspheres processed in parallel but incubated in buffer were used as control (Control-m). Bovine serum albumin (BSA) was employed to block unbound sites in both FVIII-m and Control-m. Suspensions were stored at 4°C in 1 mL of previously filtered phosphate buffered saline (PBS) pH 7.4 containing 1% BSA, 0.1% sodium azide (PBS-C) and 5% glycerol. rFVIII binding to microspheres was checked by adding biotinylated sheep IgG anti-human Factor VIII (Affinity Biologicals Inc, Ancaster, ON, Canada) followed by phycoerythrin-conjugated streptavidin (Vector, Burlingame, CA, USA). The analysis by FC was performed on FACScan cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 488 nm argon LASER and CellQuest software (Becton Dickinson, San Jose, CA, USA). We used logarithmic amplification in all parameters recorded. Microspheres were selected by gating (R1) according to size (FSC) and side (SSC) scatter profiles. A threshold of 200 was set up on SSC parameter to eliminate unwanted events. Ten thousand gated events were acquired for each determination. Mean fluorescence intensity (MFI) of FL-2 emission was recorded. MFI ratio between MFI of FVIII-m and MFI of Control-m was calculated each time after the analysis.

2.3 | Flow cytometry IgG anti-FVIII measurement

Total IgG specific to rFVIII was evaluated as we described before.[26] Plasma dilutions (1/4-1/2000) were reacted with 2.5 μL of both FVIII-m and Control-m. Captured antibodies were detected using biotinylated goat Anti-Human IgG Antibody (Vector) followed by phycoerythrin-conjugated streptavidin (Becton Dickinson). Each sample was acquired as described above. For semiquantitative results, an index was calculated multiplying the highest MFI ratio by the inverse of the corresponding plasma dilution. Values mean plus 3 standard deviations obtained with samples from 12 healthy donors, for each dilution, were considered as positive.

2.4 | IgG subclass determination of anti-FVIII antibodies by flow cytometry

We chose the dilution of the highest index for each plasma, calculated as was described above, to measure IgG subclasses. Four tubes with

TABLE 1 Groups of patients with severe hemophilia A according to the Bethesda (Bethesda Unit [BU] mL^{-1}) and flow cytometry (FC) results

Group	FVIII response	Cases (n)	BU mL^{-1}	FC positives[a]/cases	FC index range[b]
I	Without I-Ab	17	<0.6	5/17	12-255
II	Low responders	5	<0.6	1/5	
		8	0.6-1	5/8	163-1586
III	High responders	5	<0.6	5/5	4-184
		17	≤5	17/17	26-9237
		23	>5	23/23	83-79461

[a]The threshold of positivity was set at three standard deviations above the mean of FC index obtained with 12 healthy donors for each dilution.
[b]FC index range represents minimal and maximum values of the FC Indices in plasma samples of the patients belonging to each group.

2.5 μL of FVIII-m and four with Control-m were prepared. Prediluted plasma sample (47.5 μL) was added to each tube, and mixtures were incubated during 2 hour at 4°C. After washing, appropriate dilutions of biotynilated anti-human IgG1 (8c/6-39), IgG2 (HP-6014), IgG3 (HP-6050), or IgG4 (HP-6025) (Sigma-Aldrich, St. Louis, MO, USA) were added. After an incubation of 30 minute at 4°C and washing, 5 μL of a 1/100 dilution of PE-Cy5-conjugated streptavidin (Becton Dickinson) were added. Following another wash, microspheres were resuspended in FACSFlow (Becton Dickinson) and analyzed as was described above. A ratio between MFI of FVIII-m and MFI of Control-m in FL-3 was recorded for each IgG subclass. The sum of ratios was considered as 100%, and then the relative contribution of each subclass was calculated as a percentage.

2.5 | Bethesda assay

FVIII inhibitors were measured using the Nijmegen modification of the Bethesda method.[2] All inhibitor titer of more than 0.6 BU mL^{-1} was considered positive.

2.6 | Statistical analysis

Statistical tests were performed in PRISM 4.0 (GraphPad Software, San Diego, CA, USA). For comparisons between groups, the Mann-Whitney nonparametric test was applied. The coefficient of correlation to Spearman was calculated for correlation analysis of IgG anti-FVIII subclass percentages and inhibitor levels. Statistical significance was indicated when $P<0.05$.

3 | RESULTS

3.1 | Relationship between FC and Bethesda results

We investigated a total of 75 plasmas from patients with SHA categorized as was detailed in Materials and Methods. Samples were evaluated by Bethesda assay and FC (total anti-FVIII IgG). Table 1 reports the results. Unexpectedly five of 17 patients (29.4%) belonging to Group I, without history of I-Ab, were positive by FC with low index values. One of five LR patients without current I-Ab had a positive FC result. On the other hand, we only detected antibodies by FC in five of eight LR patients with very low functional titers (0.6-1 BU mL^{-1}) by Bethesda. All HR patients (45) were positive by FC assay including five patients without I-Ab at the time of the blood extraction.

3.2 | Analysis of subclasses of anti-FVIII antibodies by FC in patients with SHA

We measured IgG subclasses in each plasma with positive anti-FVIII total IgG result by FC, following the procedure detailed in Materials and Methods. There is not a suitable standard to get absolute values of each anti-FVIII IgG isotype, due to the different nature of the antibodies (affinity and specificity) in each patient. So, we have designed this assay to find out the relative contribution of the different anti-FVIII IgG subclasses in the response. Figure 1 shows one example. In this case, IgG4 was the prevalent subclass (90.8%) followed by IgG2 (6.31%), IgG3 (1.6%), and IgG1 (1.3%).

The relative contribution of each IgG subclass in the plasma from the 56 SHA patients with detected IgG anti-FVIII by FC is shown in Figure 2. Our results confirm previous reports which showing that all IgG subclasses were involved. IgG4 percentages were statistically significantly higher respect to the other isotypes only in the Group III but with different significance depending on the level of I-Ab: $P=0.02$ in patients with inhibitors between 0.6 and 1 BU mL^{-1} and $P=0.0001$ with high titer of I-Ab (5-8200 BU mL^{-1}). IgG2 was also elevated in this group of patients respect to IgG1 and IgG3 ($P=0.008$). IgG1 and IgG3 were less frequently detected in these cohorts. Correlation analysis comparing Bethesda results with the percentage of each IgG subclass shows a significative result for IgG4 ($r=0.63$, $P=0.001$) and IgG2 ($r=0.5$, $P=0.01$) in patients belonging to Group III with high levels of I-Ab (5-8200 BU mL^{-1}).

We had the chance to test two patients in different moments of the anti-FVIII immune response (Figure 3). Figure 3A shows one of them with undetectable I-Ab by Bethesda but positive by FC (index: 255) at the first time, but 4 years later, when I-Ab were detected (430 BU mL^{-1}) and the FC index increased to 4142, the profile of anti-FVIII IgG subclasses changed becoming IgG4 prevalent (23.26% to 88.32%). Figure 3B shows the second patient who was positive by both methods at first, 1 year later increased the level of antibodies and the IgG4 contribution changed from 26.17% to 68.21%.

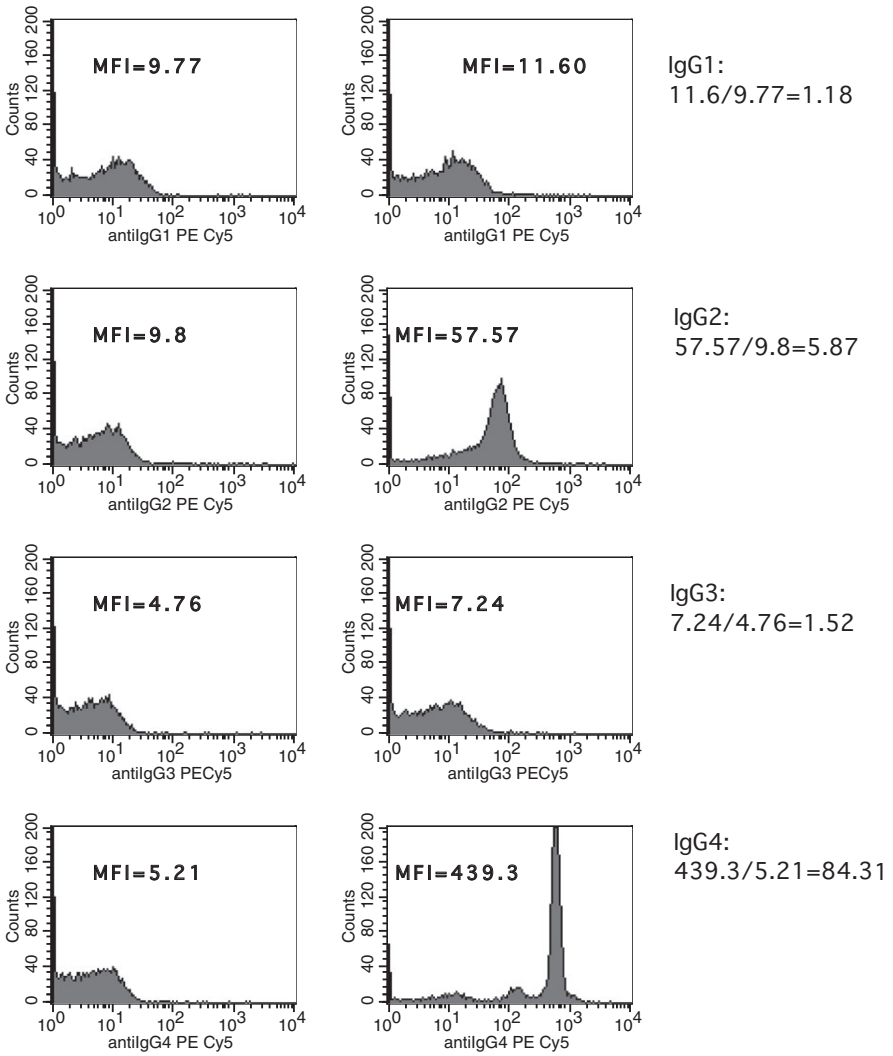

FIGURE 1 Flow cytometry (FC) analysis of the contribution of each IgG subclass in the anti-FVIII response. A sample with a FC index of 3250 and 151 Bethesda Unit (BU) mL^{-1} is shown. Plasma was diluted according to the level of total IgG (determined previously) and incubated with FVIII-m or Control-m for 2 h at 4°C. Bound antibodies were revealed using biotynilated anti-IgG1, IgG2, IgG3, and IgG4 followed by PECy5-streptavidin. Each mean fluorescence intensity (MFI) FVIII-m/Control-m ratio was recorded. The sum of ratios was considered as 100%, and then the relative contribution of each subclass was calculated as a percentage

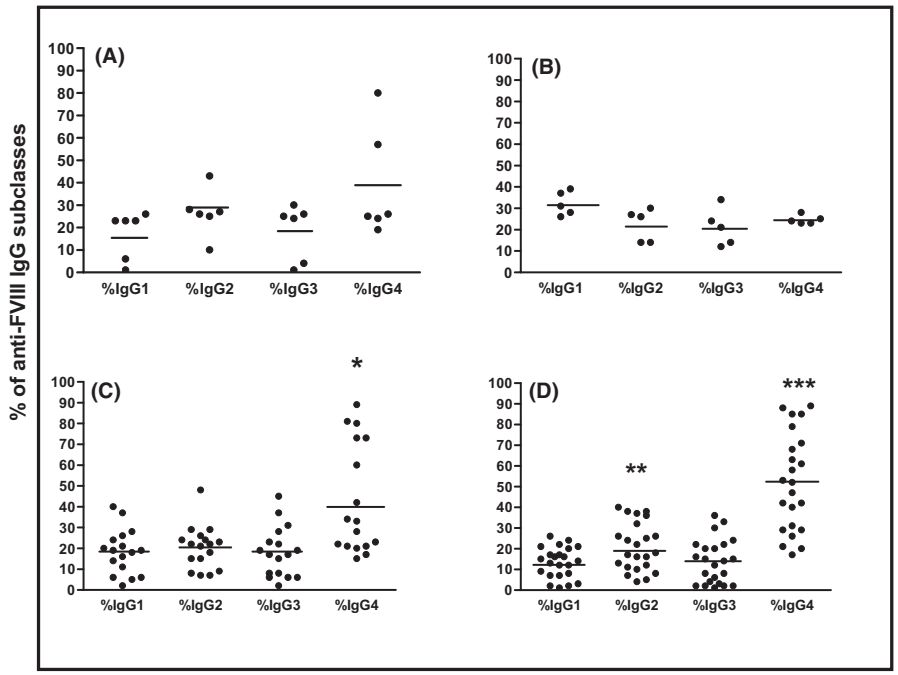

FIGURE 2 Distribution of IgG subclasses of anti-FVIII antibody in plasma from severe hemophilia A patients with inhibitors. (A) Group II: Low responder patients (n=6). (B–D) Group III: High responder patients divided as: B, without inhibitors at the moment of the assay but positive by flow cytometry (n=5); C, with 0.6-5 Bethesda Unit (BU) mL^{-1} (n=17); D, with 5-8200 BU mL^{-1} (n=23). The asterisks indicate the level of significance between values obtained for the IgG4 compared with IgG1, IgG2, and IgG3 (*P=0.02 and ***P=0.0001) and IgG2 compared with IgG1 and IgG3 (**P=0.008)

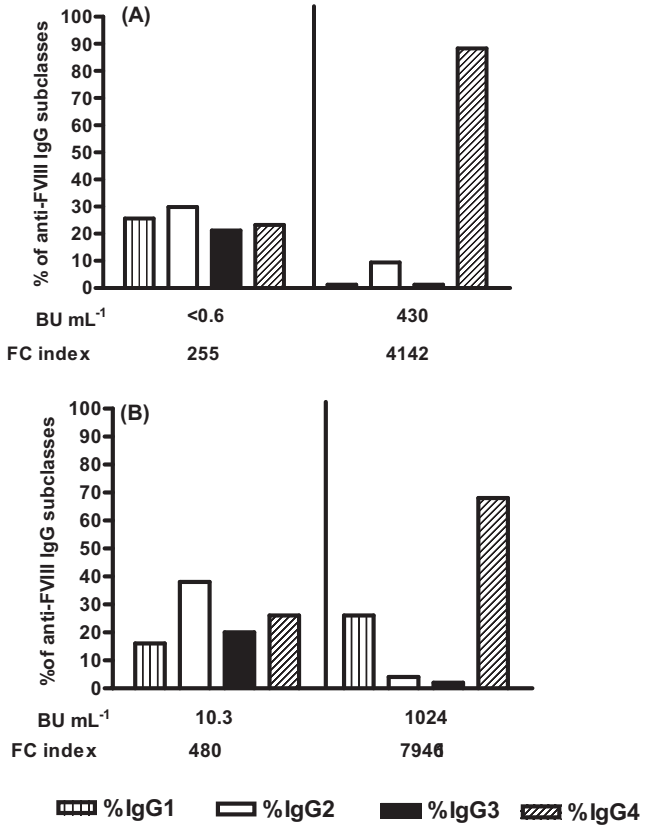

FIGURE 3 Comparison of anti-FVIII antibodies tested by the functional Bethesda and flow cytometry assays in two patients. Two patients with severe hemophilia A (A and B) were studied at different times of disease evolution. Bars indicate the percentage of contribution of each IgG subtype

3.3 | Longitudinal analysis of anti-FVIII response in patients undergoing immune tolerance induction

Eight patients were evaluated before and during ITI. Figure 4 reports inhibitor titers (BU mL^{-1}), FC index and percentages of IgG subclasses. In all patients, the four subclasses coexisted during ITI.

Inhibitors were eradicated (successful ITI) in three patients (SD, KV, and BS) but only in one of them (SD), FC index was became negative at the end of ITI (Figure 4A). In these three patients, the level of I-Ab was never high during the treatment. Five of the six patients with successful and partial response (Figure 4A,B) decreased the IgG4 contribution. It was noticeable that AA showed undetectable I-Ab between the 22nd and 29th months after starting ITI but still had detectable antibodies by FC. After that, Bethesda was positive again in month 37. The proportion of anti-FVIII antibodies of IgG4 subclass is increased in plasma of patients who failed ITI (MZ and CA) (Figure 4C).

4 | DISCUSSION

Development of anti-FVIII antibodies occurs in 20%-30% of patients with SHA after repeated administration of the factor. Hemophilic patients can develop I-Ab and NI-Ab anti-factor VIII antibodies including antiphospholipid-protein antibodies, such as LA.

The standard treatment for eradication of the inhibitors is the immune tolerance induction (ITI) therapy that is based on long-term daily injections of high concentrations of FVIII protein, with the aim of obtaining immune tolerance. Measurement of inhibitors is routinely performed by the functional Bethesda assay or its modifications. It is recognized that these assays might not detect weak but clinically significant inhibitors. There is a lot of clinically relevant information, about certain aspects of the immune response of the patients that cannot be assessed using exclusively functional assays. In an attempt to improve the evaluation of each patient, several techniques were developed.[26-32]

As it has been reported[29,31] that the fluorescence based immunoassay is 10^3 times more sensitive than the Bethesda assay, we have designed an immunomethod combining microspheres with FC to detect anti-FVIII antibodies in plasma from SHA patients, suggesting that it may be a useful alternative to complement classic functional assays. Moreover, the use of rFVIII as antigen avoids the possible interference of other antibodies in the reaction. Both I-Ab and NI-Ab can be detected by this technique. Discrepancies between Bethesda assay and FC were found especially in patients with weak level of inhibitors. In our patients, 11 of 27 (40.7%) with negative Bethesda results at the moment of the study were positive by FC, five without a history of inhibitors, and six with historically positive inhibitor titers measured by Bethesda assay. Previous studies, employing ELISA as immunomethod, have reported percentages of positivity ranging between 12% and 39% in samples from patients without inhibitors.[9,31-34] This result indicates either that the antibodies were true inhibitors undetected by the functional method, or that they were non-neutralizing antibodies. Dazzi et al.[8] reported a high incidence of anti-FVIII antibodies against noncoagulant epitopes in patients with hemophilia A. However, Ling et al.[35] have detected antibodies by ELISA in only four of 26 patients with Bethesda negative results. Technical differences (ie, FVIII purity used as antigen in ELISA plates) or diverse characteristics of hemophiliac patient cohorts may be the causes of the discordances.

We were able to characterize the IgG subclasses that participate in the response by FC using appropriate monoclonal antibodies. The limitation of the assay is the absence of a suitable gold standard calibrator to be used, so we designed a way of analysis aimed to determine the relative prevalence of each IgG subclass in the response (Materials and Methods). Studies in different groups of patients have revealed that IgG1 and IgG4 were prevalent in the response[9,22,25] but other authors have reported different distribution of IgG subclasses.[19,21,24] However, all the studies have demonstrated that IgG4 was predominant in patients with high levels of anti-FVIII antibodies. In our cohort IgG4 and IgG2, in second place, correlated with the Bethesda assay. Instead, IgG1 was found more frequently in samples with low Bethesda (0.6-1 BU mL^{-1}). It is known that the four IgG subclasses exhibit different functional activities in terms of triggering FcγR-expressing cells and activating complement.[17,18] This fact determines the quality of the ensuing immune response. Because of its characteristics, IgG4

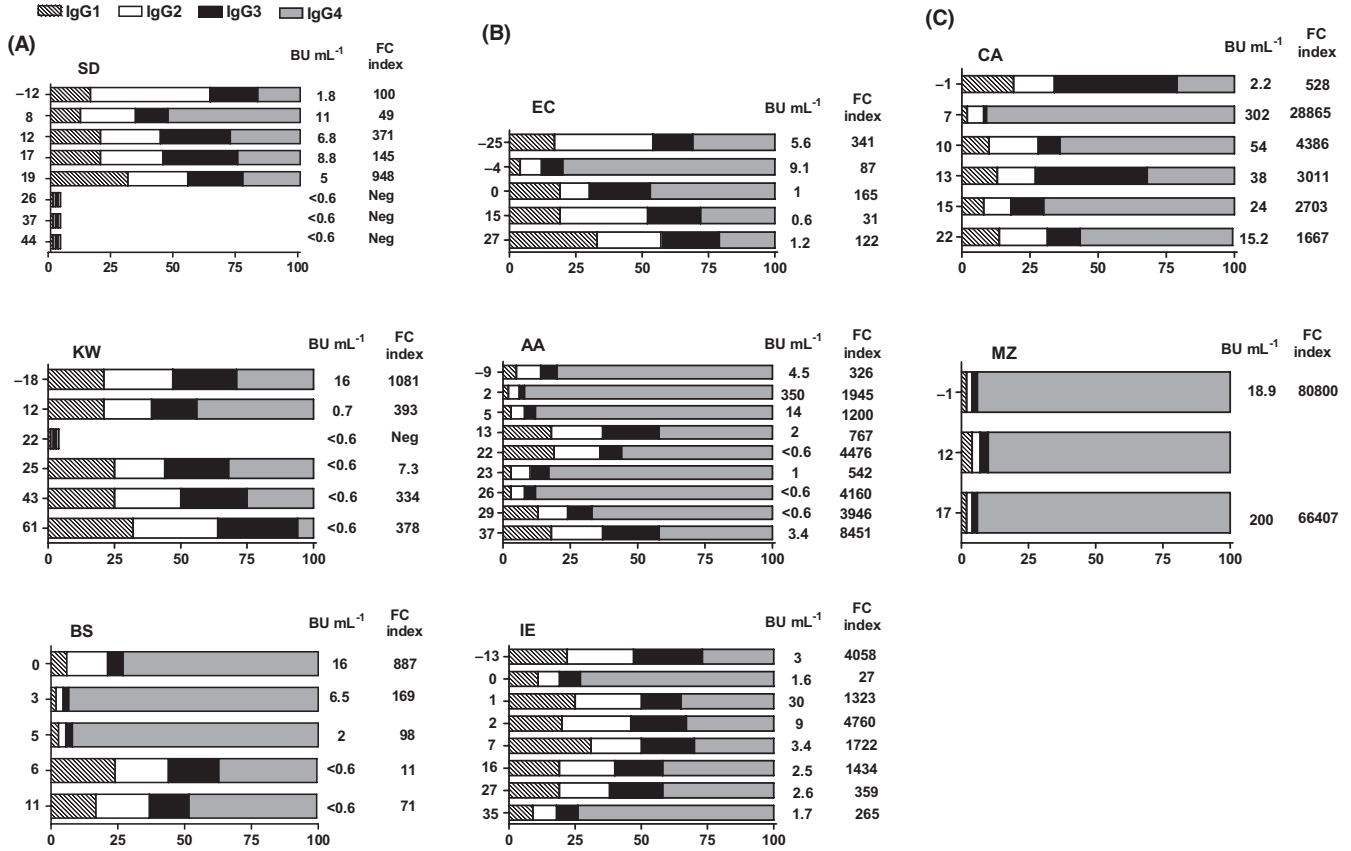

FIGURE 4 Contribution of IgG1, IgG2, IgG3, and IgG4 to the total level of anti-FVIII antibodies during immune tolerance induction (ITI). Time after the onset of the treatment is indicated on the left of each panel. The corresponding Bethesda titer and Flow Cytometry (FC) index are shown at the right side. Each graph represents values obtained from a single patient as indicated at the top left side in each graph. Patients were grouped according to their ITI outcome (A: Successful result, B: Partial response and C: Failed ITI)

is considered the more anti-inflammatory of the all subclasses and is often generated following repeated or long-term exposure to antigen in noninfectious settings.[17] Hemophilic patients receive FVIII as treatment during their whole life span. We think that anti-FVIII IgG4 is not per se a dangerous antibody. Presumably, it might have been developed by the immune system as a protective response against inflammatory damage resulting from the functional effects of the other subclasses. In this regard the results shown in Figure 3, demonstrate that in two patients the immunoglobulin anti-FVIII subclass profile changed during replacement treatment. One of them started with <0.6 BU mL^{-1}, but detectable specific antibodies by FC in the initial antibody screening, and developed a high titer of inhibitor thereafter. Recently, Hofbauer and col[36] have published a study carried out in patients without inhibitors, and they found 35.7% of the patients with specific-anti-FVIII IgG measured by ELISA. One of them, having IgG4 of high affinity, developed later FVIII inhibitors, reinforcing the usefulness of the immune assay to alert the starting of a clinically important anti-FVIII response.

We included eight patients in ITI in who measured I-Ab by Bethesda, total anti-FVIII IgG and its subclasses by FC during the treatment. In only one of them (SD), we found concordance between Bethesda and FC with negative antibodies at the end of the treatment and successful ITI. In the remaining seven patients, the FC index never became negative, despite the fact that KW and BS ended the treatment with Bethesda values <0.6 BU mL^{-1}. Importantly, the level of FC index reached during the treatment appears to be associated with the ITI outcome. Recurrent inhibitors were found in AA after 37 months of starting ITI, having a period of 11 months of Bethesda negative results, but antibodies were always detected by FC in this patient. The recurrence of inhibitors after ITI has been reported previously.[37] The question is as follows: had the inhibitors been really eradicated?

The presence of antibodies undetected by the functional method might explain the unexpected low FVIII recovery reported by others.[8,10,36] A high contribution of IgG4 during ITI appears to be associated with a difficulty to eradicate the inhibitor.[24] We found four of five patients with poor response or failure of ITI, in whom IgG4 was the predominant subclass at the end of the treatment.

According to these results and those of others, we suggest the convenience of evaluating the complete immune response in hemophilia patients in order to improve the management of the disease, especially during replacement therapy and in the process of monitoring the response of patients in ITI.

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CONFLICT OF INTEREST

None of the authors has any potential financial conflicts of interest.

AUTHOR CONTRIBUTION

MI, LP, NG, and MF performed laboratory analyzes, acquisition, and interpreted data. MC and LP provided the patients samples. MC was in charge of diagnosis and assistance of the patients. MME de B and NG designed and directed the study and wrote the manuscript.

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