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BRIEF REPORT

High levels of anti–factor VIII immunoglobulin G4 and immunoglobulin G total are associated with immune tolerance induction failure in people with congenital hemophilia A and high-responding inhibitors

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Abstract

Background: Immune tolerance induction (ITI) is the treatment of choice to eradicate neutralizing anti–factor (F)VIII alloantibodies (inhibitors) in people with inherited hemophilia A. However, it is not successful in 10% to 40% of the cases. The biological mechanisms and biomarkers associated with ITI outcome are largely unknown.

Objectives: The aim of this study was to investigate the association of plasma cytokines (interferon-γ, tumor necrosis factor, interleukin [IL]-2, IL-4, IL-5, IL-6, IL-10, and IL-

17A), chemokines (IL-8/CXCL8, RANTES/CCL5, MIG/CXCL9, MCP-1/CCL2, and IP-10/CXCL10), and anti-FVIII immunoglobulin (Ig) G total, IgG1, and IgG4 with ITI outcome.

Methods: In this cross-sectional analysis of the Brazilian Immune Tolerance Study, we assessed plasma levels of anti-FVIII IgGs using an enzyme-linked immunosorbent assay with plasma-derived FVIII and recombinant FVIII as target antigens, immobilized in microplates.

Results: We assayed 98 plasma samples of moderately severe and severe (FVIII activity, <2%) people with hemophilia A after completion of a first ITI course. Levels of anti–recombinant FVIII IgG total and IgG4 were higher in people with hemophilia A who failed ITI (IgG total optical density [OD], 0.37; IQR, 0.15-0.73; IgG4 OD, 2.19; IQR, 0.80-2.52) than in those who had partial (IgG total OD, 0.03; IQR, 0.00-0.14; IgG4 OD, 0.39; IQR, 0.09-1.11; $P < .0001$ for both) or complete success (IgG total OD, 0.04; IQR, 0.00-0.07; IgG4 OD, 0.07; IQR, 0.06-0.40; $P < .0001$ for both). Plasma cytokines, chemokines, and anti-FVIII IgG1 were not associated with ITI outcome.

Conclusion: Our results show that high levels of plasma anti-FVIII IgG4 and IgG total are associated with ITI failure.

KEYWORDS
alloantibodies, chemokine, cytokine, factor VIII, hemophilia A, immune tolerance

Essentials

- Immune tolerance induction (ITI) eradicates factor VIII inhibitors in most people with hemophilia A.
- Biological mechanisms and biomarkers associated with ITI outcome are largely unknown.
- We enrolled 98 people with severe and moderately severe hemophilia A who completed a first course of ITI.
- High plasma levels of anti–factor VIII immunoglobulin G4 and immunoglobulin G total were associated with ITI failure.

1 | INTRODUCTION

Hemophilia A is an X-linked bleeding disorder caused by mutations in the factor (F)VIII gene (*F8*). The development of neutralizing alloantibodies (inhibitors) against FVIII is the most relevant complication of FVIII replacement in people with hemophilia A. It affects about 30% of patients with severe hemophilia, leading to inefficient FVIII replacement [1]. Immune tolerance induction (ITI) is the treatment of choice to eradicate inhibitors, reaching 60% to 90% success rate among people with hemophilia A and inhibitors [2–5]. The biological bases related to ITI response are not completely elucidated. Therefore, investigation of biomarkers associated with ITI outcome is important [5].

Previous studies have shown that several cytokines, chemokines, and anti-FVIII immunoglobulins (Ig) are associated with the presence of inhibitors [6–9]. However, most of these studies were case series, had retrospective design, and assessed biomarkers in people with hemophilia A with long-standing inhibitors or in samples collected without a predefined time point related to inhibitor development or ITI [7,10–12]. The study by van Helden et al. [13] indicated that the proportion of anti-FVIII IgG4 was increased in the plasma of patients who failed ITI. However, their study included only 20 patients and was not designed to address biomarkers associated with ITI outcome. Therefore, we hypothesized that biomarkers (mainly anti-FVIII IgG) could be associated with ITI response. To test this, we assessed plasma levels of cytokines (interferon-γ, tumor necrosis factor, interleukin [IL]-2, IL-4, IL-5, IL-6, IL-10, and IL-17A), chemokines (IL-8/CXCL8, RANTES/CCL5, MIG/CXCL9, MCP-1/CCL2, and IP-10/CXCL10), and anti-FVIII IgG total, IgG1, and IgG4 in a cohort of 98 people with hemophilia A and high-responding inhibitors after completion of ITI, participants of the Brazilian Immune Tolerance (BrazIT) Study [14].

2 | METHODS

This cross-sectional analysis is a subset of the BrazIT Study, which is a cohort of people with hemophilia A with high-responding inhibitors who completed a first course of ITI [14]. For this analysis, we included people with hemophilia A who were enrolled in 10 Brazilian hemophilia treatment centers. We included people with severe (residual FVIII activity, <1.0%) and moderately severe (residual FVIII activity between 1.0% and 1.9%) hemophilia A with high-responding inhibitors

who completed a first course of ITI between February 2016 and July 2020. High-responding inhibitors were defined according to international definition [15], ie, when people with hemophilia A presented at least 1 inhibitor titer above 5 Bethesda units lifelong. Patients were treated with ITI regimen either as low- (50 international units [IU]/kg 3 times weekly) or high-dose FVIII (100 IU/kg every day), according to the Brazilian Protocol of Immune Tolerance [15]. According to this protocol, ITI was performed with the same type of FVIII concentrate used while people with hemophilia A developed an inhibitor. ITI outcome was defined following international recommendation, as complete or partial successes and failure, based on responsiveness to infused FVIII, inhibitor levels, and FVIII pharmacokinetics [2]. For a complete and detailed description of the BrazIT Study methodology, please refer to the study by Camelo et al. [14].

Cytokines interferon-γ, tumor necrosis factor, IL-2, IL-4, IL-5, IL-6, IL-10, and IL-17A and the chemokines IL-8/CXCL8, RANTES/CCL5, MIG/CXCL9, MCP-1/CCL2, and IP-10/CXCL10 were assessed using the commercial Cytometric Bead Array Kit (BD Pharmingen). Enzyme-linked immunosorbent assay (ELISA) with plasma-derived FVIII (pdFVIII; Octanate, Octapharma) and recombinant FVIII (rFVIII; Advate rurioctocog alfa, Takeda) as target antigens was used to detect specific anti-FVIII IgG total, IgG1, and IgG4. The specific activity of Octanate is ≥100 IU/mg protein; it contains von Willebrand factor (von Willebrand factor ristocetin cofactor, ≤60 IU/mL). We used these 2 brands of target FVIII because these were the types of FVIII concentrates that people with hemophilia A used for the course of ITI. Tests were performed in duplicates, and results were expressed as mean [16].

Briefly, 96-well plates were coated with 0.1 IU/well of pdFVIII or rFVIII. Plasma samples diluted 1:20 were incubated in plates with mouse monoclonal anti-human IgG-Biotin (A18821; Thermo Fisher Scientific), IgG1-Biotin (MH1515; Thermo Fisher Scientific), and IgG4-Biotin (B3648; Sigma-Aldrich). The assay was revealed using peroxidase-labeled streptavidin (Sigma-Aldrich) and *o*-phenylenediamine (Sigma-Aldrich). Optical density (OD) was measured at 492 nm in an ELISA reader. To evaluate inter- and intra-assay coefficients of variation (CVs), pools of plasma from 20 healthy individuals and 20 people with hemophilia A were titrated, and each dilution was replicated 10 times in the same assay. The interassay CV was calculated based on the results of 6 different measurements of a patient positive control sample titrated from 1:10 to 1:640 in separate assays. The inter- and intra-assay CVs for the dilution used in this work were 12% and 20%, respectively. Inversions of introns 1 and 22 were detected by using a polymerase chain reaction–based method [17,18]. Exons and intron-exons boundaries were sequenced by a whole exome sequencing approach using xGen Exome Research Kits Panel v2 and xGen CNV Backbone Panel (IDT). Sequencing was performed on the Illumina NovaSeq 6000 platform (Illumina) for those people with hemophilia A without intron 1 and 22 inversions. Inversions of introns 1 and 22, large deletions, nonsense mutations, and frameshift mutations were considered high-risk mutations, while missense and splice mutations were considered low-risk mutations for inhibitor development [19].

TABLE 1 Characteristics of the people with inherited hemophilia A included in the study according to immune tolerance induction outcome.

Characteristic	All patients	ITI outcome		
		Complete success	Partial success	Failure
No. of patients (%)	98 (100.0)	21 (21.4)	37 (37.8)	40 (40.8)
Age at the time of sample collection (y), median (IQR)	11 (7-23)	9 (7-22)	12 (7-24)	11 (9-23)
Severity, *n* (%)				
Moderately severe (FVIII activity, 1%-2%)	4 (4.1)	2 (9.5)	1 (2.7)	1 (2.5)
Severe (FVIII activity, <1%)	94 (95.9)	19 (90.5)	36 (97.3)	39 (97.5)
Type of FVIII for ITI, *n* (%)				
pdFVIII	55 (56.1)	14 (66.7)	22 (59.5)	19 (47.5)
rFVIII	35 (35.7)	7 (33.3)	15 (40.5)	13 (32.5)
pdFVIII and rFVIII	8 (8.2)	0 (0.0)	0 (0.0)	8 (20.0)
ITI regimen, *n* (%)				
Low dose	56 (57.1)	18 (85.7)	27 (73.0)	11 (27.5)
High dose	42 (42.9)	3 (14.3)	10 (27.0)	29 (72.5)
F8 mutation,[a] *n* (%)				
High risk	69 (70.4)	16 (76.2)	28 (75.7)	25 (62.5)
Low risk	17 (17.3)	2 (9.5)	5 (13.5)	10 (25.0)
Not detected/unknown	12 (12.3)	3 (14.3)	4 (10.8)	5 (12.5)

F8, factor VIII gene; FVIII, factor VIII; ITI, immune tolerance induction; pdFVIII, plasma-derived factor VIII; rFVIII, recombinant factor VIII.
[a]Nonsense, inversion, and frameshift mutations were considered high-risk mutations for inhibitor development according to Rosendaal et al. [19].

For the categorical variables, we calculated the number of events and their respective percentages. For continuous numerical variables, the median and IQR were calculated. Comparison of biomarker median levels was performed by using Mann–Whitney U-test and Wilcoxon test for unpaired and paired samples, respectively. Comparison of frequencies was analyzed by using chi-squared test. The study was approved by the Ethical Committees of each center, and all participants/guardians signed a consent form.

3 | RESULTS AND DISCUSSION

We included 98 people with hemophilia A who completed ITI, with a median age at enrollment of 11 years (IQR, 7-23 years). Most people with hemophilia A (57.1%; *n* = 56) were treated with a low-dose ITI regimen (ie, 50 IU/kg, 3 times/wk), and 56.1% (*n* = 55) were exclusively treated with pdFVIII during ITI. Complete and partial successes were achieved by 21 (21.4%) and 37 (37.8%) people with hemophilia A, respectively, and 40 (40.8%) people with hemophilia A failed ITI. The characteristics of people with hemophilia A are detailed in Table 1.

Median plasma levels of anti-FVIII IgG4 in ELISA using rFVIII and pdFVIII were significantly higher in people with hemophilia A who failed ITI (OD, 2.19; IQR, 0.80-2.52; and OD, 1.33; IQR, 0.51-2.33, respectively) than in those who had partial (OD, 0.39; IQR, 0.09-1.11; and OD, 0.15; IQR, 0.04-0.67, respectively) and complete successes (OD, 0.07; IQR, 0.06-0.40; and OD, 0.09; IQR, 0.02-0.17, respectively; $P < .0001$ for all comparisons; Figure 1B, E). There was a dose-response, with anti-FVIII IgG4 reaching the highest levels upon ITI failure > partial success > complete success (Figure 1B, E). Median levels of anti-FVIII IgG total were significantly higher in people with hemophilia A who failed ITI (OD, 0.37; IQR, 0.15-0.73) than in those who had partial (OD, 0.03; IQR, 0.00-0.14) and complete success (OD, 0.04; IQR, 0.00-0.07; $P < .0001$ for both) only when ELISA was

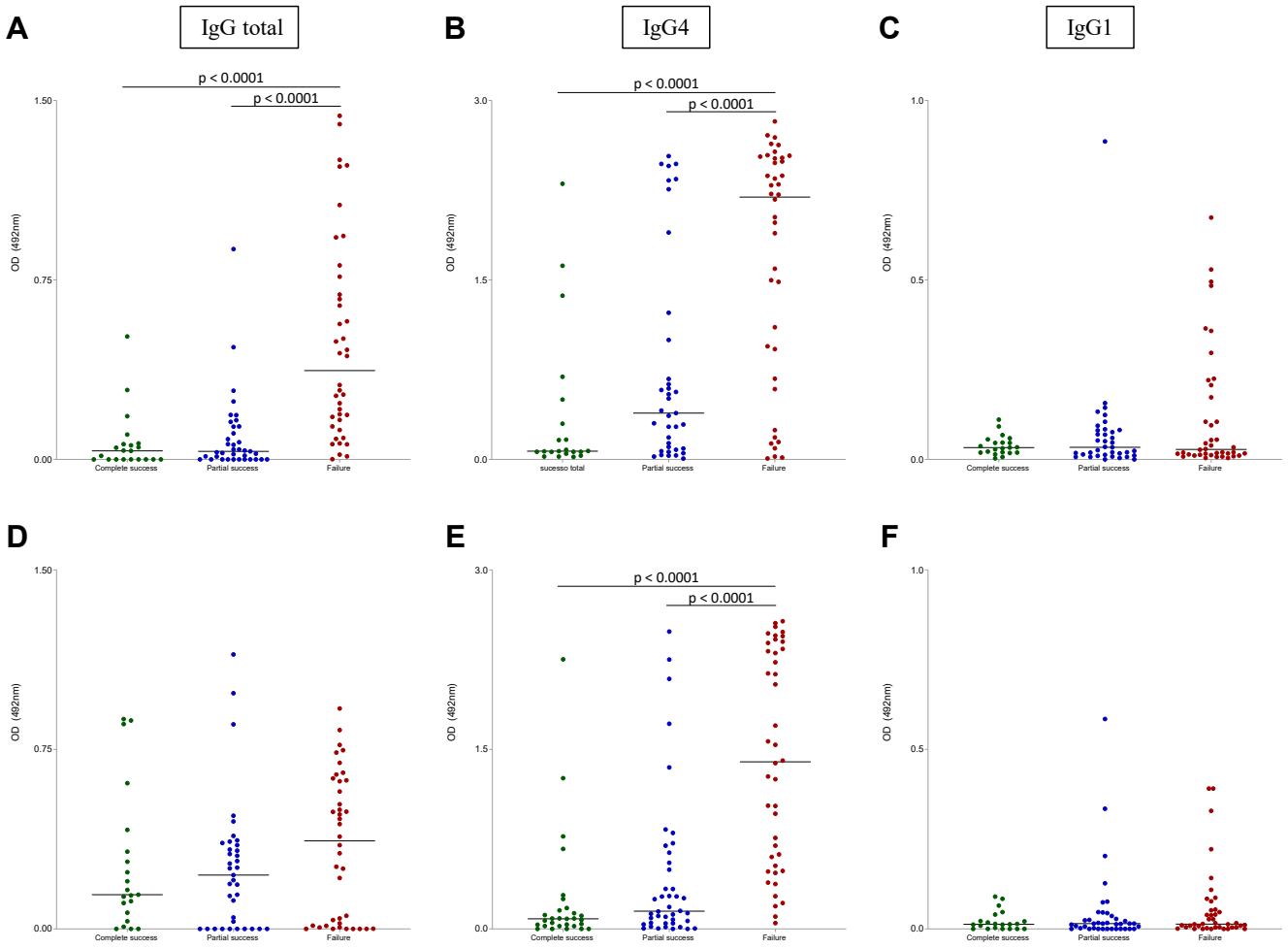

FIGURE 1 Levels of anti–factor (F)VIII immunoglobulin (Ig) G total, IgG1, and IgG4 assessed in plasma samples of people with hemophilia A and high-responding inhibitors who completed immune tolerance induction according to outcome. A, B, and C: Anti-FVIII IgG total, IgG4, and IgG1, respectively, using recombinant FVIII as target antigen. D, E, and F: Anti-FVIII IgG total, IgG4, and IgG1, respectively, using plasma-derived FVIII as target. Horizontal lines within the dots represent the median optical density (OD) of each group. Tests were performed in duplicates, and each point represents the mean of 2 measurements.

performed using rFVIII as target antigen (Figure 1A). Median levels of anti-FVIII IgG total using pdFVIII as target antigen were not significantly different in people with hemophilia A who failed ITI (OD, 0.37; IQR, 0.01-0.62) compared with those who had partial (OD, 0.23; IQR, 0.00-0.35; P = .16) and complete success (OD, 0.14; IQR, 0.05-0.37; P = .33; Figure 1D). Levels of IgG1 were not significantly different in people with hemophilia A who failed or had successful (complete or partial) ITI, either by using pdFVIII or rFVIII as the target antigen (Figure 1C, F), demonstrating that IgG1 is not a useful biomarker of ITI outcome. Plasma levels of cytokines and chemokines were also not significantly different when comparing the 3 ITI outcomes (Table 2).

Anti-FVIII antibodies consist of a polyclonal population of antibodies mainly targeting antigenic sites within A2, A3, and C2 domains of FVIII [20]. In people with hemophilia A, they comprise both neutralizing (inhibitors) and nonneutralizing antibodies [21], mainly represented by IgG4 and IgG1, respectively [16,22]. Our results demonstrated that high levels of both anti-FVIII IgG total and IgG4 are hallmarks of ITI failure. The biological explanation for this finding remains to be elucidated. However, it is likely that the presence of high levels of anti-FVIII IgG4 in people with hemophilia A who failed ITI could be due to the persistence of memory $CD4^+$ T and B lymphocytes or an imbalance between regulatory and memory cells capable of inducing a humoral response against FVIII [23–26]. Although cytokine levels did not differ between the 3 ITI outcome groups, we hypothesize that the memory response could be sustained by a microenvironment of anti-inflammatory/regulatory immune profile induced by cytokines IL-4 and IL-10 [8,9,27] that down-regulates FVIII-specific regulatory T cells, which could reduce the secretion of inhibitors by plasma cells [28,29]. Assessment of anti-FVIII IgG4 levels before starting and during ITI should be addressed in future studies to evaluate the usefulness of this biomarker as a predictor of ITI outcome.

Few studies have attempted to investigate the contribution of different IgG classes during ITI [7,10–12]. However, those studies comprised small populations of people with hemophilia A (maximum 20); most were case series, had retrospective design, and/or were not aimed at investigating the contribution of anti-FVIII Ig to ITI outcome. van Helden et al. [13] suggested that the contribution of anti-FVIII IgG4 to the total level of anti-FVIII antibodies was relatively high in people with hemophilia A who failed ITI. However, this

TABLE 2 Plasma concentration of biomarkers in people with inherited hemophilia A according to immune tolerance induction outcome.

Biomarker	Immune tolerance induction outcome			*P* value
Cytokines (ng/mL), median (IQR)	Complete success (n = 21)	Partial success (n = 37)	Failure (n = 40)	
IL-2	1.6 (0.7-2.4)	1.2 (0.1-2.2)	1.6 (0.0-2.8)	.48
IL-4	4.4 (1.1-6.9)	3.7 (1.0-6.9)	3.9 (1.3-7.1)	.96
IL-6	1.0 (0.0-2.8)	1.7 (0.2-2.9)	1.7 (0.8-3.4)	.15
IL-10	0.9 (0.0-2.2)	1.1 (0.3-2.4)	1.6 (0.0-2.7)	.85
TNF	3.9 (0.1-7.4)	1.9 (0.0-5.9)	4.3 (1.3-7.5)	.40
IFN-γ	23.8 (0.0-95.7)	23.8 (0.0-109.7)	52.9 (0.0-101.9)	.39
IL-17A	23.7 (0.0-40.7)	14.6 (3.6-32.8)	26.6 (5.1-35.1)	.45
Chemokines (ng/mL), median (IQR)				
IL-8/CXCL8	4.4 (1.9-7.3)	5.3 (0.0-6.7)	2.2 (0.0-7.0)	.39
RANTES/CCL5	2404 (1204.0-5933.6)	3833.5 (2265.7-6891.7)	2673.8 (1633.1-4311.7)	.17
MIG/CXCL9	87.4 (55.1-157.8)	99.4 (79.5-168.5)	88.8 (67.0-139.8)	.65
MCP-1/CCL2	10.5 (6.7-13.0)	8.3 (3.7-13.4)	10.7 (7.0-18.7)	.45
IP-10/CXCL10	830.9 (244.7-1089.9)	963.8 (557.1-2127.1)	670.1 (451.5-1051.2)	.09
Anti-FVIII antibodies (OD at 492 nm), median (IQR)				
IgG total (rFVIII)	0.04 (0.00-0.07)	0.03 (0.00-0.14)	0.37 (0.15-0.73)[a]	<.01
IgG total (pdFVIII)	0.14 (0.05-0.37)	0.23 (0.00-0.35)	0.37 (0.01-0.62)	.24
IgG4 (rFVIII)	0.07 (0.06-0.40)	0.39 (0.09-1.11)[b]	2.19 (0.80-2.52)[a]	<.01
IgG4 (pdFVIII)	0.09 (0.02-0.17)	0.15 (0.04-0.67)[b]	1.33 (0.51-2.33)[a]	<.01
IgG1 (rFVIII)	0.03 (0.00-0.07)	0.03 (0.01-0.08)	0.03 (0.01-0.19)	.10
IgG1 (pdFVIII)	0.01 (0.00-0.03)	0.01 (0.00-0.03)	0.01 (0.00-0.05)	.24

FVIII, factor VIII; IFN-γ, interferon-γ, Ig, immunoglobulin; IL, interleukin; OD, optical density; pdFVIII, plasma-derived factor VIII; rFVIII, recombinant factor VIII; TNF, tumor necrosis factor.

[a]Significantly different when compared with complete and partial success groups.

[b]Significantly different when compared with the complete success group.

study included a small and heterogeneous population of people with hemophilia A (n = 20), of whom only 5 underwent ITI and failed. Furthermore, assayed samples were collected over more than 20 years.

In our study, we assessed anti-FVIII IgG by using rFVIII and pdFVIII in the ELISA. We identified that rFVIII was a better target to detect anti-FVIII antibodies and discriminate their levels according to ITI outcomes than pdFVIII. We hypothesize that the protein content of pdFVIII could prevent allosteric anti-FVIII antibodies from recognizing the antigen properly.

Our study has several strengths. Firstly, it comprised a well-characterized, large population of people with severe/moderately severe hemophilia A and high-responding inhibitors who completed ITI using the same protocol. Secondly, biomarkers were assessed centrally. Thirdly, anti-FVIII IgG was tested using 2 different FVIII target antigens, which provided similar results. However, there are limitations worth mentioning. Firstly, due to insufficient volume of samples, we did not assess inhibitor titer in the same samples we assessed ELISA. Therefore, we could not correlate inhibitor levels with anti-FVIII IgG antibodies. However, ITI outcome of all people with hemophilia A were determined according to responsiveness to FVIII replacement, inhibitor levels, and FVIII pharmacokinetics. Secondly, we did not assess levels of anti-FVIII IgG2 and IgG3. However, a previous study did not find a significant correlation between anti-FVIII IgG3 and FVIII inhibitors [10]. Thirdly, we did not collect samples at different time points during ITI due to the complexity involving the collection and shipment of frozen samples from 15 hemophilia treatment centers.

We showed that high plasma levels of anti-FVIII IgG4 and IgG total are associated with ITI failure. Future studies should address levels of IgG4 and IgG total before starting and during ITI course as an attempt to determine its predictive role on ITI outcome.

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ETHICS STATEMENT

The study was approved by the Ethical Committees of each center, and all participants/guardians signed a consent form.

AUTHOR CONTRIBUTIONS

D.G.C., R.M.C., B.A.d.S.S., and M.M.D. performed the research and analyzed the data; C.S.L., A.G.d.O., M.H.C., R.d.A.R., L.Y.E., V.K.B.F., M.d.R.F.R., F.M.R.d.A.C., M.A.F.d.C., and I.P. included the patients and collected clinical data; L.W.Z. performed the molecular tests; D.G.C. and S.M.R. designed the research, contributed to data analysis, and wrote the paper. All authors revised and approved the final version of the manuscript.

RELATIONSHIP DISCLOSURE

R.M.C. received speaker fees from Bayer, Novo Nordisk, Hoffman-La Roche, and Takeda; consultancy fees from Hoffman-La Roche and Takeda; and scientific event grants from Bayer, Novo Nordisk, Hoffman-La Roche, and Takeda. A.G.d.O. received speaker fees from Novo Nordisk, Takeda, and Roche and scientific event grants from Novo Nordisk. L.Y.E. received scientific event grants from Hoffman-La Roche. V.K.B.F. received scientific event grants from Takeda. M.d.R.F.R. received speaker fees from Novo Nordisk; scientific event grants from Hoffman-La Roche, Takeda, and Novo Nordisk; and consultancy fees from the Brazilian Ministry of Health. F.M.R.d.A.C. received scientific event grants from Hoffman-La Roche and Novo Nordisk. M.A.F.d.C. received scientific event grants from Hoffman-La Roche. D.G.C., B.A.d.S.S., L.W.Z., M.M.D., C.S.L., M.H.C., R.d.A.R., I.P., and S.M.R. declare that they have no competing interests that might be perceived as posing a conflict of bias.

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