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Prevalence and Factors Associated With Inhibitor Positivity in Patients With Hemophilia at a Tertiary Care Center in Central India

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Abstract

Background: Replacement clotting-factor concentrates may induce neutralizing antibodies that reduce treatment effectiveness. This study estimated inhibitor positivity, examined its clinical associations, and evaluated whether cumulative exposure days were related to inhibitor titer among patients receiving care at a tertiary center in central India.

Methods: This hospital-based analytical cross-sectional study used consecutive total-enumeration sampling and included 152 patients with hemophilia A or B treated at Gandhi Medical College and Hamidia Hospital between August 2022 and January 2024. Eligibility required receipt of factor concentrate; patients who declined consent or had never received concentrate were excluded. Clinical characteristics, factor exposure, factor activity, and inhibitor-assay findings were recorded. Exact or fixed-margin Monte Carlo tests were used for categorical comparisons. Wilson 95% confidence intervals, exploratory odds ratios with 95% confidence intervals, and Pearson and Spearman correlation coefficients were also calculated.

Results: Eighteen of 152 participants were inhibitor positive (11.8%; 95% confidence interval: 7.6%-17.9%), and all 18 had hemophilia A. High-titer inhibitors (⩾5 BU/mL) were present in 14 participants (77.8%), whereas four participants (22.2%) had low-titer inhibitors (<5 BU/mL); the median titer was 26.0 Bethesda units per milliliter (interquartile range: 5.3-53.5). Inhibitor positivity was associated with severe hemophilia (odds ratio: 8.2; 95% confidence interval: 2.5-26.5), factor infusion more than seven times annually (odds ratio: 9.6; 95% confidence interval: 2.6-34.7), and spontaneous bleeding, which closely tracked disease severity and a family history of inhibitors. The association with spontaneous bleeding was not interpreted as independent of hemophilia severity. Within the inhibitor-positive subgroup, exposure days were not significantly correlated with titer (Pearson r = -0.31; p = 0.208; Spearman rho = -0.15; p = 0.567).

Conclusions: Inhibitor positivity clustered among participants with severe hemophilia, more frequent factor infusion, spontaneous bleeding, or a family history of inhibitors. Exposure days did not show a significant relationship with titer within the small inhibitor-positive subgroup. The cross-sectional design and imprecise estimates from sparse cells require cautious interpretation and confirmation in larger multicenter studies.

Categories: Hematology, Pathology, Pediatrics
Keywords: associated factors, bethesda assay, exposure days, factor replacement therapy, hemophilia, inhibitor positivity, risk factors

Introduction

Hemophilia A and hemophilia B result from inherited deficiencies of coagulation factor VIII (FVIII) and factor IX (FIX), respectively. Hemophilia A represents approximately 80%-85% of cases, and residual factor activity separates disease into mild, moderate, and severe categories. Lower factor activity is generally accompanied by earlier and more frequent bleeding [1-3].

Infusion of the deficient factor controls acute bleeding and can be given prophylactically to prevent recurrent bleeding and joint damage. Although modern plasma-derived and recombinant concentrates have improved survival and daily functioning, some recipients develop neutralizing alloantibodies that diminish the effect of replacement therapy [4-6]. Both inherited susceptibility and treatment-related exposure appear to contribute to this immune response [6].

Population estimates suggest that inhibitors arise in about one in five people with hemophilia A and three in 100 people with hemophilia B [7]. The Bethesda assay, or its Nijmegen modification, quantifies inhibitor activity in Bethesda units per milliliter. Inhibitor titers were classified as low-titer when <5 BU/mL and high-titer when ⩾5 BU/mL; therefore, a titer of exactly 5 BU/mL was included in the high-titer category. Because

an inhibitor can increase bleeding burden and complicate treatment, we measured inhibitor positivity and titer, compared relevant clinical characteristics, and examined the relationship between cumulative exposure days and titer in patients treated at our center.

Materials And Methods

Study design and participants

This hospital-based analytical cross-sectional study was conducted in the Department of Pathology, Gandhi Medical College, and its associated Hamidia Hospital in Bhopal, Madhya Pradesh, India. Recruitment and data collection covered 18 months, from August 2022 through January 2024. Patients with hemophilia A or B were eligible if they attended the center during this interval and had received factor concentrate. Patients who declined consent or had never received factor concentrate were excluded. Patients with previously documented inhibitors were not excluded. Participants were classified as having a previously known inhibitor when a positive inhibitor result had been documented before the study period. Participants without a previously documented positive result who first tested positive during the study period were classified as newly identified cases. Consecutive total-enumeration sampling was used: every eligible patient who presented during the predefined study period was invited to participate, and 152 eligible consenting patients were enrolled and analyzed. Because the study attempted to include the complete accessible population during the 18-month period, no separate formula-based sample-size calculation was performed.

Ethical approval and consent

Ethical approval was obtained from the Institutional Ethics Committee, Gandhi Medical College, Bhopal (Letter No. 32208/MC/IEC/2022; August 8, 2022). Written informed consent for study participation and open-access publication of de-identified aggregate findings was obtained from all adult participants and from the parents or legal guardians of minors. No directly identifying patient information is included in this article.

Data collection and variables

Recorded variables included age, religion, socioeconomic category, age at bleeding onset, hemophilia type and severity, bleeding pattern and site, family histories of hemophilia and inhibitors, treatment regimen, annual factor-infusion frequency, cumulative exposure days, and use of emicizumab or immune tolerance induction. Annual factor-infusion frequency referred to the 12 months immediately preceding the inhibitor-test blood draw. Frequency was recorded using categories defined a priori during the study design: ⩽7 infusions/year, 8-10 infusions/year, and >10 infusions/year; exact annual counts were not collected. The ⩽7 infusions/year category reflected the usual on-demand treatment burden at our center, approximately one treated bleeding episode every 1.5-2 months, whereas >7 infusions/year represented a higher-intensity bleeding and treatment pattern.

Treatment regimen was classified as prophylactic or on-demand based on the clinical record. An exposure day was any calendar day on which replacement factor was administered. Counts were abstracted from hemophilia day-care and clinic records and supplemented by participant or caregiver recall. Clinical and laboratory records were reviewed to determine whether inhibitor positivity was previously known or newly identified during the study period. Previous inhibitor results were reviewed to distinguish previously documented inhibitors from newly identified cases. However, inhibitor positivity for the present analysis was determined exclusively from current testing performed during the study period. The current Bethesda titer, which was also the most recent available titer, was used in all analyses involving inhibitor titer; historical peak titers were not used.

Laboratory methods

Laboratory testing was performed in the Department of Pathology, Gandhi Medical College and associated Hamidia Hospital. Complete blood count, coagulation profile, hepatitis B and C screening, and FVIII or FIX activity were assessed. Factor activity was measured by a one-stage activated partial thromboplastin time (APTT)-based clotting assay. Hemophilia severity was classified according to the baseline activity of the deficient factor-FVIII in hemophilia A and FIX in hemophilia B-as severe when factor activity was <1 IU/dL (<1% of normal), moderate when it was 1-5 IU/dL (1%-5%), and mild when it was >5 to <40 IU/dL (>5% to <40%). Serial dilutions of standard and patient plasma were tested for their ability to correct the APTT of plasma deficient in the relevant factor; FIX, factor XI, and factor XII assays used the corresponding factor-deficient plasma. Results were derived from a calibration graph.

Inhibitor testing followed a two-step sequential approach. All participants with hemophilia A or B initially underwent an APTT-based mixing study using patient plasma, normal pooled plasma, and a 50:50 mixture incubated at 37°C for one hour. Correction or non-correction of the APTT was then assessed. Only samples showing non-correction proceeded to Bethesda testing. In this cohort, all non-correcting samples were from participants with hemophilia A and were quantified using the FVIII Bethesda assay. Serially diluted patient plasma was mixed with normal pooled plasma, incubated at 37°C for two hours, and residual FVIII activity was measured using the one-stage FVIII assay. One Bethesda unit (BU) was defined as the amount of

inhibitor that inactivated 50% of the added FVIII after two hours at 37°C. All participants with hemophilia B had a correcting mixing study; therefore, no FIX-specific Bethesda assay was performed.

Medication histories and clinical records were reviewed, and no participant was receiving heparin, warfarin, a direct oral anticoagulant, or another anticoagulant at the time of blood sampling. Standard preanalytical procedures, including the recommended 9:1 blood-to-citrate ratio, were followed. Factor XI and factor XII activities were assessed using the corresponding factor-deficient plasma. Dedicated lupus anticoagulant testing, including dilute Russell viper venom time or phospholipid-dependent confirmatory testing, and specific laboratory testing for heparin interference, such as thrombin time or anti-Xa activity, were not performed. Inhibitor classification was based on a single study-period assessment using the manual APTT-based mixing study and one-stage Bethesda procedure; repeat testing and chromogenic confirmation were not performed.

The source protocol did not specify a single Bethesda-unit-per-milliliter threshold for inhibitor positivity. Residual FVIII activity of 80%-100% was interpreted as no inhibitor, <60% as a clear-cut inhibitor, and 60%-80% as borderline. For the analysis, borderline results were classified as inhibitor negative. Inhibitor titers were categorized as low-titer (<5 BU/mL) or high-titer (≥5 BU/mL). FVIII assay materials included FVIII-deficient plasma (<1% FVIII), platelet-poor citrated test and standard plasma, activated partial thromboplastin time reagent, 0.025 M calcium chloride, and imidazole buffer; the Bethesda assay used the same materials plus normal pooled plasma. The documented procedures were manual and used a water bath, 12 × 75-mm plastic and glass test tubes, micropipettes, an ice rack, stopwatches, and visual clot detection. No automated or semi-automated coagulation analyzer or reagent manufacturer was documented in the source record.

Statistical analysis

Data were entered in MS Excel (Microsoft Corporation, Redmond, Washington, United States) and initially analyzed with IBM SPSS Statistics for Windows, Version 20 (Released 2011; IBM Corp., Armonk, New York, United States). Categorical variables are reported as counts with percentages. Continuous variables are summarized by mean and standard deviation; median and interquartile range are also reported for skewed distributions. The 25th and 75th percentiles were calculated using linear interpolation between adjacent ordered observations. Because several contingency tables contained small or zero cells, two-sided Fisher exact or Fisher-Freeman-Halton exact tests were used for two-column tables, and fixed-margin Monte Carlo exact tests with 100,000 permutations were used for larger tables. For ordered variables, two-sided trend tests were used instead of treating categories as nominal: linear-by-linear association tests were used for ordered variables across hemophilia severity in Table *1*, and Cochran-Armitage trend tests were used for ordered variables against inhibitor positivity in Table *2*. Each variable contributed one primary test, giving nine tests in Table *1* and 11 tests in Table *2*. The Benjamini-Hochberg procedure was applied across these 20 variable-level tests to control the false discovery rate. Both unadjusted p-values and false-discovery-rate-adjusted q-values are reported; $q < 0.05$ was considered statistically significant. All 152 enrolled participants were included in the analyses, and the variables presented in Tables *1*-*2* had no missing observations. For the present analysis, inhibitor positivity required both a non-correcting APTT mixing study and a positive Bethesda result. Participants with a correcting mixing study did not undergo Bethesda testing and were classified as screening negative.

Variable	Category	Mild (7 participants)	Moderate (91 participants)	Severe (54 participants)	Primary p-value	FDR-adjusted q-value
Age (years)	1-6 years	1 (14.3%)	9 (9.9%)	4 (7.4%)	0.822	0.865
Age (years)	7-14 years	0 (0.0%)	15 (16.5%)	12 (22.2%)	0.822	0.865
Age (years)	>14 years	6 (85.7%)	67 (73.6%)	38 (70.4%)	0.822	0.865
Religion	Hindu	7 (100.0%)	81 (89.0%)	51 (94.4%)	0.381	0.448
Religion	Muslim	0 (0.0%)	10 (11.0%)	3 (5.6%)	0.381	0.448
Socioeconomic status	Lower	0 (0.0%)	26 (28.6%)	10 (18.5%)	0.139	0.252
Socioeconomic status	Middle	7 (100.0%)	65 (71.4%)	44 (81.5%)	0.139	0.252
Age at onset	<1 year	0 (0.0%)	1 (1.1%)	2 (3.7%)	0.005*	0.018*
Age at onset	1-6 years	2 (28.6%)	43 (47.3%)	31 (57.4%)	0.005*	0.018*
Age at onset	7-14 years	1 (14.3%)	24 (26.4%)	15 (27.8%)	0.005*	0.018*
Age at onset	>14 years	4 (57.1%)	23 (25.3%)	6 (11.1%)	0.005*	0.018*
Family history of hemophilia	Absent	3 (42.9%)	9 (9.9%)	1 (1.9%)	0.002*	0.009*
Family history of hemophilia	Present	4 (57.1%)	82 (90.1%)	53 (98.1%)	0.002*	0.009*
Type of bleed	Spontaneous	0 (0.0%)	70 (76.9%)	54 (100.0%)	<0.001*	<0.001*
Type of bleed	Traumatic	7 (100.0%)	21 (23.1%)	0 (0.0%)	<0.001*	<0.001*
Site of bleed	Bruises	2 (28.6%)	4 (4.4%)	0 (0.0%)	0.010*	0.027*
Site of bleed	Hemarthrosis	5 (71.4%)	78 (85.7%)	52 (96.3%)	0.010*	0.027*
Site of bleed	Intramuscular	0 (0.0%)	9 (9.9%)	2 (3.7%)	0.010*	0.027*
Factor infusion frequency	≤7/year	5 (71.4%)	60 (65.9%)	26 (48.1%)	0.084	0.168
Factor infusion frequency	8-10/year	1 (14.3%)	29 (31.9%)	26 (48.1%)	0.084	0.168
Factor infusion frequency	>10/year	1 (14.3%)	2 (2.2%)	2 (3.7%)	0.084	0.168
Hemophilia type	Hemophilia A	6 (85.7%)	85 (93.4%)	44 (81.5%)	0.083	0.168
Hemophilia type	Hemophilia B	1 (14.3%)	6 (6.6%)	10 (18.5%)	0.083	0.168

TABLE 1: Association of hemophilia severity with baseline clinical variables

FDR: false discovery rate

Values are counts with percentages in parentheses, calculated within severity categories. Age group, age at onset, and factor-infusion frequency were evaluated using two-sided linear-by-linear association tests. Other variables were evaluated using fixed-margin Monte Carlo exact tests with 100,000 permutations. Each variable contributed one primary test. The Benjamini-Hochberg procedure was applied across the 20 variable-level tests in Tables 1-2. An asterisk indicates an FDR-adjusted q-value < 0.05

Variable	Category	Inhibitor negative (134 participants)	Inhibitor positive (18 participants)	Primary p-value	FDR-adjusted q-value
Hemophilia severity	Mild	7 (5.2%)	0 (0.0%)	<0.001*	<0.001*
Hemophilia severity	Moderate	87 (64.9%)	4 (22.2%)	<0.001*	<0.001*
Hemophilia severity	Severe	40 (29.9%)	14 (77.8%)	<0.001*	<0.001*

Age (years)	1-6 years	12 (9.0%)	2 (11.1%)	0.334	0.448
Age (years)	7-14 years	22 (16.4%)	5 (27.8%)	0.334	0.448
Age (years)	>14 years	100 (74.6%)	11 (61.1%)	0.334	0.448
Religion	Hindu	124 (92.5%)	15 (83.3%)	0.186	0.310
Religion	Muslim	10 (7.5%)	3 (16.7%)	0.186	0.310
Socioeconomic status	Lower	30 (22.4%)	6 (33.3%)	0.375	0.448
Socioeconomic status	Middle	104 (77.6%)	12 (66.7%)	0.375	0.448
Age at onset	<1 year	2 (1.5%)	1 (5.6%)	0.509	0.565
Age at onset	1-6 years	67 (50.0%)	9 (50.0%)	0.509	0.565
Age at onset	7-14 years	35 (26.1%)	5 (27.8%)	0.509	0.565
Age at onset	>14 years	30 (22.4%)	3 (16.7%)	0.509	0.565
Family history of hemophilia	Absent	13 (9.7%)	0 (0.0%)	0.367	0.448
Family history of hemophilia	Present	121 (90.3%)	18 (100.0%)	0.367	0.448
Type of bleed	Spontaneous	106 (79.1%)	18 (100.0%)	0.045	0.114
Type of bleed	Traumatic	28 (20.9%)	0 (0.0%)	0.045	0.114
Site of bleed	Bruises	6 (4.5%)	0 (0.0%)	1.000	1.000
Site of bleed	Hemarthrosis	118 (88.1%)	17 (94.4%)	1.000	1.000
Site of bleed	Intramuscular	10 (7.5%)	1 (5.6%)	1.000	1.000
Factor infusion frequency	≤7/year	88 (65.7%)	3 (16.7%)	<0.001*	<0.001*
Factor infusion frequency	8-10/year	45 (33.6%)	11 (61.1%)	<0.001*	<0.001*
Factor infusion frequency	>10/year	1 (0.7%)	4 (22.2%)	<0.001*	<0.001*
Family history of inhibitors	Absent	134 (100.0%)	14 (77.8%)	<0.001*	<0.001*
Family history of inhibitors	Present	0 (0.0%)	4 (22.2%)	<0.001*	<0.001*
Hemophilia type	Hemophilia A	117 (87.3%)	18 (100.0%)	0.224	0.345
Hemophilia type	Hemophilia B	17 (12.7%)	0 (0.0%)	0.224	0.345

TABLE 2: Association of baseline clinical variables with current inhibitor status

FDR: false discovery rate; APTT: activated partial thromboplastin time

Current inhibitor positivity required both a noncorrecting APTT mixing study and a positive Bethesda result. Values are counts with percentages in parentheses, calculated within inhibitor-status categories. Hemophilia severity, age group, age at onset, and factor-infusion frequency were evaluated using two-sided Cochran-Armitage trend tests. Other variables were evaluated using two-sided Fisher exact or Fisher-Freeman-Halton exact tests. Each variable contributed one primary test. The Benjamini-Hochberg procedure was applied across the 20 variable-level tests in Tables 1 and 2. An asterisk indicates an FDR-adjusted q-value < 0.05

Exploratory supplementary analyses were performed on the same dataset. Wilson 95% confidence intervals were calculated for selected proportions, and unadjusted odds ratios with 95% confidence intervals were derived from selected two-by-two contingency tables. For the exploratory two-by-two comparison, the prespecified infusion-frequency categories were dichotomized as ≤7 versus >7 infusions/year. This cutoff was defined before analysis and was not selected after examination of the inhibitor results. When a table

contained a zero cell, the Haldane-Anscombe correction added 0.5 to each cell before calculation. Among participants who were inhibitor-positive on current study-period testing, Pearson and Spearman coefficients assessed the relationship between cumulative exposure days and the current Bethesda titer measured during the study period. Historical peak titers were not included in this analysis. Age, hemophilia severity, infusion frequency, bleeding pattern, and family history were considered potential confounding variables, but the 18 inhibitor-positive events did not support reliable multivariable adjustment; consequently, all odds ratios are unadjusted and should not be interpreted causally.

Results

All 152 enrolled participants were included in the analysis. Hemophilia A accounted for 135 participants (88.8%, 95% confidence interval 82.8%-92.9%), whereas 17 participants (11.2%) had hemophilia B. Thus, hemophilia A was nearly eight times as common as hemophilia B in this hospital sample (Figure *1*).

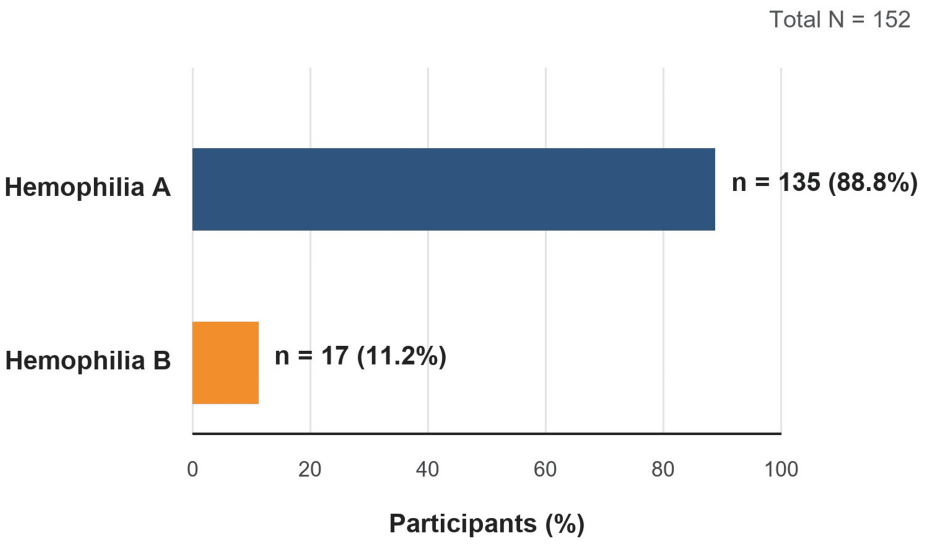


FIGURE 1: Distribution of hemophilia types among the 152 study participants

Hemophilia A included 135 participants (88.8%), and hemophilia B included 17 participants (11.2%). Percentages use the full study population as the denominator

Moderate factor deficiency was the largest severity category, comprising 91 participants (59.9%). Severe disease affected 54 participants (35.5%), while only seven (4.6%) had mild disease. The distribution shows that more than nine in 10 participants had moderate or severe hemophilia (Figure *2*).

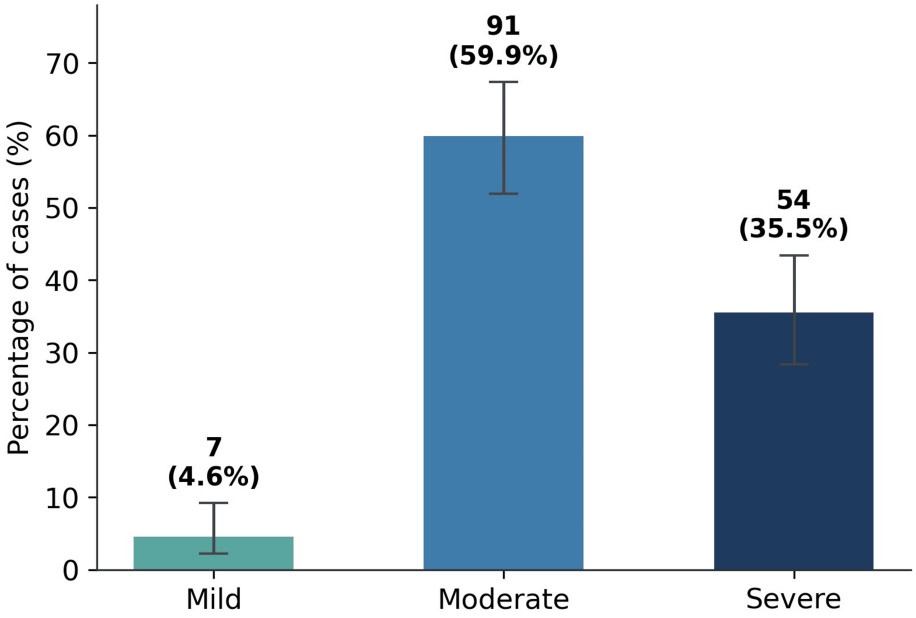


FIGURE 2: Distribution of hemophilia severity by factor activity

Error bars show 95% Wilson score confidence intervals. The total study population was 152 participants

Hemophilia severity showed clear gradients in bleeding characteristics and family history. A family history of hemophilia was recorded in four of seven mild cases (57.1%), 82 of 91 moderate cases (90.1%), and 53 of 54 severe cases (98.1%). Spontaneous bleeding rose from none of the seven mild cases to 70 of 91 moderate cases (76.9%) and all 54 severe cases (100.0%); hemarthrosis likewise became more prominent with greater severity. After ordinal trend testing and false-discovery-rate correction, earlier age at onset showed a significant trend toward greater hemophilia severity ($p = 0.005$, $q = 0.018$). Age group, religion, socioeconomic status, infusion frequency, and hemophilia type did not remain significantly associated with severity (Table *1*).

Hemophilia A remained the predominant diagnosis in every severity category. It comprised six of seven mild cases (85.7%), 85 of 91 moderate cases (93.4%), and 44 of 54 severe cases (81.5%); the relative share of hemophilia B was highest in the severe group. This comparison is displayed in Figure *3*.

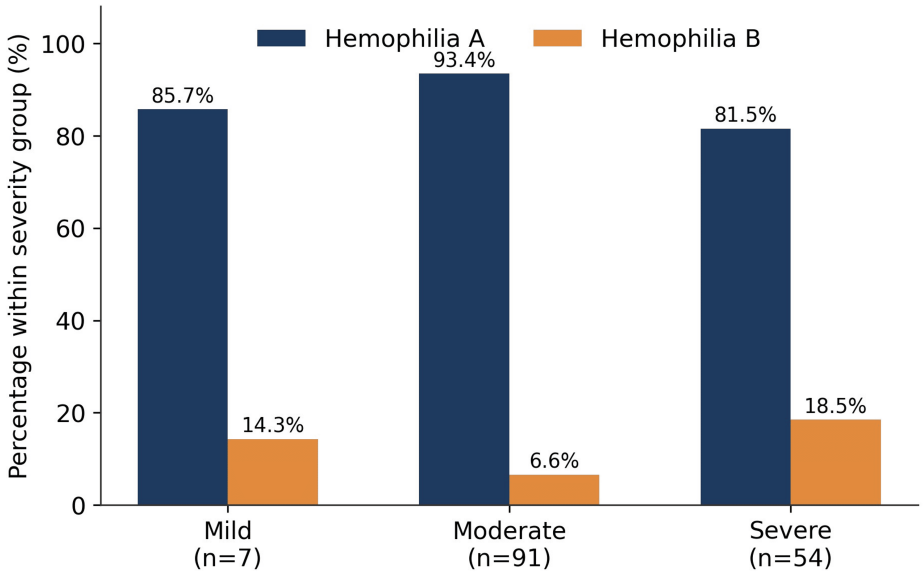


FIGURE 3: Hemophilia type within each severity category

Percentages are calculated within each severity category. The number of participants in each category is shown below the horizontal axis

All 152 participants underwent initial screening with an APTT-based mixing study. The mixing study was corrected in 134 participants (88.2%), who were classified as screening negative and did not undergo Bethesda testing. Eighteen participants (11.8%; 95% confidence interval: 7.6%-17.9%) had a noncorrecting mixing study and proceeded to Bethesda testing. All 18 had a positive FVIII Bethesda result, were classified as inhibitor positive, and had hemophilia A. Of these, two had a previously documented inhibitor but remained positive on current testing, whereas 16 were newly identified during the study period. All 17 participants with hemophilia B were among the 134 participants with a correcting mixing study and therefore did not undergo FIX-specific Bethesda testing. The inhibitor-negative group included three participants with borderline residual FVIII activity of 60%-80%. All 18 participants classified as inhibitor-positive had residual FVIII activity below 60%. All 18 inhibitor-positive participants had hemophilia A. Every inhibitor-positive participant had hemophilia A, so no inhibitor-positive result occurred among the 17 participants with hemophilia B. The overall screening distribution is shown in Figure *4*.

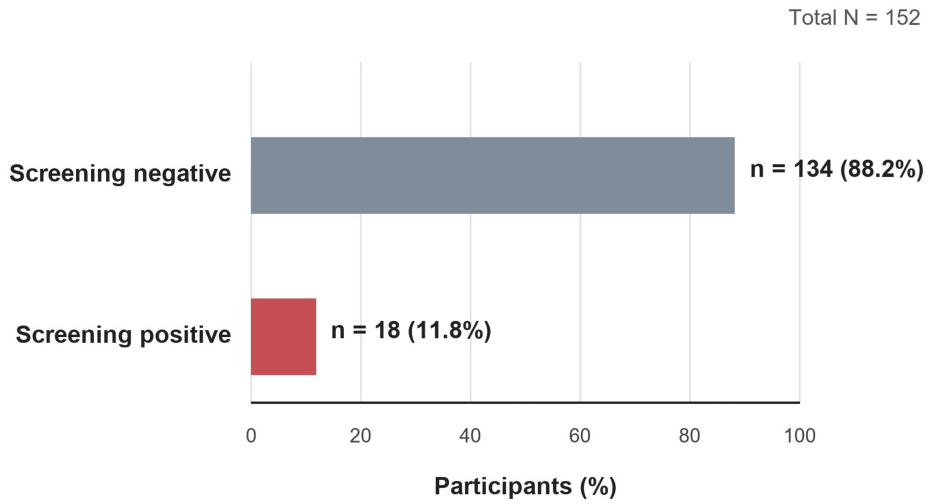


FIGURE 4: Inhibitor screening results among all study participants

APTT: activated partial thromboplastin time

All 152 participants underwent initial screening with an APTT-based mixing study. Screening was negative, with correction of the mixing study, in 134 participants (88.2%). Eighteen participants (11.8%) had a noncorrecting mixing study and underwent Bethesda testing; all 18 had a positive Bethesda result. All 18 participants with a noncorrecting mixing study had hemophilia A and subsequently had a positive FVIII Bethesda result. All 17 participants with hemophilia B were among the 134 participants with a correcting mixing study and did not undergo factor IX-specific Bethesda testing

Among the 18 inhibitor-positive participants, the mean Bethesda titer was 54.72 ± 87.91 Bethesda units per milliliter, and the median was 26.0 Bethesda units per milliliter (interquartile range: 5.3-53.5), demonstrating substantial right skew. Fourteen participants (77.8%) had high titers (⩾5 BU/mL), whereas four participants (22.2%) had low titers (<5 BU/mL). High-titer results were therefore about three and a half times as frequent as low-titer results (Figure *5*).

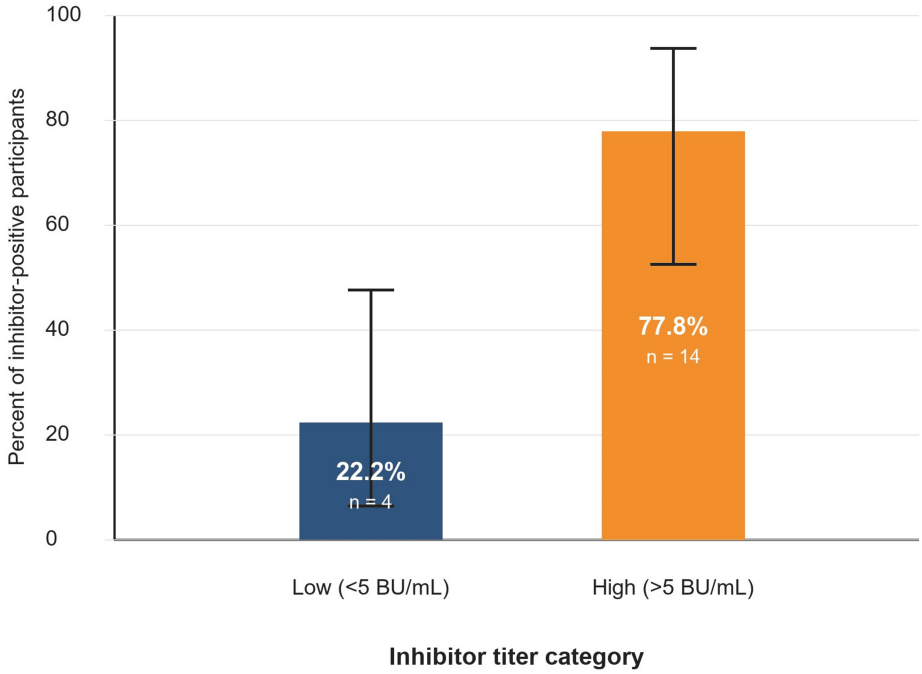


FIGURE 5: Bethesda inhibitor titer categories among inhibitor-positive participants

Error bars show 95% Wilson score confidence intervals. Titers are reported in Bethesda units per milliliter. Low-titer inhibitors were defined as <5 BU/mL, and high-titer inhibitors as ≥5 BU/mL

The inhibitor-positive group contained a larger share of severe disease than the inhibitor-negative group: 14 of 18 positive participants (77.8%) were severe, compared with 40 of 134 negative participants (29.9%). All 18 positive participants (100.0%) reported spontaneous bleeding, compared with 106 of 134 negative participants (79.1%). Infusion more than seven times annually occurred in 15 of 18 positive participants (83.3%) but in 46 of 134 negative participants (34.3%).

However, spontaneous bleeding also increased from 0% in mild hemophilia to 76.9% in moderate hemophilia and 100% in severe hemophilia. Because spontaneous bleeding closely tracked disease severity, it was not interpreted as an independent factor associated with inhibitor positivity.

A family history of inhibitors was present in four positive participants (22.2%) and in none of the negative participants; age, religion, socioeconomic category, age at onset, family history of hemophilia, bleeding site, and hemophilia type were not significantly associated with inhibitor status (Table *2*).

Exploratory association analysis

The exploratory two-by-two comparisons quantified the magnitude and precision of selected associations. Severe hemophilia and factor infusion more than seven times annually produced odds ratios of 8.2 and 9.6, respectively, with confidence intervals that excluded 1. In contrast, estimates for spontaneous bleeding and both family-history variables depended on zero-cell correction and had wide confidence intervals. Hemophilia severity ($p < 0.001$; $q < 0.001$), factor-infusion frequency ($p < 0.001$; $q < 0.001$), and family history of inhibitors ($p < 0.001$; $q < 0.001$) remained associated with inhibitor positivity. Spontaneous bleeding had a nominal p-value of 0.045 but did not remain significant after correction ($q = 0.114$). These unadjusted results identify patterns in this sample but do not demonstrate independent or causal effects (Table *3*).

Risk factor	Comparison	Odds ratio	95% confidence interval	Notes
Family history of inhibitors	**Present compared with absent**	**83.5**	**4.3-1629.8**	**Zero-cell corrected; wide confidence interval; interpret cautiously**
Spontaneous bleeding	Compared with traumatic	9.9	0.6-169.3	Zero-cell corrected; confidence interval crosses 1
Factor infusion frequency	>7/year compared with ≤7/year	9.6	2.6-34.7	Stable estimate
Severe hemophilia	Compared with mild or moderate	8.2	2.5-26.5	Stable estimate
Family history of hemophilia	Present compared with absent	4.1	0.2-72.1	Zero-cell corrected; confidence interval crosses 1

TABLE 3: Odds ratios for selected factors associated with inhibitor positivity

Zero-cell estimates use the Haldane-Anscombe correction. Comparisons and estimates were calculated from the reported contingency tables; all estimates are exploratory and unadjusted

The forest plot emphasizes the contrast between the two more stable estimates and the highly uncertain zero-cell-corrected comparisons. The horizontal intervals for spontaneous bleeding and the two family-history measures cross 1 or span several orders of magnitude, whereas the intervals for severity and infusion frequency remain entirely above 1 (Figure *6*).

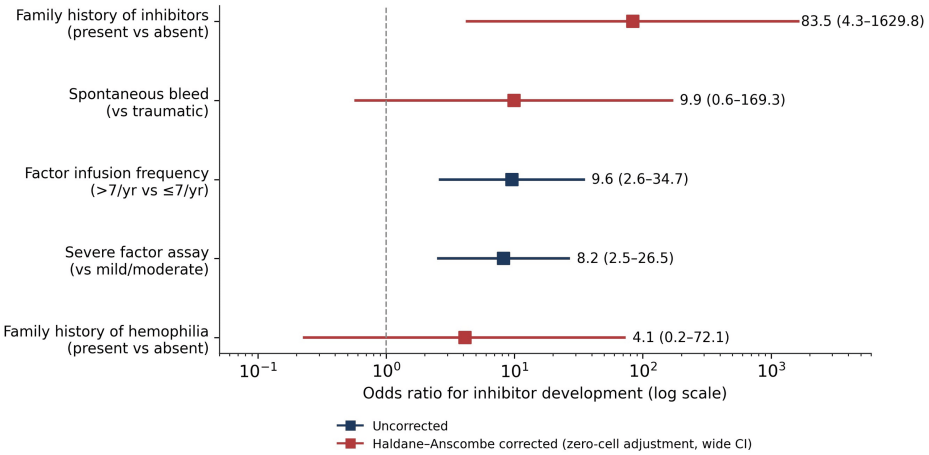


FIGURE 6: Odds ratios for selected factors associated with inhibitor positivity

Squares show point estimates and horizontal lines show 95% confidence intervals on a logarithmic scale. Red markers identify estimates that required the Haldane-Anscombe zero-cell correction

Exposure days and inhibitor titer

Among the 18 inhibitor-positive participants, the mean cumulative exposure was 106.06 ± 72.53 days, and the median was 76.5 days (interquartile range: 63.25-157.5 days). The higher mean relative to the median and the wide interquartile range reflected the right-skewed distribution, including six participants with more than 100 exposure days. Two participants (11.1%) had 10-40 exposure days, one (5.6%) had 41-50 days, five (27.8%) had 51-70 days, four (22.2%) had 71-100 days, and six (33.3%) had more than 100 exposure days (Figure *7*).

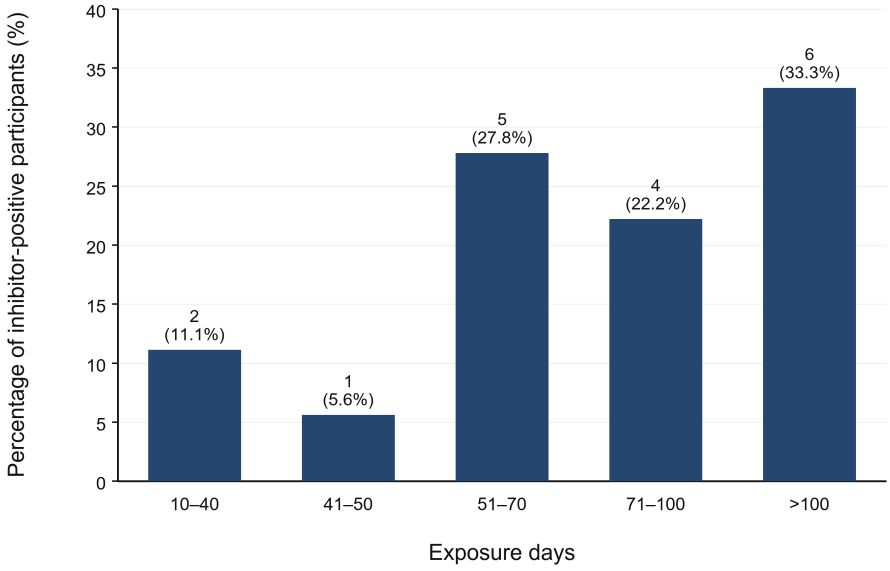


FIGURE 7: Distribution of exposure days among inhibitor-positive participants

Percentages are calculated among the 18 inhibitor-positive participants

The correlation between exposure days and Bethesda titer was weakly negative by both Pearson analysis (r = -0.312; p = 0.208) and Spearman analysis (rho = -0.145; p = 0.567). Neither coefficient was statistically significant, and the scatter included high titers at relatively modest exposure counts as well as low titers after many exposure days. This subgroup analysis describes titer variation only among participants who were already inhibitor-positive and cannot estimate the probability of developing an inhibitor (Figure *8*).

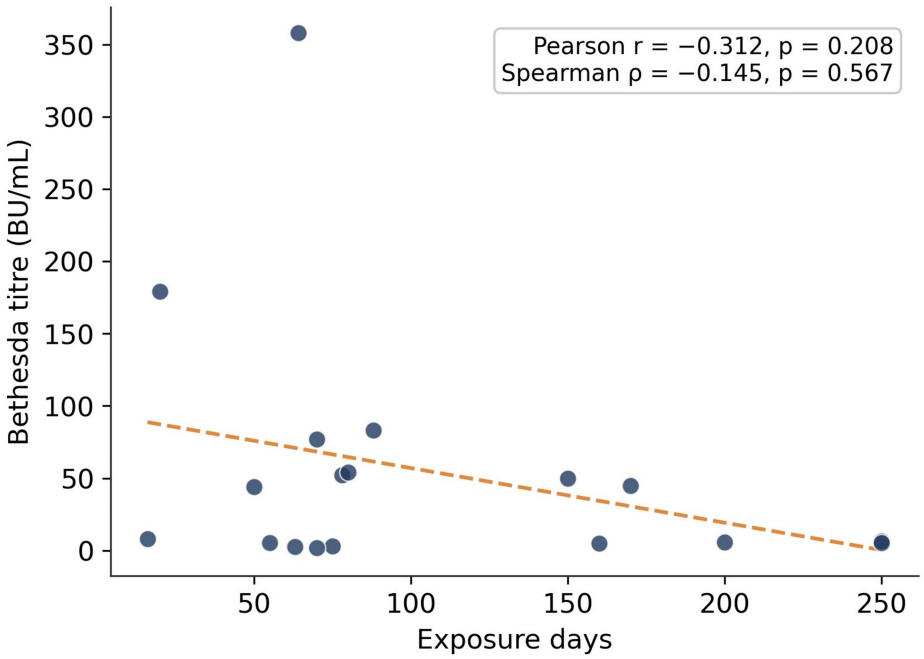


FIGURE 8: Relationship between exposure days and Bethesda inhibitor titer

The current Bethesda titer measured during the study period was used for each participant; historical peak titers were not included. The dashed line represents the least-squares trend line. The dashed line is the least-squares trend line. Inhibitor titer is reported in Bethesda units per milliliter

Discussion

This study found inhibitor positivity in 18 of 152 participants (11.8%), including two participants with previously documented inhibitors and 16 who were newly identified during the study period; all 18 had hemophilia A. Positive results were concentrated among patients with severe disease, frequent factor infusions, or a family history of inhibitors. Although all inhibitor-positive participants reported spontaneous bleeding, this characteristic closely tracked disease severity and was not interpreted as an independent associated factor. Exposure days, however, were not significantly related to inhibitor titer within the 18 positive cases. All participants underwent initial mixing-study screening, but Bethesda testing was restricted to those with a noncorrecting mixing study. Thus, the 18 inhibitor-positive cases had concordant abnormal mixing-study and positive Bethesda results.

All 17 participants with hemophilia B had a correcting mixing-study screen and therefore did not undergo FIX-specific Bethesda testing. Thus, no abnormal inhibitor screen was detected in this group, although low-level FIX inhibitors not detected by the initial screening procedure cannot be excluded [8]. Severe disease was strongly associated with positivity in this sample, with an unadjusted odds ratio of 8.2 (95% confidence interval: 2.5-26.5). This pattern is biologically plausible because severe factor deficiency often requires earlier and more intensive replacement, although inhibitors can also occur in nonsevere hemophilia A when susceptible factor VIII variants are present [9].

The titer distribution was dominated by high values: Fourteen of the 18 inhibitor-positive participants (77.8%) had high titers (⩾5 BU/mL), whereas four participants (22.2%) had low titers (<5 BU/mL). A few very high observations inflated the mean to 54.72 ± 87.91 Bethesda units per milliliter. The median of 26.0 Bethesda units per milliliter (interquartile range 5.3-53.5) therefore provides a better description of the typical positive result.

Exposure-day counts were broadly distributed, with the largest category comprising participants with more than 100 exposure days; no monotonic relationship between cumulative exposure and inhibitor titer was apparent. Previous work has placed the greatest risk of inhibitor emergence during early concentrate exposure, often within the first 20-50 exposure days, while acknowledging that later occurrence is possible [8,10]. Our correlation analysis addressed a different question: whether titer varied with cumulative exposure among patients already known to be positive. Its nonsignificant result must not be interpreted as evidence that exposure is unrelated to incident inhibitor formation. The correlation analysis used the current Bethesda titer measured during the study period rather than the highest historical titer. Accordingly, the analysis evaluated whether cumulative exposure was associated with current inhibitor magnitude among

participants who were currently inhibitor positive.

Participants receiving factor more than seven times annually had higher unadjusted odds of inhibitor positivity (odds ratio: 9.6; 95% confidence interval: 2.6-34.7). All participants in this study received factor replacement on an on-demand basis, and none received prophylaxis. Factor-infusion frequency did not show a statistically significant ordinal trend across hemophilia-severity categories after false-discovery-rate correction ($p = 0.084$, $q = 0.168$). Nevertheless, disease severity could influence both bleeding burden and the need for on-demand factor replacement. Consequently, annual infusion frequency represents treatment utilization in response to bleeding rather than a prescribed prophylactic schedule. More frequent infusion may reflect greater disease severity or bleeding burden and could also occur after inhibitor development because of reduced treatment effectiveness or recurrent bleeding. The cross-sectional design does not establish the temporal direction of this association; therefore, infusion frequency should not be interpreted as an independent causal risk factor. Disease severity, bleeding burden, and infusion frequency may represent related components of the same clinical pathway, and their separate unadjusted odds ratios should not be interpreted as independent effects. Treatment intensity and closely grouped exposures have previously been linked with inhibitor development, particularly in previously untreated patients [11]. In the present cross-sectional data, infusion frequency may also be a marker of disease severity or bleeding burden, so the observed association does not establish an independent treatment effect.

Only four participants reported a family history of inhibitors, and all four were inhibitor positive. The resulting corrected odds ratio was 83.5, but its 95% confidence interval extended from 4.3 to 1629.8 because of the zero cell and small numerator. Familial clustering has been described elsewhere and may reflect shared FVIII variants or other inherited determinants [12-15]. Because this study did not include genotyping, a genetic explanation remains a hypothesis rather than a measured mechanism.

The findings support focused surveillance in patients with severe hemophilia, frequent replacement therapy, or a family history of inhibitors. They do not justify treatment decisions based on the reported odds ratios alone, because the comparisons are unadjusted and several estimates are unstable. The hospital-based sample may be most applicable to similar tertiary-care populations in central India; broader generalization requires multicenter studies with prospective exposure records, adequate event counts, and multivariable analysis.

Limitations

The study came from one center and contained only 18 inhibitor-positive participants, which restricted precision and precluded reliable multivariable adjustment. Exact tests reduced reliance on asymptotic assumptions, but sparse and zero cells still produced wide confidence intervals for several odds ratios. Exposure histories were partly reconstructed from participant or caregiver recall and may contain classification error, while sex was unavailable in the analytic dataset. Genetic testing was not performed, and the exposure-day correlation included only positive participants; consequently, it cannot estimate incident inhibitor risk in the full population. All participants received on-demand treatment, which limits the generalizability of the findings to populations in which prophylactic factor replacement is routinely used. Bethesda testing was performed only after a noncorrecting mixing study rather than in all participants. Consequently, inhibitors not detected by the initial screening procedure could have been missed, and the reported inhibitor positivity should be interpreted in the context of this sequential testing algorithm. The cross-sectional data also could not determine whether greater infusion frequency preceded inhibitor development or resulted from increased bleeding or reduced treatment effectiveness after inhibitor development.

Classifying the three borderline results as inhibitor negative may have resulted in underestimation of inhibitor positivity. Dedicated lupus anticoagulant testing and specific laboratory testing for anticoagulant interference were not performed. Although medication records showed that no participant was receiving heparin, warfarin, or a direct oral anticoagulant at sampling, laboratory interference or another nonspecific inhibitor cannot be completely excluded. In addition, inhibitor status was based on a single manual APTT-based assessment without repeat or chromogenic confirmation, which limits the specificity of the reported inhibitor classification.

Conclusions

Eighteen of 152 participants (11.8%) were inhibitor-positive, including two with previously documented inhibitors and 16 who were newly identified during the study period; all 18 had hemophilia A. Positivity was associated with severe hemophilia, factor infusion more than seven times annually, and a family history of inhibitors. Spontaneous bleeding closely tracked hemophilia severity and was therefore not interpreted as an independent associated factor. Severity and infusion frequency yielded the most precise unadjusted estimates, whereas comparisons involving zero cells remained highly uncertain. Within the subgroup that was inhibitor-positive on current study-period testing, cumulative exposure days were not significantly correlated with the current Bethesda titer. The findings support selective surveillance but cannot establish causation or independent predictors. These findings may support consideration of closer clinical surveillance in vulnerable patients; however, because the estimates were unadjusted and the study was

cross-sectional, they do not establish independent risk factors and should not be used to define a risk-based screening strategy. Multicenter research with prospective exposure measurement, sufficient events for adjusted analysis, and genotyping is needed to test these associations.

Additional Information

Author Contributions

All authors have reviewed the final version to be published and agreed to be accountable for all aspects of the work.

Concept and design: Shelly Verma

Acquisition, analysis, or interpretation of data: Shelly Verma, Aditi Dad, Devangi Chandra

Drafting of the manuscript: Shelly Verma, Aditi Dad, Devangi Chandra

Critical review of the manuscript for important intellectual content: Shelly Verma, Devangi Chandra

Disclosures

Human subjects: Informed consent for treatment and open access publication was obtained or waived by all participants in this study. Institutional Ethics Committee, Gandhi Medical College, Bhopal issued approval 32208/MC/IEC/2022. Ethical approval was obtained from the Institutional Ethics Committee, Gandhi Medical College, Bhopal (Letter No. 32208/MC/IEC/2022; August 8, 2022). Written informed consent for study participation and open-access publication of de-identified aggregate findings was obtained from all adult participants and from the parents or legal guardians of minors. No directly identifying patient information is included in the article. **Animal subjects:** All authors have confirmed that this study did not involve animal subjects or tissue. **Conflicts of interest:** In compliance with the ICMJE uniform disclosure form, all authors declare the following: **Payment/services info:** All authors have declared that no financial support was received from any organization for the submitted work. **Financial relationships:** All authors have declared that they have no financial relationships at present or within the previous three years with any organizations that might have an interest in the submitted work. **Other relationships:** All authors have declared that there are no other relationships or activities that could appear to have influenced the submitted work.

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