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ORIGINAL ARTICLE

Effect of DNA methylation on inhibitor development in people with hemophilia A treated with FVIII concentrates

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Abstract

Background: Hemophilia A (HA) is a hereditary X-linked hemorrhagic disorder. Following the first treatment with exogenous factor (F)VIII, one-third of patients with severe HA develop anti-FVIII antibodies (inhibitors), which render treatment ineffective. Recent findings underlined the critical role of DNA methylation in several autoimmune diseases by altering gene expression profiles. This study was designed to evaluate potential differences in DNA methylation profiles of previously untreated patients (PUPs) who develop inhibitors against FVIII and those who do not, with the aim of identifying immune-regulatory genes that may contribute to the risk of inhibitor formation.

Objectives: In this study, we aimed to understand whether CpG sites are differentially methylated in peripheral blood mononuclear cells (PBMCs) of PUPs and have a role in inhibitor development to better understand the biological pathways that lead to inhibitor development.

Methods: A case-control study was performed using 45 inhibitor-positive and 67 inhibitor-negative PUPs from the Survey of Inhibitors in Plasma-Product Exposed Toddlers study cohort. Enrichment bisulfite sequencing was performed on DNA samples from PBMCs of HA patients and differentially methylated CpG sites (DMCs) were identified with bioinformatic approach.

Results: Overall, information on 621,121 CpG sites was obtained. Two thousand seven hundred seventy-two sites were significantly differentially methylated (unadjusted P value $< .05$). Association of CpG sites to a few genes involved in active immune response (*JAK1*, *CD1C*, *PIGR*, *TOLLIP*, *BLNK*, *CD44*, *IL23R*, *IFNLR1*, *SOCS2*, *TLR1*, etc.) was seen in inhibitor-positive patients, but it did not indicate specific pathways associated with inhibitor development.

Conclusion: DMCs were identified in PBMC samples from HA patients with inhibitors. However, our data could not confirm the role of these CpG sites in affecting immune-regulatory pathways.

KEYWORDS
antibodies, neutralzing, DNA methylation, blood coagulation disorders,inherited, factor VIII/immunology, hemophilia A

Essentials

- People with Hemophilia A (HA) may develop inhibitors against FVIII causing ineffective treatment.
- This study analyzed DNA methylation differences in HA patients with and without inhibitors.
- 2772 sites with significant difference were identified indicating differences in immune response.
- Findings suggest that few immune-related genes may contribute to inhibitor risk, but further study is needed.

1 | INTRODUCTION

Hemophilia A (HA) is a recessive X-linked hemorrhagic bleeding disorder caused by mutations in *F8* gene. HA patients suffer from spontaneous bleeding manifestations within muscles, joints, and sometimes vital organs caused by the absence or defect in coagulation factor (F)VIII. To manage and prevent bleeding episodes in people with hemophilia A, exogenous FVIII concentrates are commonly administered as a standard replacement therapy [1]. Although this treatment has significantly improved the quality of life of patients, following the treatment with exogenous FVIII, a major challenge arises in the development of neutralizing antibodies, known as inhibitors, against the administered FVIII. Despite the significant advancements in hemophilia management, roughly one-third of patients with severe HA develop anti-FVIII antibodies (inhibitors) during the first 50 exposure days to FVIII concentrates that render treatment ineffective [2].

The development of inhibitors in previously untreated patients (PUPs) with HA remains a complex and poorly understood phenomenon. It is a multifaceted process with intricate involvement between genetic factors and external environmental factors. Several genetic risk factors (for instance, the type of *F8* gene mutation, family history, human leukocyte antigen type, and variants in immune-related genes [eg, interleukin 10]) and treatment-related risk factors (such as the source of FVIII [3] and the intensity of FVIII treatment at first exposure) have been discussed [4] as triggers of the immune response against FVIII in some HA patients. Lacroix–Desmazes et al. [5] highlight mechanisms driving inhibitor development, including innate and adaptive immune responses; strategies to induce immune tolerance, such as immune tolerance induction therapy; and innovative approaches such as engineered FVIII products or tolerogenic nanoparticles. However, the main causal pathways and additional causal factors leading to inhibitor development remain unknown.

The cellular immune response against FVIII is complex involving both T-cell and B-cell responses. Studies also exhibit the vital role of activated T cells in the memory B-cell response to FVIII and the requirement of direct T-cell contact to restimulate these cells [6]. Recent studies have suggested that genetic factors, environmental influences, and immune responses play pivotal roles in shaping this phenomenon [7,8]. However, one emerging epigenetic mechanism that has gained attention in various diseases is DNA methylation.

DNA methylation modification is characterized by the addition of a methyl group to cytosine residues by DNA methyltransferase enzymes, which play a critical role in regulation of gene expression. Changes in DNA methylation patterns are linked with immune dysfunction and autoimmune disorders exhibiting improper gene expression. It has been demonstrated in several studies that immune components like $CD4^+$ T cells and co-stimulatory molecules undergo epigenetic modifications and play a crucial role, leading to dysfunction and regulation issues in cellular immune system in systemic autoimmune rheumatic diseases [9]. In patients with systemic lupus erythematosus (SLE) studies have described hypomethylation of neutrophils and granulocytes, especially at the gene locus of the interferons *MX1* and *IF144* [10]. In rheumatoid arthritis, early methylation studies revealed that patients' T cells, similar to SLE, are characterized by global hypomethylation [11,12].

Studies involved in the association of the role of DNA methylation in inhibitor development are limited. In hemophilia research, only 1 non–peer-reviewed study, published on a preprint server in 2019, has assessed the effect of changes in methylation on inhibitor development [13]. This study found that a differentially methylated CpG site (DMC) in the *IL4* gene was associated with inhibitor development. However, the study only assessed the methylation status of 13 CpG sites in several regulatory regions that were previously shown to be associated with inhibitor development.

Our current study seeks to identify DNA regions in peripheral blood leukocytes that are differentially methylated in patients with and without an inhibitor, using a genome-wide approach, and understand how DNA methylation may influence inhibitor development in people with hemophilia A undergoing FVIII replacement therapy. Utilizing next-generation sequencing methods, we intend to investigate the functional implications of differentially methylated genes, allowing us to gain a deeper understanding of the underlying biological pathways contributing to inhibitor development and possibly to identify biomarkers that could predict the occurrence of inhibitor development.

2 | METHODS

2.1 | Sample population

DNA samples of 111 patients were obtained from the Survey of Inhibitors in Plasma-Product Exposed Toddlers (SIPPET) randomized trial [3]. SIPPET study was performed on PUPs or minimally treated patients suffering from severe HA (FVIII < 0.01 IU mL^{-1}). Minimally treated patients were the patients that received blood components as a form of treatment for <5 times, were not treated with investigational drugs, and tested negative for FVIII inhibitors [3]. Administration of either plasma-derived FVIII or recombinant FVIII as replacement therapy was randomly assigned to each patient. Follow-up on these patients was done

for 50 exposure days or 3 years for patients that did not develop inhibitors. For inhibitor-positive patients, end of study was considered when inhibitor development was confirmed. Among 111 patients, 45 were inhibitor-positive (cases) and 66 were inhibitor-negative (controls). For this study, DNA samples were obtained from end-of-study samples of citrated whole blood stored at −80 °C.

2.2 | Sample preparation

Genomic DNA was extracted from peripheral blood leukocytes (PBMCs). 2 μg of high-quality genomic DNA was used for methylation assay.

2.3 | Methylation assay

Target enrichment bisulfite sequencing was performed using Agilent SureSelectXT Human Methyl-Seq assay. Genomic DNA was sheared into smaller fragments using the Covaris E-series system. The recommended methyl-Seq protocol and specific enzymes were used to repair the ends, adenylate the 3′ ends, and ligate methylated adaptors. Genomic DNA library hybrids were prepared. The hybrids were captured on biotinylated RNA baits and purified using streptavidin beads (New England BioLabs), and this step was performed to capture target-enriched genomic DNA library, which consisted only of CpG sites and genomic regions susceptible to methylation in the whole genome. The target sequences were bisulfite-converted using the EZ DNA Methylation-Gold kit (Zyma Research) as described in the methyl-Seq protocol. During the bisulfite conversion step only, the unmethylated cytosine bases get converted to uracil, but the methylated cytosine bases remain unchanged. Target sequences were then indexed. The sequencing of libraries was then performed on Illumina NextSeq2000. This assay captures about 3.7 million CpGs, which consist of about 84 Mb of the human genome.

2.4 | Bioinformatic pipeline

2.4.1 | Preprocessing of bisulfite sequencing data

Binary base call files obtained from the sequencer were converted to FASTQ format using bcl2fastq conversion software. Quality check was performed with FastQC [14]. Low-quality reads (phred < 20) were removed, and adapters were trimmed from the fastq files using Trim Galore [15] to increase the quality of sequences. Adapter trimmed fastq files were aligned to bisulfite-converted human reference genome (Hg38), and methylation call files were generated using Bismark [16]. Quality check on alignment files was performed with Qualimap [17] to get information about the coverage of reads for each sample.

2.4.2 | Data cleaning

Methylation call files (cov format) were used for data analysis using the MethylKit [18] R package. A quality check was done on all the methylation call files for any low coverage. Filtering of bases with coverage of <10× was done to avoid unreliable statistical results. Similarly, bases with very high coverage were also removed in case of overamplification and/or PCR bias. All the files were normalized taking the median of coverage as the scaling factor.

2.4.3 | Differentially methylated CpG sites detection

After normalization, CpG sites that were present across all the samples were merged into a MethylKit data frame. Differential methylation was calculated using the beta-binomial model: this model considers the variability of the sample as well as epigenetic variability and corrected for multiple testing using the Benjamini–Hochberg method. Then, all the CpG sites with very low methylation differences among them were filtered out. A new data frame was created where CpG sites were selected with *P* values of <.05 and methylation difference of at least 5%. The Reactome database was used to perform overrepresentation analysis on the differentially methylated sites Enrichr web-based tool was used to get Gene Ontology (GO) molecular functions [19].

3 | RESULTS

3.1 | Patient population

The patient population of our study comprises individuals diagnosed with HA who were previously untreated with minimal (<5) or no prior exposure to FVIII concentrates or blood components, and they exhibit various mutations of the *F8* gene, as shown in Table 1. Moreover, the study encompasses patient samples from different ethnic backgrounds, with a majority hailing from Egypt (39.64%%) and India (36.03%; Table 1).

3.2 | General data quality

Quality control of the data revealed read fragment sizes from 70 to 151 bp long. The average Bismark alignment rate of the reads to the converted genome was ∼80%. The mean coverage to the targeted sites was found to be 50×. Reads with at least 10× coverage were found to be between 51% and 77% with a mean of 2 million CpG sites in the data.

3.3 | Exploratory analysis result

Principal component analysis performed on the whole data revealed no distinction between cases and controls, suggesting little variation between the 2 groups. The density distribution of CpG sites for both cases and control showed very little difference between the 2 groups (Figure 1). The graph shows how the CpG sites are distributed across its range (0%-100%, where 0% depicts unmethylated CpG sites while 100% depicts methylated CpG sites) and gives insight into the

TABLE 1 Characteristics of *F8* gene mutations and ethnicity of people with hemophilia A (*n* = 111)

	Inhibitor-positive (*n* = 44)	Inhibitor-negative (*n* = 67)	Percentage of total study population (*n* = 111)
***F8* gene mutation (*n* = 111)**			
Int 22 Inv	25	28	47.75%
Missense	2	6	7.21%
Splicing	2	4	5.40%
Frameshift	6	9	13.52%
Large deletion	3	4	6.30%
Polymorphism		2	1.80%
Nonsense	5	10	14.42%
Int 1 Inv	1	3	3.60%
Ethnicity (*n* = 111)			
Argentina		1	0.90%
Austria	1	1	1.80%
Brazil	2		1.80%
Chile	2	1	2.70%
Egypt	23	21	39.64%
India	12	28	36.03%
Iran	1	5	5.40%
Italy		3	2.70%
Mexico		1	0.90%
Spain	3	1	3.60%
Turkey		1	0.90%
USA		4	3.60%

distribution of methylation among the population. From this graph, we can interpret the differential methylation among inhibitor-positive and inhibitor-negative patients on a wider scale. Density represents the relative likelihood of methylation values of CpG sites at different levels of methylation. This graph gives 2 peaks: one peak at 0% denoting unmethylated CpG sites and the other peak at 100% giving information on methylated CpG sites. However, it indicated that the inhibitor-positive group (cases) was less methylated than the inhibitor-negative group (control). Overall information on 621,121 CpG sites was obtained. From the statistical beta-binomial testing method, 2772 differentially methylated CpG sites (DMCs) were selected based on *P* value < 0.05 and at least a 5% methylation difference.

From the obtained DMCs, 1680 sites were hypermethylated and 1092 CpG sites were hypomethylated in inhibitor-positive samples. However, these sites were found to be statistically insignificant on a genome-wide level (*Q* value $> .05$). Of the total DMCs, 30% were found in the introns, 13% in the intergenic region, 48% in promoters, and 9% in the exon region (Figure 2). Most of these DMCs were outside the CpG island regions (Figure 2). The plot indicates that intergenic regions exhibit differential methylation, with hypomethylation predominantly observed in CpG shelves and hypermethylation in CpG islands. This suggest that methylation changes are most notable in non-CpG island regions, potentially influencing gene regulation and contributing to the differences between the 2 sample groups.

3.4 | Overrepresentation analysis

An association with a gene was found for 2762 out of 2772 DMCs. Association with a gene is determined if DMC is present in the regulatory region or gene body region (Figure 4). We then used the biomaRt package [20] to annotate these DMCs to the gene it is associated with. Reactome pathway analysis identified 144 genes involved in the immune pathway and 59 genes involved in hemostasis. Keeping aside the *P* value, hypermethylation of 105 genes and hypomethylation of 52 genes involved in the immune system were found, while 46 hypermethylated and 22 hypomethylated genes related to hemostasis were observed. Genes involved in the immune system were selected from the Reactome database for both hypermethylated and hypomethylated sites, and the Enrichr web-based tool was used to see enriched molecular functions of the related genes (Tables 2 and 3). From this analysis, we identified GO of molecular functions performed by genes that match the genes annotated to a specific molecular function term in the GO database. It also highlights molecular functions that are enriched in our gene set. GO Molecular Function revealed many hypomethylated genes to be involved in cytokine receptor binding (GO:0005126; *P* value, 0.000442 [*SOCS2*, *IL23R*, *TLR9*, and *JAK1*]), toll-like receptor binding (GO:0035325; *P* value, 0.000669 [*TLR1*, *TOLLIP*]), and cytokine receptor activity (GO:0004896; *P* value, 0.002504 [*IL23R*, *IFNLR1*, and *CD44*], suggesting a possible role of these genes in inhibitor-positive patients.

3.5 | Subpopulation analysis

Since our patient population consisted of different ethnic backgrounds, a substudy using the same methodology was also performed to observe any methylation difference among the patient samples based on their ethnicities as environmental factors play a major role in epigenetic modification of an individual. Clustering of patient samples from Egypt and India based on CpG sites was performed using Ward's method. The analysis did not reveal any pattern in the cluster, suggesting that the geographic origin of an individual does not play a major role in DNA methylation leading to inhibitor development.

No new enriched pathway was observed when performing a similar analysis on samples with Int22Inv mutation of the *F8* gene (*n* = 53). This was performed to observe whether any enrichment of a particular pathway could be involved in relation to a specific mutation.

Although the analysis did not provide any methylation pattern among inhibitor-positive and inhibitor-negative groups based on their ethnic background or *F8* mutation, we acknowledge that the cohort

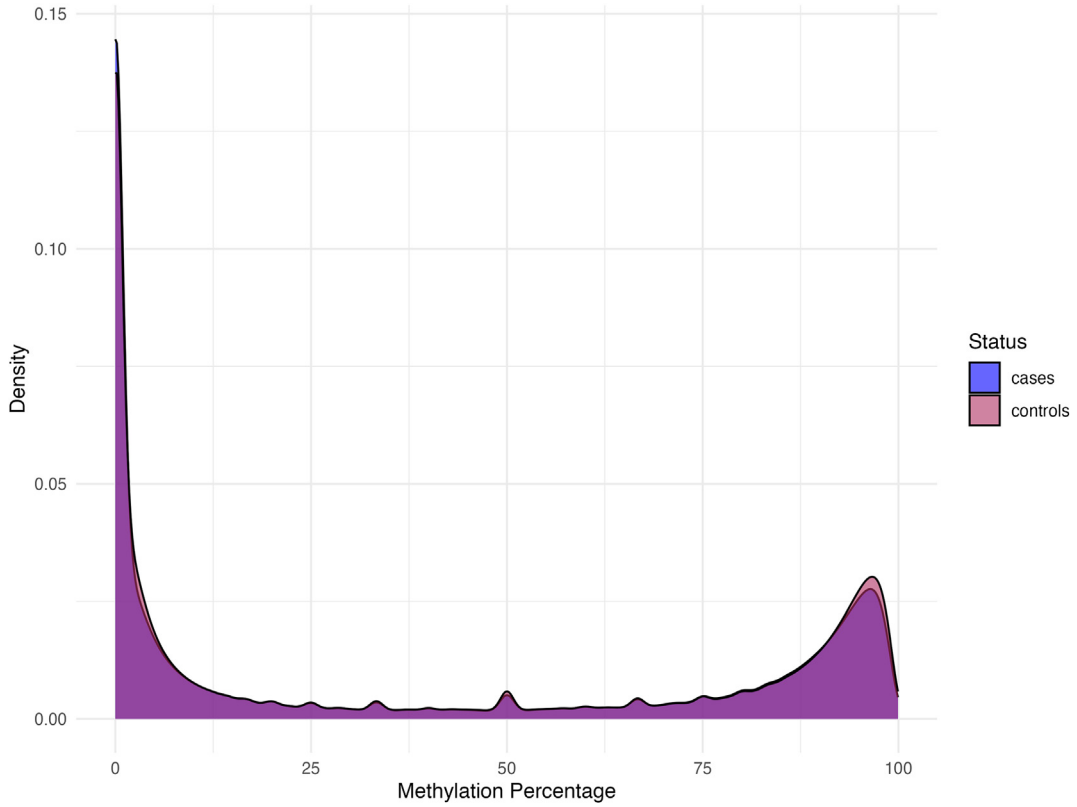

FIGURE 1 Distribution plot of percentage methylation of CpG sites showing the control group (inhibitor-negative) having slightly higher methylation levels than the case group (inhibitor-positive). The *x*-axis represents percentage methylation of CpG sites, ranging from 0% (completely unmethylated) to 100% (fully methylated). The *y*-axis represents probability density, indicating the relative frequency of CpG sites at different methylation levels.

size is limited. This small sample size may reduce the statistical power to comprehensively evaluate the contribution of certain genetic or epigenetic factors, such as population-specific combinations of single nucleotide polymorphisms.

4 | DISCUSSION

The process of inhibitor development in HA remains an intricate puzzle, involving the complex interaction of genetics and environmental factors. This study presents a comprehensive genome-wide analysis of the potential role of DNA methylation in influencing inhibitor development among people with hemophilia A undergoing FVIII replacement therapy. Additionally, our focus on exploring the functional implications of differentially methylated genes enabled us to delve deeper into the underlying biological pathways that might underpin inhibitor development. The study comprises PUPs with different ethnicities and different *F8* gene mutations. Our results demonstrate the complexity of the epigenetic landscape concerning inhibitor development. While the influence of genetic factors and external triggers on inhibitor development has been recognized, the involvement of epigenetic mechanisms such as DNA methylation has been only studied in recent years. The role of DNA methylation in the regulation of gene expression adds new complexity to the already multifaceted process of inhibitor development. The study demonstrates a slight difference in the methylation status between 2 groups. The inhibitor-positive group was moderately less methylated as compared with the inhibitor-negative group, indicating that patients who develop inhibitors have higher gene expression of certain genes. This study also showed that several CpG sites associated with genes related to immune regulation and hemostasis were either hypomethylated or hypermethylated in patients who developed inhibitors as compared with those who did not. While most of the hypomethylated genes are a part of the signaling cascade, a few genes that are part of the immune system are also included; for example, *JAK1*, *CD1C*, *PIGR*, *TOLLIP*, *BLNK*, *CD44*, *IL23R*, *IFNLR1*, *SOCS2*, and *TLR1*. These genes are an indicator of the involvement of DNA methylation in a stronger immune response in patients who develop inhibitors against FVIII. Hypermethylation of genes involved in ubiquitin-related pathways (eg, *UBE2H*, *TRIM6*, *TNFAIP3*, *CUL3*, and *TRAF3*; Table 2) indicates increased inflammatory responses, dysregulated immune system activation, and impaired antigen presentation. Genes such as *TRIM6*, *TNFAIP3*, and *TRAF3* are also involved in NF-κB immune signaling pathway indicating immune dysregulation since NF-κB is one of the major regulators of inflammation and immunity.

Our findings revealed specific differentially methylated CpG sites in relation to genes that could be associated with the formation of

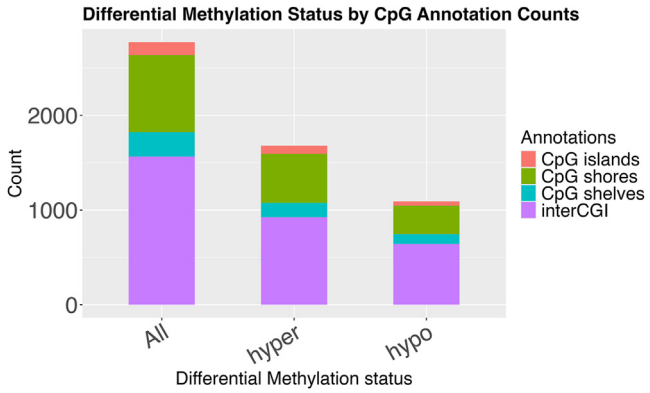

FIGURE 2 Annotation of CpGs according to their locations in the context of CpG islands. The bar plot depicts the distribution of DMCs across various genomic regions (CpG islands, shores, shelves, and inter-CGI) based on their methylation status. The *y*-axis represents the count of CpG sites annotated to each genomic region. The *x*-axis categorizes the data into 3 groups: "all" represents the total number of differentially methylated CpG sites irrespective of their methylation status; "hyper" refers to CpG sites that exhibit increased methylation in the inhibitor-positive group compared with that in the inhibitor-negative group; "hypermethylation" refers to CpG sites with significantly higher methylation percentages in the inhibitor-positive group than that in the inhibitor-negative group, based on a predefined statistical threshold ($P < .05$, methylation difference > 5); "hypo" refers to CpG sites that exhibit decreased methylation in the inhibitor-positive group compared with that in the inhibitor-negative group; and "hypomethylation" refers to CpG sites with significantly lower methylation percentages in the inhibitor-positive group than in the inhibitor-negative group ($P < .05$, methylation difference < -5). CpG shores are defined as 2 Kb upstream/downstream from the ends of the CpG islands, CpG shelves are defined as another 2 Kb upstream/downstream of the farthest upstream/downstream limits of the CpG shores, and the remaining genomic regions make up the inter-CGI annotation. DMC, differentially methylated CpG sites.

inhibitors like genes associated with cytokine receptor binding, toll-like receptor binding, and cytokine receptor activity, although these associations were not statistically significant at the genomic level. This suggests that although DNA methylation may play a role in increasing the gene expression of genes that heighten the immune response, it is likely part of a larger network of factors contributing to inhibitor development. However, no clear mechanism or pathway has been determined.

Importantly, our study highlights the diversity of the patient population, which includes people of different ethnic origins and presents with different *F8* gene mutations. This diversity could be extremely important because genetic background plays a role in the modulation of the manifestation and severity of HA. However, the observed lack of distinct clustering based on geographic origin indicates that ethnicity might not be a predominant factor in influencing inhibitor development in our study population or it could be due to our small sample size to provide statistical power to address the possibility. While not predominant, such factors could potentially influence inhibitor susceptibility and warrant further investigation in larger, more diverse cohorts. Future studies with expanded sample sizes could help validate these findings and explore the role of genetic variability in greater detail.

In a study published by Liu et al. [21] in 2022 comparing gene expression and methylation levels between people with hemophilia A with and without inhibitors, it was found that differentially expressed genes were significantly enriched in immune response regulation, particularly involving pathways such as toll-like receptor and tumor necrosis factor signaling. Additionally, methylation analysis revealed lower DNA methylation status in certain gene regions in inhibitor-positive patients, potentially implicating a role for epigenetic modifications in inhibitor development [21]. However, in our study involving 111 patients, despite the lack of significant *Q* values in the analysis, enrichment analysis revealed several hypomethylated genes to be involved in crucial functions such as cytokine receptor binding and toll-like receptor binding, suggesting a potential role of these genes in inhibitor-positive patients.

The data in Tables 2 and 3 provide important insights into the molecular functions linked to hypermethylated and hypomethylated genes in inhibitor-positive samples, shedding light on potential immune system implications. Table 2 points to significant pathways like ubiquitin-protein transferase activity, ubiquitin-like protein transferase activity, and ubiquitin ligase activity, which are central to processes such as protein degradation, immune signaling, and maintaining cellular balance. Changes in these pathways could disrupt antigen processing and presentation, potentially impairing immune surveillance. Table 3 highlights pathways enriched in hypomethylated genes, including cytokine receptor binding, toll-like receptor binding, and PI3K regulation—all of which are critical for immune signaling, inflammatory responses, and communication between immune cells. Genes like *SOCS2*, *JAK1*, and *TLR9* within these pathways may reflect disruptions in immune regulation specific to inhibitor-positive samples. These findings provide a starting point for future studies to explore how such pathway alterations might contribute to immune system dysregulation, potentially leading to weakened immune responses or increased susceptibility to autoimmune issues. Investigating these pathways further, especially their roles in antigen presentation and immune activation, could help identify new therapeutic targets. Overall, the results underscore the importance of DNA methylation as a regulator of immune-related pathways in disease, offering valuable leads for clinical research.

Despite its contributions, this research is not without limitations. Our sample size, though modest, takes into consideration a lot of CpG sites that might have no role in inhibitor development, contributing to higher *Q* values and thereby increasing insignificance. A larger sample size might impact the generalizability of findings. Moreover, while our data provide a preview of DNA methylation patterns, a longitudinal study design of how methylation of DNA changes over time and in response to treatment could offer a better understanding as described by Verlinden et al. [22]. Additionally, since the primary FVIII immune response is T-cell dependent, focusing on peripheral blood leukocytes might not fully capture methylation patterns that could play a role in inhibitor development. DNA methylation study targeting T lymphocytes and B lymphocytes might

TABLE 2 Top 10 significant gene ontology molecular function of hypermethylated genes in inhibitor-positive samples as observed by Enrichr web-based tool.

Term	*P* value	*Q* value	Overlap_genes
ubiquitin-protein transferase activity (GO:0004842)	3.064843×10^{-9}	6.865249×10^{-7}	UBE2H, LRSAM1, TRIM41, UBE3D, CUL3, MIB2, TNFAIP3, LMO7, FBXL14,FBXL22, TRIM6, TRAF3, TRIM5, RNF182, UBE2O, RNF130, HERC6
Ubiquitin-Like Protein Transferase Activity (GO:0019787)	1.013918×10^{-8}	1.135588×10^{-6}	UBE2F, UBE2H, LRSAM1, CUL3, MIB2, TNFAIP3, LMO7, FBXL14, TRAF3, TRIM5, RNF182, UBE2O, RNF130
Ubiquitin-Protein Ligase Activity (GO:0061630)	1.469754×10^{-4}	1.071939×10^{-4}	LRSAM1, TRIM6, TRIM41, UBE3D, TRAF3, TRIM5, CUL3, MIB2, UBE2O, RNF130, FBXL22, HERC6
Ubiquitin-Like Protein Ligase Activity (GO:0061659)	1.914176×10^{-6}	1.071939×10^{-4}	LRSAM1, TRIM6, TRIM41, UBE3D, TRAF3, TRIM5, CUL3, MIB2, UBE2O, RNF130, FBXL22, HERC6
Phosphotyrosine Residue Binding (GO:0001784)	6.456476×10^{-6}	2.892501×10^{-4}	SYK, SLA, PIK3R2, GRB2, PIK3R1
Cytokine Receptor Activity (GO:0004896)	1.309272×10^{-5}	4.243176×10^{-4}	IFNAR2, IL1RL2, IL18RAP, CCR6, IL17RC, CRLF1
Protein Phosphorylated Amino Acid Binding (GO:0045309)	1.325992×10^{-5}	4.243176×10^{-4}	SYK, SLA, PIK3R2, GRB2, PIK3R1
Kinase Binding (GO:0019900)	3.121649×10^{-4}	8.740617×10^{-3}	IFNAR2, TRIM6, CDKN1A, STK11IP, TNIP2, TRIM5, CASP1, GRB2, PRKACA, AP2A2, PTPN2
Neurotrophin TRK Receptor Binding (GO:0005167)	4.528891×10^{-4}	1.014472×10^{-2}	GRB2, PIK3R1
Neurotrophin TRKA Receptor Binding (GO:0005168)	4.528891×10^{-4}	1.014472×10^{-2}	GRB2, PIK3R1

Tables 2 and 3 summarize the results of the enrichment analysis.

Term: The specific GO molecular function term describes the activity performed by gene products associated with the input gene list. Each term represents a molecular function enriched among the gene list provided.

P value: The statistical significance of the enrichment for the corresponding GO term. A lower *P* value indicates a stronger association between the input gene set and the GO term.

Q value: The false discovery rate–adjusted *P* value, which accounts for multiple testing correction.

Overlap_genes: The list of genes from the input dataset that overlap with the genes annotated to the specific GO molecular function term in the reference database. These are the genes contributing to the observed enrichment and are potentially involved in the processes described by the term.

GO, gene ontology; TRK, tropomyosin receptor kinase; TRKA, tropomyosin receptor kinase A.

provide a finer differential methylation pattern of immunoregulatory genes. Furthermore, while the study explores DNA methylation as a potential mechanism, it is important to recognize that other epigenetic modifications, such as histone modifications, could also contribute to the observed outcomes.

Concluding the study, although the observed associations were not genome-wide significant, differentially methylated sites associated with the immune system and hemostasis especially the hypomethylation of genes involved in cytokine receptor activities and toll-like receptor activity suggest the complexity of the underlying mechanisms and highlight the need for larger and more comprehensive investigations. It also indicates that patients with DNA methylation of genes involved in active immune response might be associated with the development of inhibitors against FVIII.

Acknowledging the limitations of our study, further research and a more comprehensive methodology are required to elucidate the intricate connections between genetics, epigenetics, and inhibitor development, potentially revealing novel avenues for intervention and personalized therapeutic approaches.

APPENDIX

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TABLE 3 Top 10 significant gene ontology molecular functions of hypomethylated genes in inhibitor-positive samples as observed by Enrichr web-based tool.

Term	*P* value	*Q* value	Overlap_genes
Growth hormone receptor binding (GO:0005131)	.000343	.022875	SOCS2, JAK1
Cytokine receptor binding (GO:0005126)	.000442	.022875	SOCS2, IL23R, TLR9, JAK1
Regulatory RNA binding (GO:0061980)	.000446	.022875	TRIM71, HNRNPA2B1, TLR9
Toll-like receptor binding (GO:0035325)	.000669	.025771	TLR1, TOLLIP
1-Phosphatidylinositol-3-kinase regulator activity (GO:0043274)	.001100	.032500	SOCS2, SOCS3
Phospholipase binding (GO:0043274)	.001266	.032500	BLNK, FYN
Phosphatidylinositol-3-kinase regulator activity (GO:0035014)	.001633	.035274	SOCS2, SOCS3
Hormone receptor binding (GO:0051427)	.001832	.035274	SOCS2, JAK1
Cytokine receptor activity (GO:0004896)	.002504	.042839	IL23R, IFNLR1, CD44
Ubiquitin-conjugating enzyme binding (GO:0031624)	.004425	.058792	RNF144B, TOLLIP

Tables 2 and Table 3 summarize the results of the enrichment analysis.

Term: The specific GO molecular function term describes the activity performed by gene products associated with the input gene list. Each term represents a molecular function enriched among the gene list provided.

P value: The statistical significance of the enrichment for the corresponding GO term. A lower *P* value indicates a stronger association between the input gene set and the GO term.

Q value: The false discovery rate–adjusted *P* value, which accounts for multiple testing correction.

Overlap_genes: The list of genes from the input dataset that overlap with the genes annotated to the specific GO molecular function term in the reference database. These are the genes contributing to the observed enrichment and are potentially involved in the processes described by the term.

GO, gene ontology.

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ETHICS STATEMENT

The study was approved by the relevant medical ethics committee at each participating study center. Written informed consent was obtained from all parents or legal guardians of the patients. All procedures were conducted in accordance with the ethical standards of the institutional and national research committees and with the 1964 Declaration of Helsinki and its later amendments.

AUTHOR CONTRIBUTIONS

H.C., A.C., and S.H. were involved in the study design, sequencing, data analysis, and manuscript development. S.H., R.P., and F.P. designed and supervised the work. R.P. and F.P. critically revised the manuscript. All authors have read and approved the final manuscript.

RELATIONSHIP DISCLOSURE

F.P. is a member of advisory boards of Biomarin, Sanofi, Sobi, CSL Behring, and Roche. R.P. has received speaker fees from Novo Nordisk for an educational workshop. The other authors have no competing interests to disclose.

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