NOVEL APPROACHES TO STUDY FVIII INHIBITOR DEVELOPMENT IN INFANT PATIENTS: HEMOPHILIA INHIBITOR PUP STUDY

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INTRODUCTION

Neutralizing antibodies against FVIII (FVIII inhibitors) as the major challenge in the treatment of hemophilia A patients with FVIII products have been studied for many years. Despite progress in explaining the regulation of anti-FVIII immune responses in experimental animal models, the regulation of FVIII inhibitor development in patients is still poorly understood. The objective of this study was to establish a comprehensive set of technologies to detect new biomarkers that could provide further insight into the regulation of FVIII-specific immune responses in patients during early exposure days to FVIII, optimized for small blood volumes. This poster illustrates first "proof of principle data".

STUDY DESIGN

HIPS is an investigator-initiated, prospective clinical study funded by Baxter Healthcare Cooperation. The primary objective of this study is to monitor biomarkers of FVIII-specific immune responses within the first 50 exposure days to recombinant human FVIII (ADVATE®) in previously untreated patients (PUPs) suffering from severe Hemophilia A. Before first treatment and at regular intervals thereafter peripheral blood samples will be taken for immune monitoring (Table 1).

METHODS

Ig isotypes and IgG subclasses of FVIII-binding antibodies (Ab)
Multi-step analysis of Ab titers in citrated plasma is done using semi-quantitative ELISA assays for the detection of human FVIII-binding IgM, IgA, IgG1, IgG2, IgG3 and IgG4 [1].

Apparent affinity of FVIII-specific binding antibodies
Apparent affinity of FVIII-binding Ab in citrated plasma is assessed by ELISA based competition assays as described by Bobrovnik et al [2].

FVIII-specific CD4⁺ T cell signatures
PBMCs are isolated from citrated blood samples and *in vitro* restimulated with human recombinant FVIII (10µg/ml) for 6 hours. Activation of FVIII-specific memory CD4⁺ T cell responses is detected via analysis of gene expression patterns using microarray technology (Agilent). Data analysis is done using IPA® software (Ingenuity Systems).

Epigenetic assay for general immune status
Total T cells, Treg and Granulysin positive cells were assessed in EDTA blood using Q-PCR based epigenetic assay [3].

Patients and healthy blood donors
Peripheral blood samples were received after written informed consent from patients with severe hemophilia A and from healthy blood donors with local ethical committee approval.

RESULTS

FVIII-binding IgG4 Ab are exclusively found in patients with FVIII inhibitors
FVIII-binding antibodies are not only found in patients with FVIII inhibitors (HA-INH, 100%), but also in non-inhibitor patients (HA-noINH, 34%), patients after successful ITI (HA-ITI, 39%) and in healthy individuals (19%) (Figure 1). Ig isotypes and IgG subclass distributions differ between the different study cohorts.

Different apparent affinities of FVIII-specific Ab found in patients with and without FVIII inhibitors and in healthy individuals
FVIII-specific antibodies found in healthy individuals and in non-inhibitor patients are of medium or low apparent affinity, whereas patients with FVIII inhibitors express high apparent affinity Ab (Figure 2).

Gene expression patterns indicate involvement of Th17 cells in inhibitor formation
In vitro restimulated PBMCs of a FVIII inhibitor patient have a FVIII-specific induction of CD4⁺ T cell signature gene expression patterns indicating the potential involvement of Th17 cells in inhibitor pathophysiology (Figure 3).

Epigenetic markers are able to identify changes in immune status
Analysis of epigenetic markers reveal distinct features of the immune status (Figure 4).

DISCUSSION

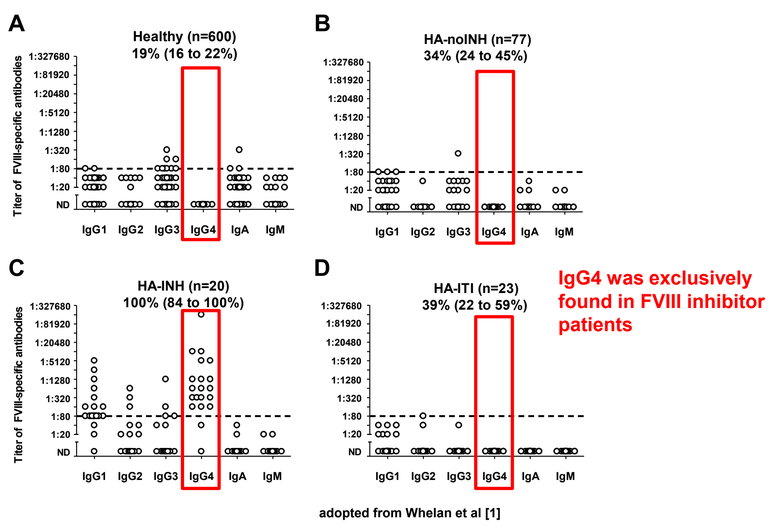
Recent evidence in the literature has suggested that Ab class switch from IgM to IgG or IgA can occur both, in the presence and absence of CD4⁺ T cell help (Figure 5) [4]. However, affinity maturation is still believed to require CD4⁺ T cell help.

Based on these literature data, we believe that the development of high-affinity, class-switched neutralizing antibodies (FVIII inhibitors) requires help by FVIII-specific CD4⁺ T cells, an assumption which is supported by our data (Figures 2 & 3). The FVIII-specific up-regulation of Th17 signature genes detected in the FVIII inhibitor patient supports data published by Ettinger et al. [5] and highlights the potential relevance of this CD4⁺ T cell subset in FVIII inhibitor development.

The role of IgG4 in FVIII inhibitor patients (Figure 2) [1] is interesting and gives rise to the question which regulatory pathways induce the development of FVIII-specific IgG4. Th2 cells and IL-10 producing regulatory T cells have been described to promote the differentiation of B cells to IgG4 producing plasma cells. It remains to be investigated if this also applies to the development of FVIII-specific IgG4.

Epigenetic markers for immune cells like total T cells, regulatory T cells and granolysin-producing killer cells are a useful tool to monitor patients immune status during a multi-center clinical study.

Figure 1: Ig isotypes and IgG subclasses of FVIII-binding Ab

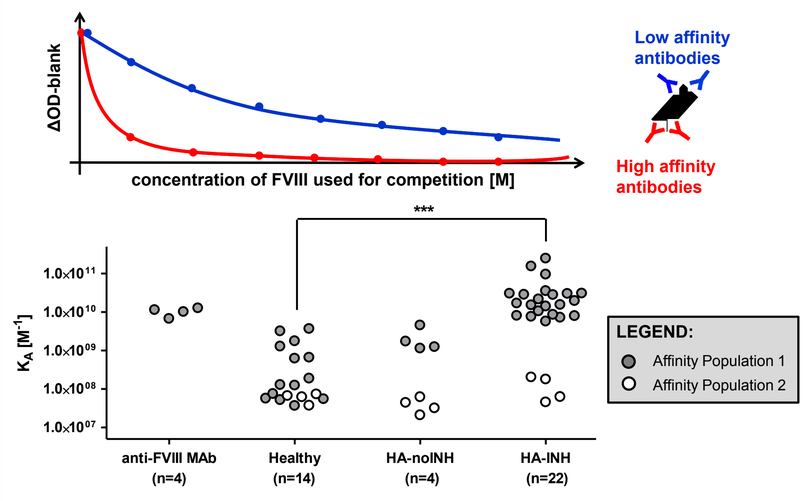


adopted from Whelan et al [1]

Table 1: Panel of HIPS laboratory assessments

Assessment	Pre-FVIII Exposure	Post-FVIII Exposure
FVIII activity	✓	
FVIII inhibitor		✓
CD4⁺ T-cell signatures and FVIII-binding Ab	✓	✓
General immune status	✓	✓
RNA expression profile of whole blood	✓	✓
Analysis of FVIII mutations and polymorphisms of specific genes		✓

Figure 2: Apparent affinity of FVIII-binding IgG



Cohort comparison via one way ANOVA and Bonferroni post test (p<0.001=***)

Figure 3: Example of a patient with FVIII inhibitors

Inhibitor [BU/ml]	FVIII-binding antibody titer						Apparent affinity [1/M]
	IgG1	IgG2	IgG3	IgG4	IgA	IgM	
1	1:40	-	-	1:40	-	-	IgG1: 68% 1.52E+10 32% 6.69E+07 IgG4: 100% 2.22E+11

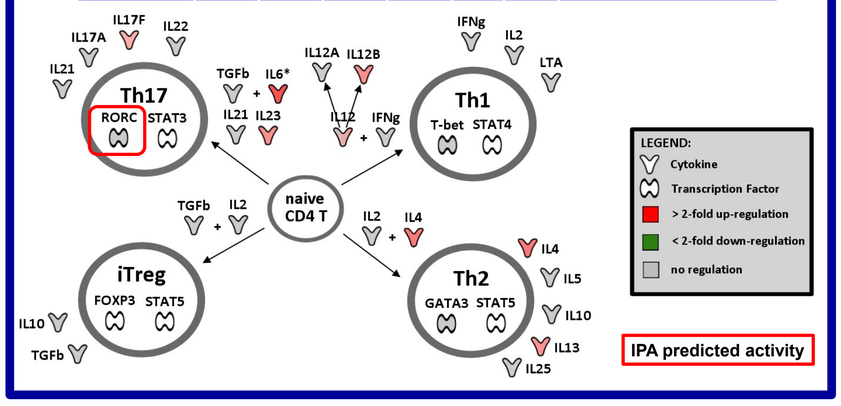
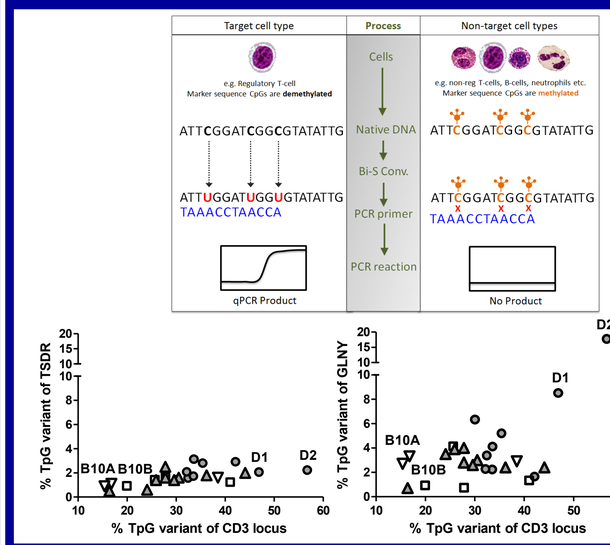
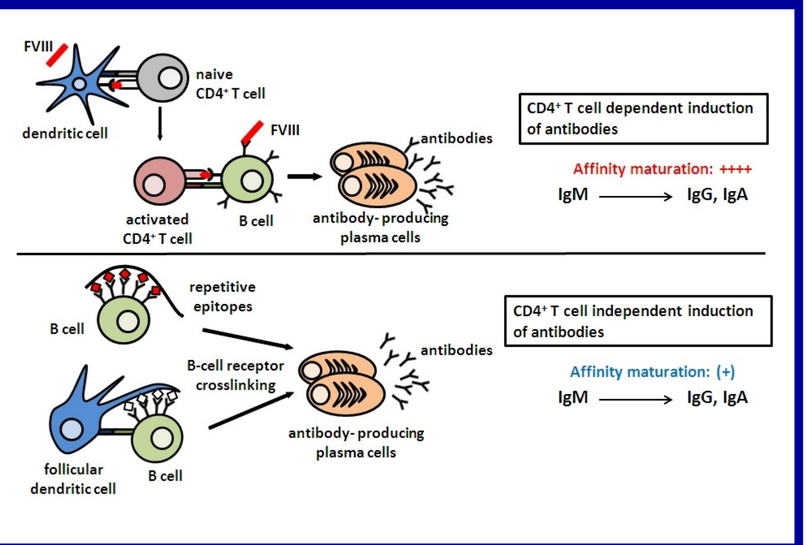


Figure 4: General immune status via epigenetic assays



Schematic figure courtesy of Epiontis GmbH

Figure 5: Regulation of FVIII-specific antibody responses



CONCLUSIONS & FUTURE OUTLOOK

We developed state-of-the-art ELISA technologies for the characterization of FVIII-binding antibodies, their Ig isotypes, IgG subclasses and apparent affinities. In addition, we established technology for detecting FVIII-specific gene expression patterns in *in vitro* restimulated PBMCs, indicating the direct and/or indirect activity of FVIII-specific memory CD4⁺ T cells.

This methodology to monitor FVIII-specific immune responses is part of the analytical approach in the upcoming HIPS clinical study. Epigenetic markers for immune cells help to identify patients with infections, which are regarded as a risk factor for FVIII inhibitor development.

We hope that the longitudinal data to be collected in this study will contribute to a better understanding of the immune systems decision whether or not to develop FVIII inhibitors.

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