200µL PBS on day 7. On day 4, mice received hydrodynamic injection of 50µg FVIII plasmid. Flow cytometry was used to characterize peripheral blood cell populations, while ELISA was used to assess inhibitor concentrations in plasma. At week 20, mice received a secondary hydrodynamic injection of 100µg FVIII plasmid.

Results: Mice receiving Mut. 24 showed a dramatic increase in the population of activated Tregs (Table 1). FVIII gene therapy via hydrodynamic injection resulted in high anti-FVIII inhibitor concentration in control mice, while mice treated with Mut. 24 produced low or non-existent inhibitor levels (Figure 1). This difference persisted after the second hydrodynamic injection.

Conclusions: Mut. 24 significantly increased and activated the Treg population in mice, which prevented formation of high-titer anti-FVIII antibodies when administered in coordination with FVIII gene therapy. This tolerance persisted over 6 months, even after a second administration of FVIII gene therapy, implying a potential method for induction of long term immune tolerance of FVIII.

TABLE 1 Characterization of lymphocytes 7 days after Mut. 24 (n=5) or PBS (n=4) injection

Treatment Group	% CD4+/ Lymphocytes	% CD25+Foxp 3+/ CD4+	% CTLA-4+Helios+/ CD4+CD25+Foxp3+
PBS	24.8	8.3	66.4
Mut. 24	25.5	18.9	78.4

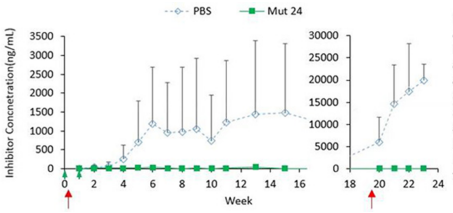

Figure 1. Anti-FVIII inhibitor concentration in blood serum measured by ELISA. On day 0 and 7, control mice (*n*=5) received injections of plain PBS, while experimental mice (*n*=8) received injections of 6µg and 3µg Mut. 24 in PBS, respectively (green arrows). Red arrows indicate dates of hydrodynamic FVIII plasmid injections. Note the axis break at week 17 to allow for higher values at later time points.

FIGURE 1 Anti-FVIII inhibitor concentration in blood serum measured by ELISA

OC 51.2 | High-throughput Immunoprofile Mimotope Variation Analysis in Previously Untreated Patients with Severe Haemophilia A before and after FVIII Exposure

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Background: In recent years, quantitative immunomics has developed rapidly, offering immune response analysis at high-throughput. Mimotope Variation Analysis (MVA) is a powerful tool to characterize the immune response profiles in a sample against millions of synthetic peptide antigens simultaneously.

Aims: To delineate immunoprofiles of patients with haemophilia A and to predict inhibitor occurrence upon response to therapy.

Methods: MVA was used to analyse plasma samples of 80 haemophilia A patients (37 with inhibitor and 43 without) from the SIPPET cohort. Plasma samples before and after treatment of each patient were analysed.

Results: The analysis of Top2500 peptide repertoire across individuals revealed a high dissimilarity in immunoprofiles derived from the pre- and post-treatment samples of the same individual. This dissimilarity suggested that immunoprofile differences might reflect upon the effects of the specific treatment product, environment or maturation of the immune system.

In the pre-treatment stage, clustering analysis of group-specific peptides selected from the entire dataset resulted in 59 motifs exclusive to patients who developed inhibitor and 747 motifs exclusive to patients who did not develop inhibitors. Logistic regression analysis resulted in the selection of 20 most relevant epitope motifs able to predict an inhibitor with 82.9% accuracy.

FVIII-specific immunoprofiles revealed immunogenicity/immunoreactivity "hotspots" in A1, A2 and C2 domains of FVIII protein regardless of the used FVIII product, confirming the dominant epitopes of FVIII from previous studies. Five FVIII epitope motifs were found to correctly classify all inhibitor cases with 85.4% accuracy in the post-treatment stage.

Inhibitor-positive patients treated with rFVIII displayed a much broader immune response targeting FVIII than those treated with pd-FVIII, whereas the situation was quite reverse concerning VWF.

Conclusions: MVA analysis exposed novel, unknown features of immune response in patients with haemophilia, that could provide predictive and prognostic parameters for inhibitor development at early stage of treatment.

OC 51.3 | Exploring Spontaneous Readthrough over Recurrent *F8* Nonsense Mutations: Potential Correlation with Inhibitor Risk?

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Background: Nonsense mutations, relatively frequent (>10%) in Hemophilia A (HA), are considered as "null conditions". However, they have been found also in moderate/mild HA patients and, as compared with large gene alterations, seem to be associated with lower inhibitor risk. This observation suggests residual factor VIII (FVIII) expression

levels that could arise from ribosome readthrough that would produce full-length proteins through mis-recognition of "leaky" nonsense triplets.

Aims: To investigate the occurrence of spontaneous readthrough over the most recurrent *F8* nonsense mutations.

Methods: Transient expression studies in HEK293 cells with the chimeric FVIII-GL protein, where FVIII is fused to the naturally-secreted Gaussia (GL) luciferase, and evaluation of luciferase activity in media and cell lysates.

Results: The FVIII-GL chimeric construct, bearing the selected nonsense mutations, was designed to release the GL moiety alone, to magnify the output arising from readthrough due to the luciferase high sensitivity. Appreciable luciferase activity was detectable in cell lysates from cells expressing all FVIII variants. Noticeably, analysis of media revealed three groups of mutations differing in the secreted luciferase activity, namely i) low (0.15-0.34% of wild-type FVIII-GL) such as the p.R15X (patients' number 14), p.R355X (27), p.R446X (22), p.R1715X (13) and p.R1985X (31), ii) medium (0.58-1.48%) such as the p.R602X (20),v p.2166X (34) and p.R814X (32) and iii) high (3.9-4.9%) such as the p.R1960X (20), p.R2135X (27), p.R2228X (41), and p.R2326X (34).

Conclusions: These preliminary data provide a pioneer indication that spontaneous readthrough occurs over all the investigated *F8* nonsense mutations and identify three classes of mutations. In turn, this would result in different levels of full-length FVIII in HA patients, which could influence the risk for immune response after therapy and help diagnosis and treatment. The relationship of these data with the presence of inhibitors is in progress.

OC 51.4 | A Dysbiotic Gut Microbiota Diminishes Short-Chain Fatty Acid Production and Increases Factor VIII Immunogenicity in Haemophilia A Mice

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Background: Anti-factor VIII antibodies develop in ~30% of haemophilia A (HA) patients however the basis of this adverse event is incompletely understood. The gut microbiota significantly influences the immune system but its mechanistic action on the anti-FVIII immune response remains unresolved.

Aims: To determine the mechanistic basis of changes to the anti-FVIII immune response documented in association with the gut microbiota.

Methods: A microbiologically-confirmed model of gut dysbiosis has been established in HA mice through oral administration of ampicillin. Ampicillin-administered and control mice were given 50 ng of recombinant FVIII biweekly for two weeks and sacrificed two weeks later. Anti-FVIII antibodies were quantified by ELISA and Bethesda assay. Caecal contents harvested before FVIII challenging were sequenced for 16s rRNA or analyzed via nuclear magnetic resonance for short-chain fatty acid (SCFA) quantification. Splenic lysates were analyzed for cytokine profiles and spleens and mesenteric lymph-nodes were evaluated for immune cell frequencies by flow cytometry.

Results: There was no difference in the incidence of anti-FVIII antibodies between ampicillin and control mice, but ampicillin-treated mice produced significantly higher titres of total anti-FVIII IgG (P=0.0023). Elucidation of anti-FVIII IgG antibody subclasses revealed elevated titres of IgG1 (P=0.002), IgG2a (P=0.0003), and IgG2b (P< 0.0001) but not IgG3. Anti-FVIII inhibitory antibodies were also elevated in this group (P< 0.0001). SCFA investigation revealed that prior to FVIII challenging, ampicillin-treated mice had a 1.6-, 1.3-, and 8.1-fold reduction in butyrate, acetate, and propionate (P=0.0035, P=0.0010, and P< 0.0001, respectively) in the caecum. Between both study groups, there were no significant differences in either splenic cytokine profiles nor frequencies of $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^-LAP^+$ T lymphocytes or $B220^+$ B lymphocytes before FVIII challenging.

Conclusions: Significant reductions in immunomodulatory SCFAs are seen in association with the dysbiotic gut microbiota of HA mice. Diminished SCFA levels precede FVIII exposure and are associated with increased FVIII immunogenicity.

OC 51.5 | Recombinant Canine and Human FXa-I16LProvide Safe and Efficacious Hemostasis during Major Bleeding Events in Hemophilia A Dogs with High-titer Inhibitory Anti-FVIII Antibodies

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Background: Recombinant FXa-I16L (Ile16→Leu) is a zymogen-like variant of activated factor X (FXa) that shows enhanced resistance to inactivation by endogenous inhibitors as compared with wild-type FXa. It corrected the hemophilic coagulopathy in animal models and was safe in normal subjects (Haemophilia 2012;18:87; JTH 2017;15:931) but has not been tested in the presence of high titer, circulating anti-FVIII inhibitory antibodies.

Aims: To determine the safety and hemostatic efficacy of FXa-I16L in hemophilia A dogs with high titer Bethesda Inhibitors during major bleeds.

Methods: Recombinant canine and human FXa-I16L were produced in HEK293 cells and purified by column chromatography. Hemophilia A dogs with an intron 22 inversion (n=5) or a multi-exon deletion (n=1, exons 22-25 deleted) with Bethesda Inhibitors ranging from 6.5 to > 200 BU received canine and/or human FXa-I16L on demand to treat spontaneous muscle, throat, nose, and central