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Pharmacological analysis of liver-directed AAV-fVIII gene therapy and anti-fVIII inhibitor incidence in a preclinical model of hemophilia A

By

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Doctor of Philosophy
Master of Science in Clinical Research

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An abstract of a dissertation submitted to the Faculty of the
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Abstract

Pharmacological analysis of liver-directed AAV-fVIII gene therapy and anti-fVIII inhibitor incidence in a preclinical model of hemophilia A

By Taran Shea Lundgren

Hemophilia A is an X-linked, monogenic bleeding disorder caused by mutations in the *F8* gene leading to defective or deficient production of coagulation factor VIII (fVIII). Hemophilia A is estimated to occur in 1 of 4,000 males globally and approximately 1 in 5,000 males in the United States. 10 in 100,000 of these males have the severe phenotype of this disease, bringing with it severe morbidity and mortality risks and major impacts to quality of life. Those patients that develop anti-fVIII neutralizing antibodies (inhibitors) to their fVIII replacement protein therapeutic incur similar risks as those of untreated patients along with the increased cost of non-fVIII alternative therapeutics and immune tolerance induction strategies, which are not always successful. While the immune response to exogenous fVIII replacement therapy remains poorly understood, even less clarity exists regarding the immune response to fVIII after adeno-associated viral (AAV) vector gene therapy delivery encoding functional fVIII (AAV-fVIII) for endogenous expression by transduced cells. Most preclinical programs do not include formal immunogenicity testing and no previously untreated patients (PUPs) have received AAV-fVIII.

The goal of the current project is to define the mechanisms and parameters that govern the anti-fVIII inhibitor response following AAV-fVIII gene therapy and help guide the development of safe and effective AAV gene therapies. Animal models of disease remain our premiere resource for preclinical candidate evaluation prior to clinical testing, and this study utilizes an established murine model of hemophilia A to evaluate the efficacy and transgene product immunogenicity of four AAV-fVIII vectors over a range of clinically relevant doses. The results provide a pharmacokinetic model of fVIII protein product expression kinetics and anti-fVIII inhibitor outcomes providing reliable predictions of immunogenicity risk and efficacy associated with vector potency and dose. This study provides a platform for future investigations of mechanisms of immune tolerance to fVIII following gene therapy, for testing strategies to promote tolerance, and for further establishment of frameworks that are appropriate for development of genetic medicines in order to more properly assess their safety and efficacy. Greater understanding of these areas is critical for the potential of AAV-fVIII gene therapy to reach the entire hemophilia A patient population and for progress in the field of gene therapy in general.

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List of Abbreviations

AAV	adeno-associated viral (vector)
ADA	anti-drug antibodies
ADME	absorption, distribution, metabolism, excretion (pharmacokinetic parameters)
AICD	antigen induced cell death
An53	ancestral sequence 53 fVIII
APC	activated protein C
APC	antigen presenting cell
aPCC	activated prothrombin complex concentrate
ARI	attributable risk increase
ASH	American Society of Hematology
ASR	ancestral sequence reconstruction
AT	antithrombin
AUC_{fVIII}	total plasma fVIII protein exposure/production
BAR	B cell antibody receptor
BCR	B cell receptor
BDD	B-domain-deleted (within the fVIII protein)
BHK	baby hamster kidney cell
bp	base pairs
Breg	regulatory B cell
BU	Bethesda assay units (functional fVIII inhibition)
C	concentration
C_0	initial concentration
Ca	Calcium
CAR	chimeric antigen receptor
CDC	Centers for Disease Control
cDNA	complementary DNA
CDR	complementarity determining region
cfVIII	canine fVIII
CHAMP	CDC Hemophilia A Mutation Project
CHO	Chinese hamster ovary cell
CL	clearance
CRM	cross-reactive material
cryo-EM	cryogenic electron microscopy
CSW	class-switched
CTL	cytotoxic T lymphocyte
Cu	Copper
DC	dendritic cell
DNA	deoxyribonucleic acid
E16 (-/-)	exon 16 disrupted (mice)

ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ET3	human-porcine chimeric fVIII
ET3i	recombinant, purified ET3 protein
Expi293F	high expression human cell line derived from HEK 293 cells
F8	human factor VIII gene
Fab	antigen-binding fragment
FACT	human factor assay control plasma
Fc	fragment crystallizable region
FcR	Fc receptor
FcRn	neonatal Fc receptor
FcγR	Fc gamma receptor
FDA	Food and Drug Administration
FFP	fresh frozen plasma
fI	coagulation factor I (fibrinogen)
fIa	activated factor I (fibrin)
fII	coagulation factor II (prothrombin)
fIIa	activated factor II (thrombin)
fIX	coagulation factor IX
fIXa	activated coagulation factor IX
FLUC	firefly luciferase
fV	coagulation factor V
fVa	activated factor V
fVII	coagulation factor VII
fVIIa	activated factor VII
fVIII	coagulation factor VIII
fVIIIa	activated factor VIII
fX	coagulation factor X
fX	coagulation factor X
fXa	activated coagulation factor X
fXa	activated factor X
fXIII	coagulation factor XIII
GC	germinal center
GFP	green fluorescent protein
GMA	Green Mountain Antibodies
HEK	human embryonic kidney cell
HEL	human erythroleukemia cell line
hfVIII	human factor VIII (specifically)
HLA	human leukocyte antigen
HLP	hybrid liver-specific promoter
HSPC	hematopoietic stem and progenitor cell
HSQ	B-domain-deleted human fVIII
HUH-7	immortalized human cell line derived from hepatoma (abnormal liver) tissue

IC	immune complex
ID_{50}	dose at 50% inhibitor development
IgG	immunoglobulin G
IgH	immunoglobulin heavy chain
IgL	immunoglobulin light chain
IgM	immunoglobulin M
IND	investigational new drug
ITAM	immunoreceptor-based activation motifs
ITI	immune tolerance induction
ITR	inverted terminal repeat
iTreg	inducible Treg
IU	International Units (for fVIII activity)
k	rate constant
k_0	general exposure/production rate of a drug/molecule
k_e	elimination rate constant
k_{fVIII}	rate of production of, or plasma exposure to, fVIII protein
LLPC	long-lived plasma cell
LSEC	liver sinusoidal endothelial cell
LV	lentiviral (vector)
(c)-MPL	thrombopoietin receptor
MBC	memory B cell
MHC	major histocompatibility complex
mRNA	messenger RNA
NAB	neutralizing antibody (to the AAV capsid)
NHP	non-human primates
NIH	National Institutes of Health
NSG	NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NOD-scid) immunodeficient mice
nt	nucleotides
nTreg	natural Treg
OVA	ovalbumin
P2A	ribosomal cleavage skipping amino acid sequence
PD	pharmacodynamics
PEG	polyethylene glycol
pfVIII	porcine fVIII
PK	pharmacokinetics
PTM	post-translational modification
PUP	previously untreated patient
(RT)-qPCR	(reverse transcriptase) quantitative polymerase chain reaction
rfVIIIa	recombinant activated fVIII
RNA	ribonucleic acid
RR	risk ratio
scFv	single-chain variable fragment

SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SLPC	short-lived plasma cell
STAT5	signal transducer and activator of transcription 5
t	time (as in a timepoint or time interval)
$T_{1/2}$	half-life
TCR	T cell receptor
TF	tissue factor
TFPI	tissue factor pathway inhibitor
TKO	total F8 knock-out
TPO	thrombopoietin
Treg	regulatory T cell
TTR	transthyretin
UPR	unfolded protein response
VCN	vector genome copy number
V_d	volume of distribution
vg	vector genomes
vWF	von Willebrand factor
Xase	tenase complex
XTEN	a bioengineered flexible 16 amino acid linker
Zn	Zinc

Chapter 1

Introduction

1.1 Coagulation Factor VIII (fVIII)

1.1.1 FVIII: the protein

The factor VIII gene (*F8*) is 186,000 base pairs (bp) long and generates an mRNA molecule that is 9,048 nucleotides (nt). F8 was the longest gene ever cloned when scientists from a collaboration between the Royal Free Hospital in England and the biotech company Genentech in the United States first achieved it in 1984. The encoded factor VIII protein (fVIII) sequence is 2,351 amino acids long with six domains identical in designation to coagulation factor V (fV): A1-A2-B-ap-A3-C1-C2. The mature 2,332 amino acid fVIII protein is generated after excision of an N-terminal 19 amino acid signaling peptide that traffics fVIII into the conventional secretory pathway. The synthesis and secretion of fVIII is a complicated process requiring interaction with multiple chaperone proteins.[1-3] FVIII is cleaved by the intracellular trans-Golgi convertase furin at R-1313 and/or R1648 to form a heterodimer composed of one heavy chain (A1-A2-B-domain) and one light chain (A3-C1-C2). FVIII circulates mostly in this heterodimer form, which remains associated due to non-covalent and metal ion-dependent interactions (Fig. 1.1).[4] The A domains share homology with ceruloplasmin and participate in metal ion binding, with the A1 and A3 domains mainly binding Zn^{2+} and Cu^{+}, respectively. The C domains share homology with discoidin/milk-fat globule-binding proteins and participate in lipid binding. The B domain serves no known functional role and fVIII recombinant proteins have indeed been shown to retain full coagulation function after deletion of the B domain.

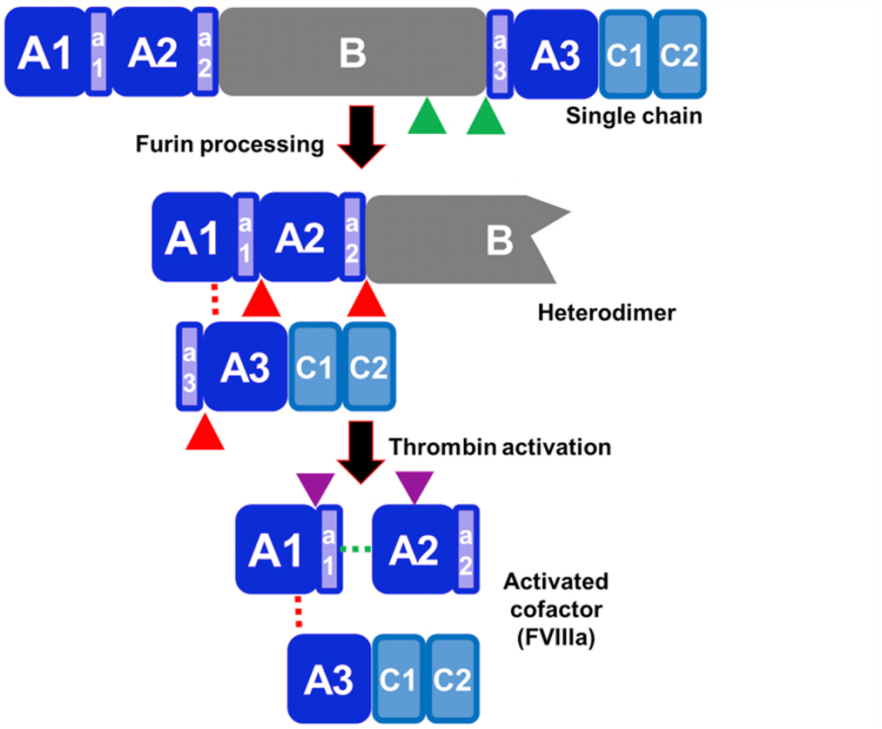

Figure 1.1 The domain structure and activation of fVIII.
FVIII protein is initially produced as a single chain with the domain structure: A1-A2-B-ap-A3-C1-C2, which is then intracellularly converted to the heterodimer form via furin cleavage. FVIII circulates mostly as this heterodimer with various additional cleavages in the B domain. After initiation of the coagulation cascade, fVIII becomes activated (fVIIIa) via cleavage by thrombin, creating a heterotrimeric fVIII wherein the domains remain associated by non-covalent interactions. [This figure was reprinted with permission from the American Society for Gene & Cell Therapy and Elsevier SD Cell Press (CC BY-NC-ND 4.0): Samelson-Jones BJ, Arruda VR. Protein-Engineered Coagulation Factors for Hemophilia Gene Therapy. Mol Ther Methods Clin Dev. 2018 Dec 31;12:184-201. doi: 10.1016/j.omtm.2018.12.007. PMID: 30705923; PMCID: PMC6349562.]

Endogenous production of fVIII occurs in liver sinusoidal endothelial cells (LSECs) that line the vessels composing the network of liver sinusoids, or capillary beds, through which blood is filtered in the liver. Once fVIII is secreted into the bloodstream, it binds to its chaperone protein, von Willibrand Factor (vWF). The high-affinity interaction (k_d = 0.2 – 0.5 nM) occurs between the acidic a3, C1, and C2 regions of fVIII and the N-terminal D'-D3 region of vWF once the propeptide (vWF D1-D2) has been cleaved. vWF circulates at 50 nM, which is 50 M excess to fVIII circulating at 1 nM. However, normal physiological levels of vWF and fVIII are each defined

as 1 IU/mL, and hence they circulate at a 1:1 ratio even though the molar concentrations are so vastly different. About 2 – 5% of fVIII circulates as free protein (i.e., not bound to vWF).[5-14] The interaction of fVIII with vWF serves multiple functions in maintaining normal hemostasis and clot formation after injury. Firstly, vWF is critical for maintaining normal pharmacokinetics of fVIII in circulation by regulating stability and clearance. The half-life of fVIII bound to vWF is approximately 12 hours in human patients; however, without vWF, the half-life of fVIII drops drastically to only about 2.5 hours.[15] One way that vWF helps maintain the longer half-life is by stabilizing the fVIII heterodimer structure, wherein binding of vWF to the fVIII light chain facilitates association of the fVIII heavy and light chains.[16-19] Another way is by protecting fVIII from cleavage by serine proteases other than thrombin, such as fXa, APC and fIXa, which are also involved in coagulation and could aberrantly activate or inactivate fVIII. This protection is mediated by vWF blocking fVIII binding to phospholipids or platelets as well as directly blocking protease binding sites on the fVIII light chain.[5] Free fVIII is also cleared at a much higher rate than fVIII bound to vWF. There are multiple receptors in the liver and spleen that have been investigated for their role in fVIII clearance.[20-27] Additionally, these receptors may differ in their preference for clearance of free fVIII vs. bound fVIII vs. fVIIIa, with fluctuations in the pool of free fVIII directly affecting the amount of bound fVIII.[28,29]

1.1.2 FVIII in hemostasis and hemophilia A

The primary function of fVIII is to maintain hemostasis as a key component of the blood coagulation signaling cascade triggered after injury (Fig. 1.2).

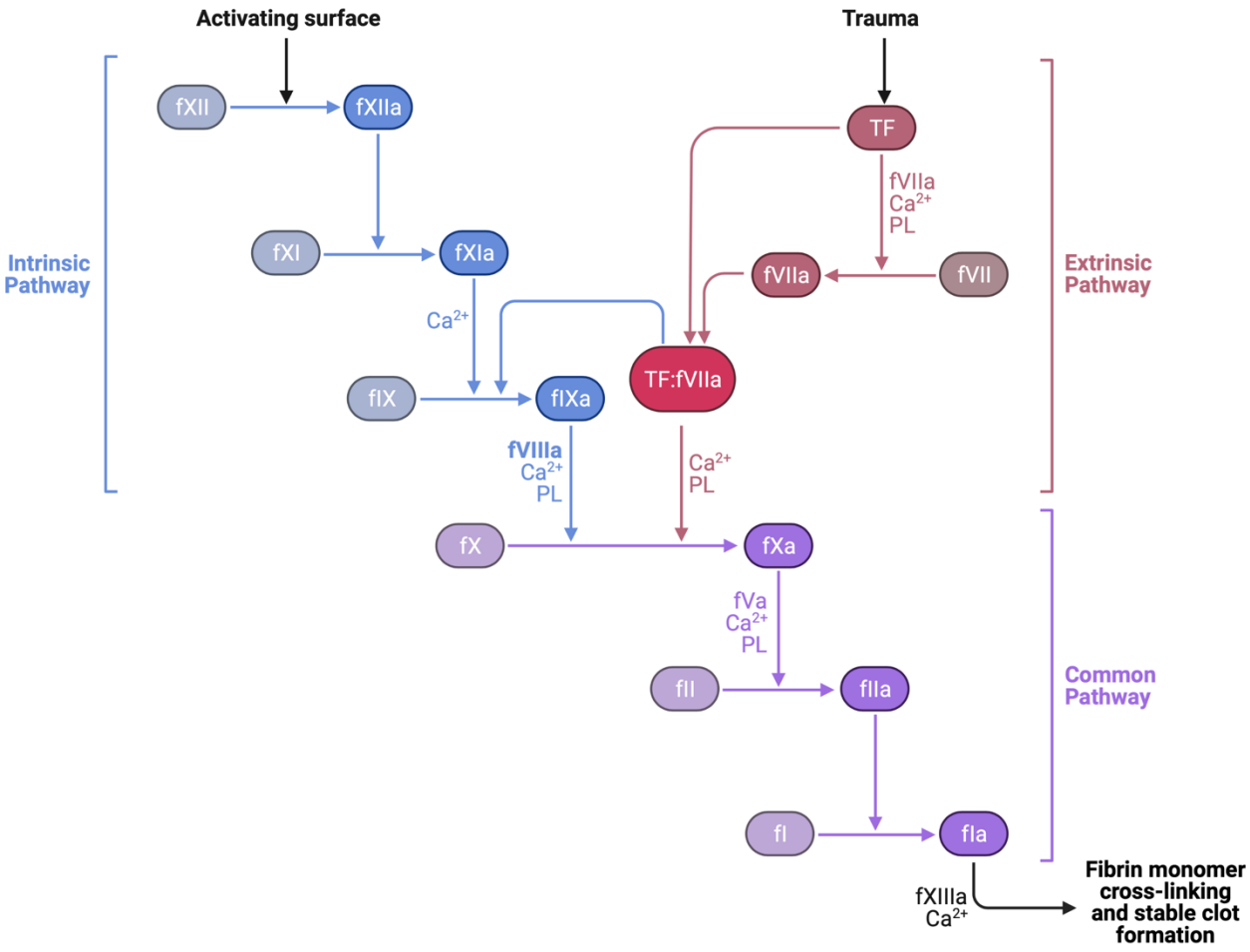

Figure 1.2 The coagulation cascade.

There are three main pathways that participate in the overall coagulation cascade responsible for formation of a stable blood clot. Most participants in the cascade circulate as zymogens and the signaling proceeds via a series of cleavage events by these serine proteases. However, tissue factor (TF), fV and fVIII are glycoproteins that participate as cofactors in complex formation and fXIII is a transglutaminase. The coagulation pathways are primarily initiated via the extrinsic (or tissue factor) pathway following injury to blood vessel walls. The intrinsic and extrinsic pathways converge on formation of activated FX, which occurs via the prothrombinase complex from the extrinsic pathway and the tenase complex from the intrinsic pathway, in which fVIII participates. The common result (i.e., the common pathway) is formation of a fibrin clot. [This figure was created using Biorender.com]

FVIII is a coenzyme that localizes to the site of injury with the help of vWF, which binds the subendothelial matrix at that site to mediate platelet adhesion. FVIII is activated via cleavage by thrombin (fIIa) at residues R-372, R-740, and R-1689. This heterotrimeric fVIIIa dissociates from

vWF to then associate with activated factor IXa (fIXa) on the platelet surface and form the tenase (Xase) complex, a process which requires access to phospholipids on the activate platelet surface and calcium ions (Ca^{2+}). FVIIIa increases the catalytic activity of fIXa by several orders of magnitude. The tenase complex potentiates the generation of fXa, which subsequently leads to generation of more thrombin (Fig. 1.3). Activated thrombin cleaves fibrinogen for fibrin clot formation. FVIIIa is inactivated via proteolytic cleavage (usually by activated protein C, APC), or the A2 domain spontaneously dissociates.[4]

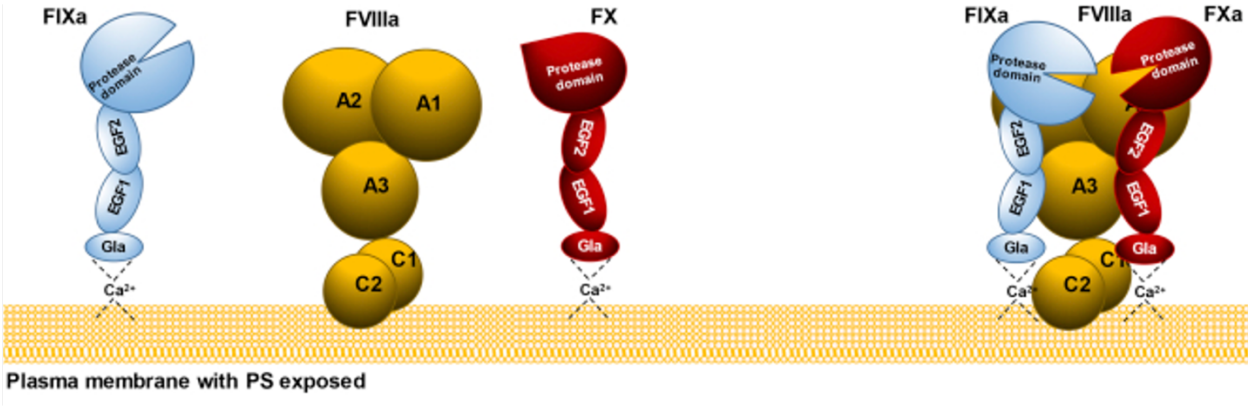

Figure 1.3 Formation of the tenase complex.
Activated fVIII (fVIIIa) interacts with the activated serine protease fIXa and the zymogen fX to form the tenase complex. This interaction allows fIXa to activate fX, with the catalytic efficiency of this activation greatly augmented by the presence of the fVIIIa cofactor. [This figure was reprinted with permission from the American Society for Gene & Cell Therapy and Elsevier SD Cell Press (CC BY-NC-ND 4.0): Butterfield JSS, Hege KM, Herzog RW, Kaczmarek R. A Molecular Revolution in the Treatment of Hemophilia. Mol Ther. 2020 Apr 8;28(4):997-1015. doi: 10.1016/j.ymthe.2019.11.006. Epub 2019 Nov 13. PMID: 31843450; PMCID: PMC7132613.]

Hemophilia A is an X-linked, monogenic bleeding disorder resulting from genetic defects at chromosome position Xq28 that lead to defective or deficient fVIII production. There are currently 2,537 mutations in the *F8* gene reported to cause hemophilia A according to the CDC Hemophilia A Mutation Project, or CHAMP (https://www.cdc.gov/ncbddd/hemophilia/champs.html).

These genetic alterations occur by multiple mechanisms, including deletion, duplication, insertion, insertion and deletion, inversion, and substitution.[30] An individual is diagnosed with hemophilia A when their circulating fVIII levels fall below 50% of normal, and severity of the disorder phenotype is categorized based on the percent of functional fVIII remaining: <1% of normal is severe, 1 – 5% of normal is moderate, and 5 – 49% of normal is mild hemophilia A. It is estimated that 40% of severe hemophilia A cases are caused by inversions in the *F8* gene, with substitutions being the second most common type of gene mutation. As the fVIII levels decrease (i.e., disease severity increases), patients have increased risk of morbidity and mortality following a major injury or trauma. Indeed, many patients in the mild hemophilia A category are not diagnosed until such an event or surgery occurs. Patients with the moderate phenotype tend to have multiple bleeding episodes following a significant injury, trauma, or surgery. However, patients with the severe phenotype have frequent spontaneous hemorrhagic episodes, ranging from microbleeds during normal activity to life-threatening bleeds after significant injury or trauma. Bleeding often occurs into joints and muscles, causing permanent damage over time, but bleeds can also occur in other locations like the brain. If left untreated, severe hemophilia A is usually fatal by young adulthood. The current estimate of global hemophilia A prevalence at birth is 1 in 4,000 males (approximately 1 in 5,000 in the United States.), with 10 of 100,000 males having the severe phenotype. This currently results in over 1.1 million males living with hemophilia A worldwide and over 400,000 of those estimated to be severe cases, many of which are undiagnosed and going untreated. In addition to being the most common severe congenital bleeding disorder, the life expectancy disadvantage for patients living in high-income countries is 30% for hemophilia A and 37% for severe hemophilia A.[31]

1.2 Treating hemophilia A

1.2.1 Advances in fVIII protein replacement therapeutics

While severe hemophilia A is generally uniformly fatal, luckily only small increases in fVIII levels are required to significantly improve the disease phenotype and associated morbidity/mortality risk. However, before discovery of the fVIII protein and sequencing of the F8 gene, treatment options still resulted in high mortality rates and a life expectancy of about 30 years. Healthy donor fresh frozen plasma (FFP) was needed in such large volumes that, if it was even available, it often only partially corrected the bleeding phenotype. The situation improved in 1965 with the discovery that cryoprecipitate from matched whole blood that could be stored in a freezer for on-demand use and increased treatment potency by 5 – 20-fold when properly administered. The 1970s and 80s were characterized by the discovery and purification of the fVIII and vWF proteins. But the first major advancements in recombinant fVIII protein engineering and drug development resulted from the cloning and sequencing of the F8 gene in 1984. Initially, recombinant fVIII proteins were co-produced with vWF and the vWF was subsequently removed, and first recombinant fVIII products brought to market in the early 1990s were produced in Chinese hamster ovary (CHO) and baby hamster kidney (BHK) cells. Recombinate was produced by the Genetics Institute in Boston with Baxter in 1992, and Kogenate was produced by Bayer (previously Genentech) in 1993. The initial recombinant full-length fVIII preparations were made with human or bovine serum albumin plus other animal-derived cell culture supplements. But as fVIII drug development evolved, manufacturing also moved from production in animal cell lines with animal or human protein supplements to production in human cell lines with no protein supplements.[32]

The 21st century would bring with it great effort in developing recombinant fVIII products. Prophylactic dosing of recombinant fVIII protein therapeutics is historically based on units of fVIII activity per kg body weight (IU/kg) and the severity of the patient's hemophilia A phenotype, which determines the units of fVIII required to bring fVIII activity to safe levels (>50% of normal). While the half-life of human fVIII bound to vWF is around 12 hours, and most recombinant fVIII products have a half-life of around 8-12 hours, the actual pharmacokinetics (PK) of recombinant hfVIII and other engineered fVIII protein products varies widely between individual hemophilia A patients, with half-lives ranging between 6 – 25 hours.[33-36] This presents a problem when dosing based only on body weight and disease severity, which led to more personalized dosing based upon the half-life of the fVIII product and the tolerability of troughs in fVIII activity, with critical levels considered to be between 1 – 3% of normal (0.01 – 0.03 IU/mL). FVIII PK has a known association with levels of vWF antigen and age; however, a recent study has concluded that fVIII PK is predominantly based on vWF clearance from circulation and fVIII-vWF binding.[37-40] FVIII is also highly immunogenic and, like all protein therapeutics, brings with it the risk of developing a humoral immune response to the treatment. Bioengineering approaches to fVIII therapeutic development have looked to improve the pharmacological properties of fVIII, such as improving its biosynthetic efficiency, increasing the half-life, and decreasing its immunogenicity.

The first B domain deleted (BDD) fVIII product (Refacto) was developed in the year 2000 and produced in CHO cells. While the B domain deletion had no effect on the fVIII PK or immunogenicity, it did increase protein production efficiency. Refacto was replaced by Xyntha in 2008, with the improvement being that it no longer used albumin in the cell culture production process. Over the next several years, multiple recombinant fVIII products were approved for use by the FDA, both B domain modified and full-length. One of these, Nuwiq, was the first approved

fVIII product to be produced in a human cell line (human embryonic kidney cells, HEK). Nuwiq has a longer half-life (mean 17.1 hours, median 13.7 hours) due to differences in post-translational modifications that improved binding to vWF. Production in human cell lines also removed N-linked glycans that could contribute to increased immunogenicity risk. In 2016, another novel recombinant fVIII product was developed called Afstyla, which was expressed in CHO cells. Deletions in the B domain and a3 domain of this fVIII protein led to creation of a unique N-linked glycosylation site at the junction of the heavy and light chains, resulting in fVIII expression as a single chain. Once activated, Afstyla is identical to endogenous activated fVIII, and it demonstrated improved vWF binding and stability in storage. However, there was only a slight improvement in half-life (mean 14.5 hours).

Standard recombinant fVIII products usually require dosing every 2 – 3 days, which is a significant burden to the patient, increases cost, and increases morbidity/mortality risks from noncompliance. Therefore, there has been great effort to bioengineer fVIII proteins with increased half-lives. One method is to create fusion proteins with the IgG Fc domain. This strategy looks to take advantage of the neonatal Fc receptor (FcRn) that recycles IgG back into circulation, giving it a half-life of 21 days. However, this approach has not been very successful. One such product approved by the FDA is a BDD-fVIII-Fc fusion called Eloctate, and it has a half-life of 19 hours. Another strategy is to attach polyethylene glycol (PEG) to the protein. Many fVIII-PEG products did not make it to clinical trials due to interference with the clotting activity of fVIII. The ones that did show a similarly modest increase in half-life as the Fc fusion proteins, with mean half-lives of 15 – 19 hours.[41] FVIII PEGylated products currently used in treatment include Adynovate, Esperoct, and Jivi, and they are recommended for administration every 3 – 7 days depending on the individual patient.[42] BIVV001 is a fVIII product in clinical testing that attaches the D'D3

domain of vWF to fVIII via two IgG Fc domains and two XTEN polypeptide linkers. The rationale behind this design is to take advantage of the extended half-life potential of an Fc domain without the restrictions on half-life imposed by clearance of vWF. Preclinical evaluation of BIVV001 in mice and non-human primates resulted in half-lives of 25 – 31 hours and 33 – 34 hours, respectively.[43] Phase 1 and 2 clinical trials of BIVV001 have thus far shown a half-life of 37.6 hours with an average of 5% fVIII activity remaining on day 7 post-infusion. A phase 3 trial for efficacy and safety in both adult and adolescent patients with severe hemophilia A is ongoing.[42]

1.2.2 Non-fVIII alternatives for hemophilia A treatment

Alternative treatments are being developed to control or treat bleeding in hemophilia A patients when it can no longer be managed with fVIII replacement, usually due to a pathogenic immune response against the recombinant fVIII protein (Table 1.1 and Fig. 1.4).[4]

Protein	Variants	Mechanism	Development Stage
fVIIa			
VEAY	L305V S314E K337A F347Y	10-fold increase in fX activation due to an optimized active site	Preclinical efficacy in mice
DVQ, NN1731, vatreptacog alfa	V158D E296V M298Q	50-fold increase in fX activation due to partial mimicking of the TF-bound conformation	11% (n = 8) of subjects in a phase 3 clinical study developed ADA
BAY86-6150	P10Q K32E A34E R36E T106N V253N	P10Q shows increased affinity for activated membranes K32E has a prolonged half-life	10% (n = 1) of subjects in a phase 3 clinical study developed ADA
marzeptacog alfa	T128N P129A Q286R M298Q	T128N shows a 7-fold increase in fX activation P129 has a prolonged half-life	A phase 2 study is underway for subcutaneous delivery
fVa			
^{SUPER}fVa	H609C E1691C R306Q R506Q R679Q	H609C has an engineered disulfide bond between the A2 and A3 domains E1691C possesses APC resistance	Preclinical efficacy in mice
fXa			
zymogen-like fXa	I195L	Extends the biological half-life of activated protease by limiting inhibition by serpins	Early-phase clinical studies
A1AT			
KRK Serpin PC	P357K M358R S359K	P357K specifically and irreversibly inhibits APC	Preclinical efficacy in mice

Table 1.1 Non-fVIII alternatives to management of hemostasis in hemophilia A.
This table outlines current development of non-fVIII alternative protein therapeutics that participate the coagulation cascade in order to control bleeding in the context of hemophilia A. ADA: anti-drug antibodies, TF: tissue factor. FXa-I195L is typically referred to as I16L in the literature, but the numbering is given here based on the mature zymogen. [Adapted from: Samelson-Jones BJ, Arruda VR. Protein-Engineered Coagulation Factors for Hemophilia Gene Therapy. Mol Ther Methods Clin Dev. 2018 Dec 31;12:184-201. doi: 10.1016/j.omtm.2018.12.007. PMID: 30705923; PMCID: PMC6349562.]

A relatively new therapeutic with significant efficacy in controlling bleeding that is approved for use in patients with and without inhibitors is the humanized bispecific monoclonal antibody-based fVIII mimetic, emicizumab (Hemlibra, Genentech/Roche). This antibody serves as a bridge between fIXa and fX, bypassing the need for fVIII to form the tenase complex. Emicizumab can be delivered every 7, 14, or 28 days. Another alternative treatment is activated prothrombin complex concentrate (aPCC). aPCC contains a mixture of clotting factors, such as prothrombin and activated FX. High doses of recombinant activated factor VII (rfVIIa, NovoSeven, Novo Nordisk) are proposed to control bleeding via its tissue factor-independent activation of fX. SerpinPC is an engineered serine protease inhibitor that has been shown to effectively increase thrombin generation *in vitro* and in murine models of hemophilia A via inhibition of activated protein C. This drug is currently under investigation in phase 1 and 2 trials with healthy subjects. Other non-fVIII alternatives to control hemostasis that are still under investigation include anti-TFPI (tissue factor pathway inhibitor) antibodies that block TFPI binding to the early prothrombinase complex formed by binding of TF (tissue factor) to activated factor II (fIIa), or thrombin. Three such products are currently in clinical trials: Concizumab (Novo Nordisk, phase 2), Marstacimab (Pfizer, phase 2), MG1113 (Green Cross, phase 1). One anti-TFPI antibody from Bayer was recently pulled from phase 2 clinical trials due to serious adverse events. A therapeutic that has completed phase 1 trials and has ongoing phase 2 and 3 trials for patients with hemophilia A or B (with or without inhibitors) is Fitusiran (Sanofi), which is a double-

stranded small interfering RNA that inhibits synthesis of antithrombin by inducing degradation of the *SERPINC1* gene transcript, which prevents formation of antithrombin (AT), a serine protease that inactivates thrombin (fXa) and other proteins participating in the coagulation cascade (Fig. 1.4).[42,44]

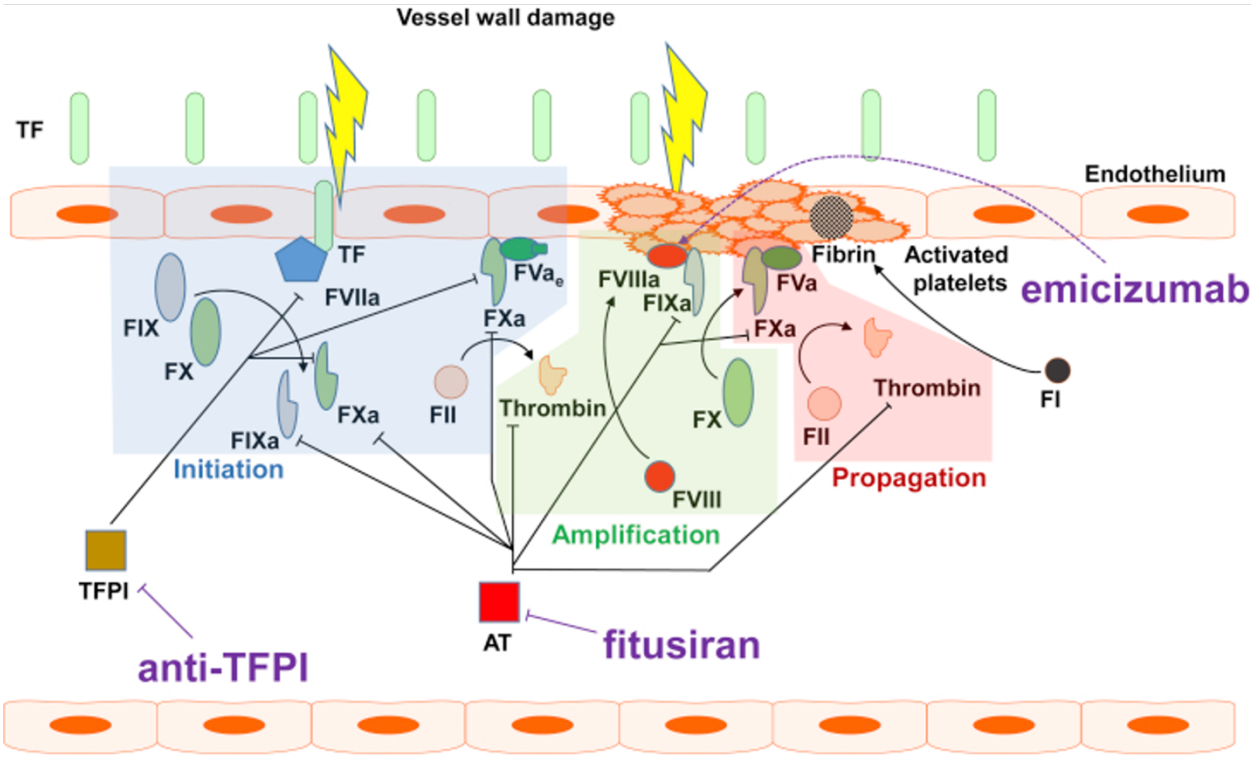

Figure 1.4 Mechanisms of non-fVIII therapeutics for hemophilia A.
This schematic outlines the mechanisms of action of anti-TFPI therapeutics, fitusiran siRNA, and the monoclonal antibody fVIII mimetic emicizumab within the context of different phases of the coagulation cascade after injury. [This image was reprinted with permission from the American Society for Gene & Cell Therapy and Elsevier SD Cell Press (CC BY-NC-ND 4.0): Butterfield JSS, Hege KM, Herzog RW, Kaczmarek R. A Molecular Revolution in the Treatment of Hemophilia. Mol Ther. 2020 Apr 8;28(4):997-1015. doi: 10.1016/j.ymthe.2019.11.006. Epub 2019 Nov 13. PMID: 31843450; PMCID: PMC7132613.]

1.2.3 Preclinical models for hemophilia A drug development

Prior to clinical trial initiation, product candidate evaluation in animals for efficacy, safety and dose finding is a standard part of the drug development pipeline for small molecule and protein

therapeutics. These animals may or may not be preclinical disease models; however, animal models of disease play an extremely important role not only in understanding the disease pathogenesis but also in drug target identification, mechanism of action, and initial pharmacokinetic/pharmacodynamic studies that kick off the drug development process. Species used in testing of novel therapeutic candidates for hemophilia A include mice, rats, dogs, sheep and non-human primates (NHP).[45-47] Dogs and sheep have naturally occurring mutations that cause hemophilia A and, along with their more complex genetics, this makes them ideal as large animal models for studying hemophilia A and evaluating treatment candidates. Most disease mechanism studies and testing and characterization of potential therapeutics begin on a larger scale in mice or rats, which have been genetically engineered to harbor mutations causing a hemophilia A phenotype. Mutations that cause hemophilia A in NHP have yet to be observed and characterized. Therapeutic candidates are instead evaluated in wild-type animals. Interestingly, in spite of the high degree of sequence similarity between endogenous NHP fVIII and human fVIII, recombinant fVIII products are basically uniformly immunogenic in NHP (i.e., they cause a pathogenic antibody response to the exogenous therapeutic fVIII protein). Due to the high degree of immunogenicity of fVIII protein products, discussed in detail below, these product candidates are often evaluated in immunocompromised animals, most commonly NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ). Despite the degree of protein sequence similarity between human fVIII and NHP fVIII (99%) or murine fVIII (85%), and the fact that immunocompetent murine models of hemophilia A have been shown to replicate many aspects of the human immune response to exogenous recombinant fVIII,[48] no model of immune tolerance to human fVIII has been established for preclinical studies in any species. Therefore, all animals administered these xenogeneic bioengineered proteins (i.e., the recombinant fVIII protein therapeutics not genetically

matched to the animal model that they are evaluated in) can be expected to develop an anti-fVIII immune response.

1.3 The Immune Response to Exogenous fVIII

1.3.1 The anti-fVIII inhibitor response

Indeed, antibody formation is a major complication associated with protein drug treatment. The patients at highest risk for developing this adverse immune response are those with severe protein deficiencies resulting from monogenic disorders, like hemophilia A. Around 30 – 40% of severe hemophilia A patients develop a humoral response to the therapeutic fVIII protein after the first 20 exposures that results in anti-fVIII neutralizing antibodies, or "inhibitors", that bind fVIII and render it nonfunctional. [49-53] Other diseases in this category include Pompe disease, which results from a deficiency in acid α-glucosidase, and over 90% of these patients develop neutralizing antibodies to the exogenous therapeutic acid α-glucosidase.[54-56] Therapeutic proteins are also an invaluable approach to treating other types of health conditions, such as autoimmune disorders and cancer, and these treatments can also be impeded by a humoral immune response. [57-59] The antibodies developed against protein drugs can bind the protein and impede its activity, increase protein clearance and alter the drug's pharmacokinetics, and patients can also develop potentially deadly hypersensitivity reactions to the protein drug. Lifelong treatment of hemophilia A with fVIII protein replacement products is attached to a high financial and quality of life cost for the patients and their families, and development of a humoral response to these products only increases that burden. Therefore, it is essential to elucidate what is yet unknown about the immune

response mechanisms to protein therapeutics like recombinant fVIII, why and when the antibody response occurs, and how to prevent or eradicate it.

As previously stated, only a subset of hemophilia A patients develop anti-fVIII antibodies to replacement fVIII proteins. However, not only is fVIII inhibitor incidence varied, but there is also inter-individual variability in multiple facets of an established inhibitor response,[60,61] including:

1. The response severity as indicated by the functional inhibitor titer in Bethesda Assay Units, or BU/mL, which measures the concentration at which inhibitor plasma reduces the fVIII activity in normal human plasma to approximately 50% of normal levels.
2. The response may be persistent, or it may be transient and resolve spontaneously (usually low severity).
3. Persistent responses differ in terms of whether they can be resolved using current inhibitor treatment options, even among patients treated with the same strategy, and with what degree of difficulty resolution is achieved.
4. If tolerance to fVIII is reestablished, there is also variability in the stability of this immune tolerant state.

These observations indicate that fVIII exposure can result in induction of multiple different immune response pathways during fVIII protein prophylaxis or tolerance induction protocols. It remains unclear why this inter-individual variation in the fVIII immune response occurs among hemophilia A patients; however, multiple genetic and environmental factors have been proposed to influence fVIII inhibitor risk. [50,62-69] One hypothesis for the cause of inhibitor development is the “danger” signals hypothesis. These signals refer to those required by antigen presenting cells (APCs) to activate, mature and present antigen to members of the adaptive immune system, like CD4 T cells. In other words, systemic inflammation from a secondary independent source

produces a high enough concentration of pro-inflammatory signals that it indirectly triggers an antibody response to the exogenous fVIII by providing the co-stimulation required to activate fVIII-specific T cells.

Another hypothesis is that genetics play the dominant role in fVIII immunogenicity risk. Different hemophilia A causing mutations in the *F8* gene result in different amounts of residual, defective or truncated fVIII protein still secreted into circulation, which is referred to as cross-reactive material (CRM). This CRM is seen by immune cells and can be used to educate the immune system to a certain degree. So, patients with mutations that result in complete or near complete absence of fVIII CRM in circulation are thought to be more likely to develop inhibitors during fVIII protein replacement therapy since this protein is basically seen as a "non-self" antigen.[50,62] Another factor to consider is genetic variation in genes that encode immune system components, such as human leukocyte antigen (HLA) haplotype or polymorphisms in cytokines and other signaling molecules important to immunity (e.g., TNF-alpha, IL-10). These foundational differences between individual patients may alter the mechanism(s) by which the immune system responds during inhibitor formation, or the stimulation threshold required to trigger an antibody response, or the ability for the immune system to reestablish tolerance.[63-69] Additionally, clinical data indicate that patients with African and/or Hispanic ancestry demonstrate a higher incidence of inhibitor formation than Caucasian patients, pointing to markers of population genetics that could alter susceptibility to inhibitor formation.[70-72] Preclinical studies in mice also support these hypotheses. The fVIII immunogenicity threshold appears lower for more severe *F8* mutations (less CRM) within the same strain of mice, but the threshold also appears lower for the same mutation on different genetic backgrounds (possible immune polymorphisms).[73,74] These two rationales based in genetics also make sense given that patients have developed inhibitors in the absence of

significant independent inflammatory events. However, it should be noted that both monozygotic twins and siblings with the same causal *F8* mutation have developed different immune responses to fVIII.[75,76] Taken together, it is likely that the immune response to fVIII in hemophilia A patients is likely either a multifaceted, complex process that can proceed differently at any step, or that there is one (or more) fundamental, unifying factor causing the different inhibitor response outcomes that has yet to be discovered or correctly understood.

1.3.2 The T and B cell response

The immune system cell type responsible for producing antigen-specific antibodies are a subset of B cells called plasma cells. However, there cannot be a proper, robust antibody response without the participation of T cells. All B cells are initially produced in the bone marrow and start out as with many copies of a single, unique B cell receptor (BCR) on each newly produced, naïve B cell. Once the BCR on a circulating naïve B cell comes into contact with its cognate antigen, providing the initial activation signal, in the absence of sufficient levels of BCR cross-linking, further signaling help is required for the B cell to become fully activated, replicate, and produce antibodies. The predominant way this process is triggered is via CD4 T cell help. The prevailing understanding of how fVIII inhibitors form begins with endocytosis of circulating fVIII by antigen presenting cells (APCs), such as dendritic cells (DCs) and macrophages. The endocytosed fVIII is processed into peptides that are then presented on the cell surface by major histocompatibility complex (MHC) molecules to other immune system cells. Typically, endogenous (or "self") proteins are expressed by MHC class I to CD8 T cells. However, in the case of a viral infection for example, presentation of foreign peptides after viral entry can target the infected cell for destruction by a CD8 T cell. Exogenous (or "non-self") proteins are loaded onto MHC class II molecules and presented to CD4 T cells by APCs. This educates the CD4 T cells about what is

going on in the body, such as a bacterial invader causing an infection, and the CD4 T cell can initiate multiple immune response outcomes depending upon the information it receives. FVIII-specific CD4 T cells can recognize the MHCII-fVIII, become activated, proliferate, and provide the additional signals required for fVIII-specific B cells to become fully activated.[77-85] Also not to be overlooked is the role that the innate immune system can play in orchestrating an adaptive immune response. Complement C3 with the help of its activation fragment C3b have been shown to facilitate the uptake of fVIII by DCs, with the prediction that fVIII interacts directly with C3b on the cell surface. And DCs have been shown to be the key player in antigen presentation during fVIII inhibitor development.[86]

Once B cells become sufficiently activated, they can proceed down a number of pathways involved in immunity depending upon the signals that the cells receive and the avidity and affinity of the BCR. The two main pathways available to these newly activated IgM expressing B cells are either: (1) the B cells remain extrafollicular and differentiate into either IgM memory B cells (MBC) or into short-lived plasma cells (SLPC) that produce IgM, or (2) the B cells enter a germinal center (GC) in which a process occurs to select for high affinity BCR clones that then expand into populations of class-switched (CSW, IgM → IgG) MBCs and/or long-lived plasma cells (LLPC).[87] These LLPCs are IgG antibody producing factories and, in the case of fVIII inhibitors, these antibodies are mostly high-affinity IgG1 and IgG4 isotypes that have undergone somatic hypermutation.[61,88-90] This population of LLPCs are thought to be the main cause of a sustained anti-fVIII inhibitor response; however, this has not been definitively proven. For as long as a particular population of MBCs is stable after the initial antigen exposure and humoral response, these cells stay behind as sentinels to ensure much more rapid antibody production and SLPC and LLPC generation upon repeat antigen exposure compared to after the original exposure.[91-99]

However, fVIII specific MBCs have been detected in only a portion of inhibitor patients.[100-102] It has yet to be concluded whether sustained persistent or recurrent inhibitor responses are the result of established LLPC populations, stimulation of a memory T and B cell response that triggers further antibody production by SLPCs and LLPCs, or whether fVIII antigen is cyclically restimulating a naïve B cell response that continually induces transient antibody titers, or some combination of these general pathways. It is also possible that the success of tolerance induction protocols is partly dependent upon the pathway(s) used to establish and sustain the fVIII inhibitor response (Fig. 1.5).

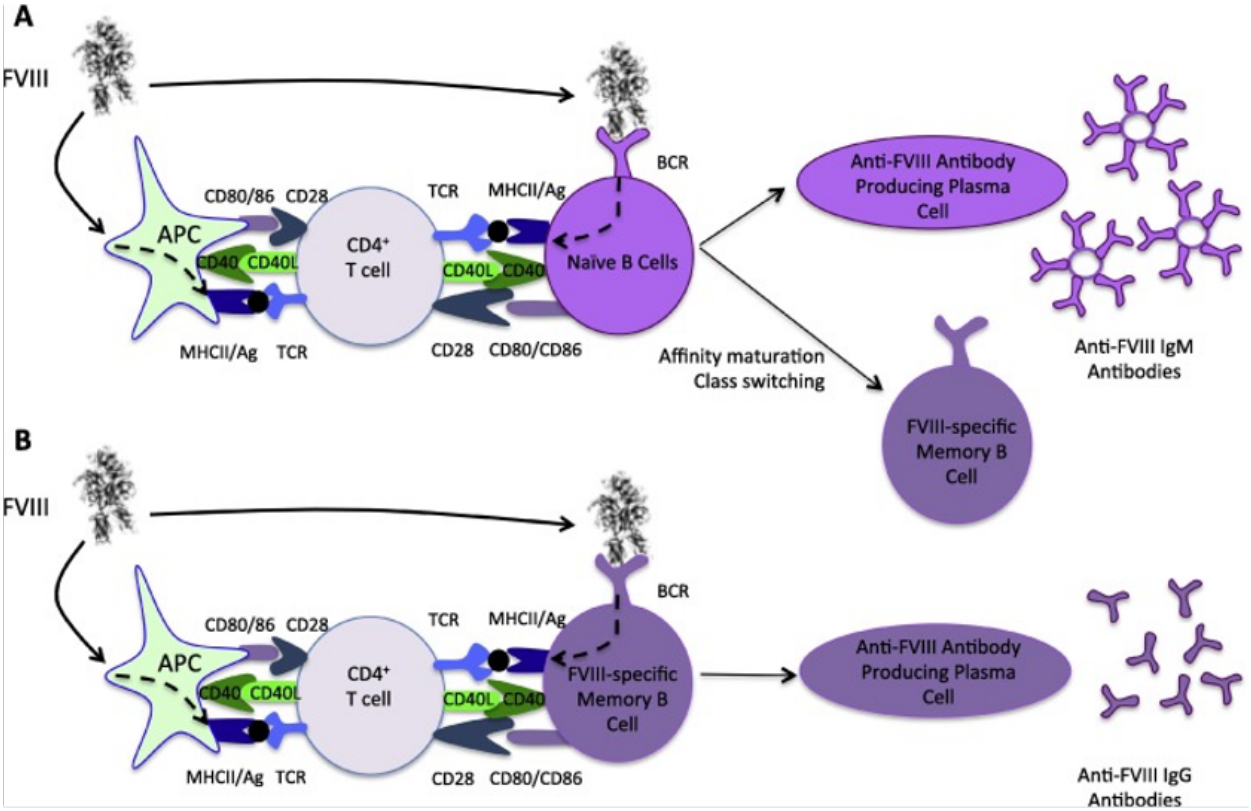

Figure 1.5 Progression of the anti-fVIII antibody response.
The anti-fVIII antibody response is initiated by uptake of fVIII protein by antigen presenting cells (APCs), which is thought to be primarily dendritic cells. (A) APCs present fragments of fVIII to circulating CD4 T cells, which, with proper co-stimulation, can help activate circulating naïve B cells. The initial response is generation of anti-fVIII IgM. However, these activated B cells can also undergo affinity maturation and class switching to form fVIII-specific memory B cells. (B) Upon continued stimulation or restimulation

with fVIII, these memory B cells have the potential to develop into antibodies producing factories called plasma cells. These plasma cells can be short-lived (SLPCs), or they can become long-lived plasma cells (LLPCs) with the potential to continue producing anti-fVIII IgG after fVIII antigen has been removed. [This figure was reprinted with permission from Elsevier SD Academic Press: Georgescu MT, Lai JD, Hough C, Lillicrap D. War and peace: Factor VIII and the adaptive immune response. Cell Immunol. 2016 Mar;301:2-7. doi: 10.1016/j.cellimm.2015.11.008. Epub 2015 Nov 28. PMID: 26676073.]

Although humoral immunity to exogenous protein drugs is thought to be predominantly dependent on the classical CD4 T cell response, it is also possible that initial B cell responses occur independent of T cell help. The first activation signals for B cells arise from naïve B cell BCR binding to the antigen. And, to emphasize the obvious, B cells are the cell type that can produce antibodies. However, while B cell independent contributions to humoral immunity have been studied in the contexts of autoimmune and infectious diseases, the recombinant protein therapeutics and hemophilia A fields have been dominated by research into the T cell response. There is precious little information on the early B cell response to fVIII or the role that the B cell profile and B cell independent responses play in inhibitor formation. One avenue of investigation underway by collaborators is the contribution of the naïve B cell repertoire to the mechanisms of inhibitor formation and inter-individual differences between the fVIII immune response. Naïve B cells released into circulation each express their own specific, genetically predetermined BCR in high amounts on the cell surface. This BCR is essentially the Fab fragment tethered to the cell by the immunoglobulin C domain. The Fab fragment is made up of segments of both the immunoglobulin heavy (IgH) and immunoglobulin light (IgL) chains, and multiple versions of the protein domains (V, D and J) that form these segments are encoded in the germline DNA. The diverse antigen specificity of naïve BCRs is generated by somatic recombination of these V-D-J segments that randomly joins different combinations of these segments together. Two of the regions important for antigen recognition (called Complementarity Determining Regions, CDRs)

are encoded in the V domain germline DNA. The third CDR exists only in the IgH chain D domain (IgL chains lack a D domain and only assemble various combinations of V and J domains). CDR3 is the strongest contributor to BCR diversity and the specificity of antigen recognition, as this domain is not entirely encoded in the germline DNA but also generated by nucleotide sequences that link the D domain to the V and J domains, and these nucleotide substitutions can vary in base composition and length. The result is a high degree of variation in IgH polymorphisms and gene copy numbers between individuals and at the population level.[103-105] It seems logical that different immune responses could be observed among hemophilia A patients based on their individual naïve B cell repertoires if one collectively considers the following points:[95,106-112]

1. The naïve BCR must bind fVIII antigen to mount an antibody response prior to any degree of T cell help.
2. There is a high degree of genetic variation between individuals and even populations in the DNA sequences that encode the CDRs of BCRs (especially CDR3).
3. A combination of the B cell precursor frequency, affinity, and avidity have been demonstrated to collectively regulate GC fitness, which would directly affect the cognate antigen specific antibody response (i.e., the fVIII inhibitor response).
4. The anti-fVIII antibodies produced after proper stimulation of fVIII reactive B cells show variation in their fVIII epitopes and binding affinities.

Indeed, the functional inhibitor titer (Bethesda titer) does not always correlate to the anti-fVIII IgG titer. This indicates that antibodies developed against some fVIII epitopes may be more potent (have higher affinity) and disrupt fVIII activity by targeting regions essential to cofactor function compared to antibodies against other epitopes. In fact, preclinical studies suggest that B cells initially expressing a high affinity BCR are more likely to engage the extrafollicular pathway and

generate IgM MBCs or SLPCs, while the GC reaction pathway appears open to newly activated naïve B cells with a wide range of affinities/avidities for the cognate antigen. This precursor naïve B cell repertoire hypothesis also tracks with the predicted importance of the individual patient's immune genetics and the availability of CRM that could possibly contribute to education of "self" vs. "non-self" antigens during naïve B cell development. It is also possible that the identity of the fVIII precursor BCR could contribute to the nature of the antibody response after B cell activation, meaning the types of B cell populations that form, the longevity of these populations, the severity and duration of the inhibitor response, the types of antibodies produced, and whether the B cell populations can ultimately contribute to reestablishment of immune tolerance. For the purposes of translational immunology research, it is also important to note that production of the four IgG subclasses is not the same in mice compared to humans. In fact, mice do not produce IgG subclass 4 and instead produce IgG1, IgG2a, IgG2b and IgG3. [87,91-93,95-99,113-116]

1.3.3 Protein drug factors contributing to the anti-fVIII immune response

There are also factors particular to the recombinant fVIII protein sequence and structure that could be contributing to its immunogenicity. For example, post-translational modifications (PTMs), particularly N-linked glycosylation. Glycans have been implicated in fVIII protein assembly and trafficking, as well as in uptake by APCs. When recombinant fVIII is produced in cell lines from different species, these cells confer different glycans, and preclinical studies have demonstrated that different fVIII products delivered to the same strain of hemophilia A mice have resulted in differential immune responses.[117] It is possible that different bioengineered fVIII protein sequences when produced in different animal cell lines vs. in human cell lines or *in vivo* via gene therapy, result in different PTMs that might affect fVIII immunogenicity.

vWF binding is another important factor to consider in the immune response to fVIII, and its role in fVIII inhibitor formation or tolerance is an active area of research.[5] Studies have shown that vWF binding to fVIII can play a role in antigen recognition by immune cells and the ability of certain anti-fVIII antibodies to bind to their epitopes.[118-121] In fact, initial preclinical studies of a fusion protein containing BDD-fVIII fused with the D'D3 domain of vWF demonstrated both increased fVIII half-life and a reduction in the fVIII inhibitor response as compared to BDD-fVIII alone.[122] Additionally, single nucleotide polymorphisms have been identified in VWF and other protein encoding genes, like the ABO gene for blood group, that associate with changes in vWF:fVIII binding, as well as vWF and fVIII levels and pharmacokinetics. It was also recently demonstrated for the first time in a pediatric population that mutations in the D'D3 region of the VWF gene alter observed fVIII pharmacokinetic parameters.[37] It is therefore possible that variation in multiple genes could have downstream effects on the pharmacokinetics of both vWF and fVIII, and hence on the immune response to recombinant fVIII therapeutic proteins among hemophilia A patients.

Infusion of soluble protein antigens like fVIII can also result in immune complex (IC) formation. Immune complexes vary in composition and are formed by association of one or more antigen molecules with antigen-specific antibodies and possibly other proteins as well. ICs occur more frequently upon infusion of high concentrations of antigen and can affect both how the immune system detects and responds to that antigen.[123,124] FVIII-ICs can contain both anti-fVIII antibodies that do and do not inhibit fVIII activity, and these ICs have been shown to modulate both fVIII pharmacokinetics (half-life and clearance) and the fVIII immune response. It has also been demonstrated that mainly DCs take up fVIII-ICs for MHC-II presentation to CD4 T cells and that this uptake is enhanced for fVIII-ICs compared to free fVIII, with different murine Fc receptor

family members binding different IgG isotypes with different affinities.[79,125-131] It follows then that polymorphisms in genes encoding receptors that internalize fVIII-ICs could contribute to variability in the fVIII inhibitor response.[132] Even though the fVIII protein and its various engineered forms are all immunogenic, with hotspots found along the entire peptide, these data suggest that some engineering approaches could be useful for decreasing the immunogenicity risk of fVIII protein drugs. For example, if modifications could be made to the more immunodominant regions of fVIII that decreased the overall inhibitor risk and/or generated a less robust inhibitor response, this would be clinically helpful. As mentioned above, fVIII fused to the vWF binding region D'D3 may reduce the risk of inhibitor formation due to an increased half-life and reduced chances of antigen presentation. Additionally, D'D3 binding to fVIII could reduce the risk of BCR cross-linking by decreasing the number of fVIII molecules available to bind physiological vWF. FVIII fusion to an IgG Fc domain may also decrease immunogenicity risk due to increased half-life but also due to potential tolerogenic properties conferred by the Fc domain.[133] Another approach could be to discern if there are any protein modifications that reduce the formation of ICs, which could in turn reduce immunogenicity risk.

Given the diversity of factors that could possibly influence the immune response to fVIII and the prevalence of inhibitor development to recombinant fVIII products, it is disconcerting that evaluation in immunocompromised animal models is often the penultimate study supporting initiation of clinical testing in humans. This also runs contrary to the desire to discover fVIII products that are highly efficacious at eradicating inhibitors and reestablishing immune tolerance.

1.4 Immune Tolerance to fVIII

1.4.1 Antigen-specific immune tolerance

The purpose of the immune system is to detect foreign, pathogenic invaders or endogenous cells that have become unhealthy and potentially threatening (tumor cells, for example), and eliminate them for the overall health and survival of the organism. In order to do this effectively without harming the organism in the process, immune system responses must be highly regulated, and the immune system must be able to discern between what antigens belong ("self") and what antigens do not ("non-self"). The term "tolerance" refers to this ability of the immune system to recognize antigens that belong in the healthy organism and avoid mounting a destructive anti-self immune response. The immune system does this through two major, distinct, and yet complementary pathways: central tolerance and peripheral tolerance (Fig. 1.6).

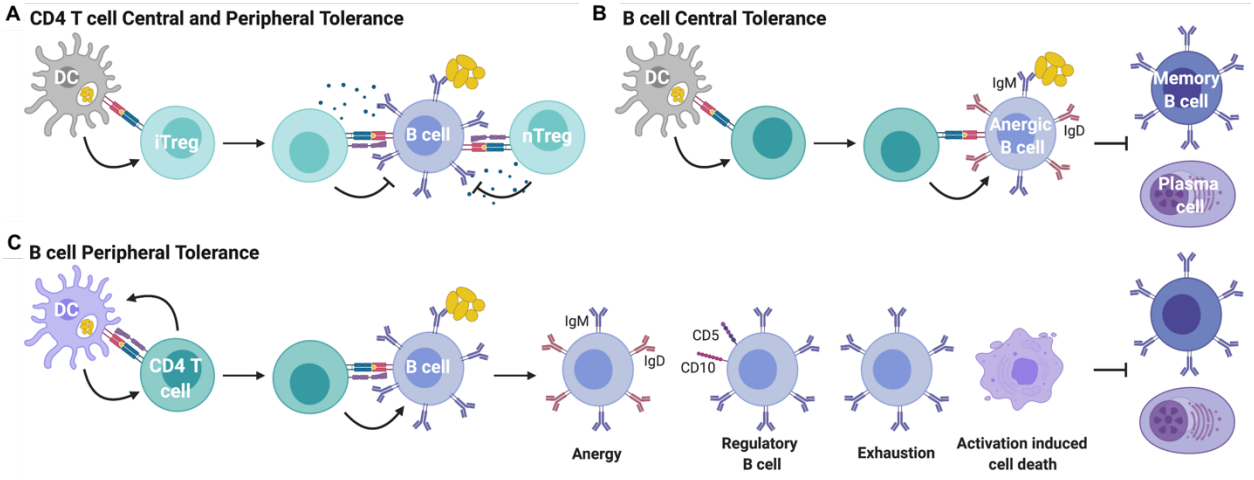

Figure 1.6 Immune tolerance mechanisms of CD4 T cells and B cells.

Tolerance to an antigen can develop via several mechanisms involving T and B cells. (A) T cells can be directed to differentiate into nTregs during development in the thymus. If released into the periphery, CD4 T cells can develop into iTregs after exposure to antigen without proper co-stimulation. These Treg populations can then act to suppress B cell activation and downstream antibody formation. (B) B cells develop in the bone marrow and can be exposed to some antigens in that compartment. If stimulation is too intense, or if the stimulation is too weak and the B cells become anergic, they undergo apoptosis. Anergic B cells that happen to be released into the periphery will not be able to activate in response to cognate antigen stimulation. (C) B cell tolerance can also be generated in the periphery by several types of B cell

responses that depend upon different scenarios of antigen stimulation, T cell interactions, and co-stimulatory signals. [This figure was created using Biorendender.com.]

Central tolerance is generated during T cell development in the thymus and B cell development in the bone marrow and fetal liver. Developing thymic T cells (thymocytes) are exposed to ubiquitous antigen as soon as they have expressed a functional T cell receptor (TCR). Once the T cell has committed to either a CD4 or CD8 lineage, they can be exposed to antigens with restricted expression via APCs. T cells expressing TCRs with very high affinity and/or avidity for self-antigen are deleted from the repertoire. Those TCRs with intermediate affinities and lower pMHC ligand densities may be directed to differentiate into regulatory T cells (Tregs), and those with the lowest affinities for self-antigen can be released into the periphery as conventional T cells. A similar process occurs for B cells. As mentioned above, there is extreme diversity in assembly of the BCR. Each B cell that can form a functional IgM isoform expresses many copies of a membrane form of the IgM that is anchored via immunoreceptor-based activation motifs (ITAMs) for signal transduction. This is the functional BCR and it samples self-antigens in the bone marrow microenvironment during precursor B cell development. When a BCR encounters its cognate antigen and the affinity and avidity of this interaction produces an acceptable intermediate level of signaling through the ITAMs, then this B cell is promoted to positive selection. If the signal is too weak, then the B cell has time to continue L-chain recombination to create a better BCR. If it cannot do this within a span of approximately 3 days, the cell dies. Likewise, if the BCR has too high an affinity and avidity for its cognate self-antigen, surface expression of the IgM is reduced via internalization, and the cell remains in the pre-B cell stage for ongoing L-chain recombination. If this cell cannot produce a BCR with acceptable signaling strength within a few days, it too will

die. These processes help ensure that autoreactive T and B cells are deleted, and only cells with acceptably low levels of self-reactivity are released into the peripheral circulation.

However, once naïve T and B cells are released into the periphery, it is possible that they were not properly educated on certain self-antigens during development or that autoreactive cells managed to escape detection and/or deletion. These failures to properly establish central tolerance are proposed to lay the groundwork for autoimmune disease development. It is also detrimental for a humoral immune response to occur long after the pathogenic invader has been eliminated. Additionally, there are times when development of tolerance to environmental non-self antigens is desirable, for example, in the case of developing tolerance to allergens. These situations are where the mechanisms of peripheral tolerance come into play. And these peripheral tolerance mechanisms are also important in controlling the unwanted pathogenic immune responses to exogenous protein therapeutics like recombinant fVIII. There are multiple pathways that T and B cells can follow that can contribute to generating peripheral tolerance. Naïve T cells can exist in a state of quiescence where they are actively arrested in the G0 stage of the cell cycle, maintain a small size and low metabolism, thus creating a higher threshold of activation by an antigen and co-stimulation. Both T and B cells can maintain ignorance, wherein the TCR or BCR cannot seem to recognize or be activated by their cognate antigen. This could be because the antigen is being sequestered or it is present at levels too low to trigger a response. The cells can also become anergic, which is a state in which the cell can recognize its cognate antigen but is hyporesponsive even in the presence of sufficient co-stimulation. The anergic response arises early after initial antigen exposure and maintenance of anergy requires prolonged exposure to the cognate antigen in the absence of sufficient co-stimulation. Responsiveness can recover very slowly after long-term absence of the antigen and, conversely, prolonged anergy can also lead to deletion (although

when and why this occurs is unclear). If T and B cells are exposed to sufficient antigen and co-stimulation such that a robust immune response occurs, there are also multiple ways that tolerance can be re-established. If the cells are completely overwhelmed by a sudden increase to extremely high antigen levels and co-stimulation signals, it can trigger a type of apoptosis called antigen induced cell death (AICD), thus deleting peripheral antigen-specific immune cells. This can also occur in effector T cells when they are highly restimulated after initial activation (restimulation-induced cell death). If T and B cells are not overwhelmed enough to cause AICD, but they are exposed to high levels of antigen for a long period of time, the cells can eventually shut down and stop responding. This is called exhaustion. Although the nature of the response appears very similar to anergy, it arises much later after antigen exposure and is the result of chronic stimulation in the presence of proper co-stimulation, for example in the context of a chronic infection. In an exhausted state, memory cells fail to develop properly and effector cell functions are compromised, characterized by severely attenuated responses to antigen, decreased cytokine production, altered metabolic and transcriptional states, and increased inhibitory receptor expression.

Another option for how T and B cells can actively participate in suppressing an immune response to an antigen is by differentiation into regulatory cells. The generation and role of regulatory B cells (Bregs) remains an extremely new and almost incompletely understood facet of immune tolerance. However, much more is known about regulatory T cells (Tregs). In fact, Tregs are not only a subset of CD4+ T lymphocytes that play a key role in immune system regulation and suppression of autoimmunity, but they are also currently thought to play the predominant role in establishing and maintaining tolerance to protein drugs.[134-142] There are two populations of peripheral regulatory Tregs: natural Tregs (nTregs) and inducible Tregs (iTregs). nTregs are generated in the thymus and constitute a fixed 2 – 8% of peripheral CD4 T cells in a healthy human.

They are identified mainly by high surface levels of CD25 (high-affinity IL-2 receptor alpha subunit) and low to negative levels of CD127 and CD45RA, along with the transcription factors Foxp3 and Helios. In contrast, the circulating iTreg population are generated and expanded from conventional peripheral CD4 T cells after antigen recognition in the presence of insufficient co-stimulatory signals and sufficient immunomodulatory cytokines, such as TGF-beta and IL-2, and do not necessitate the constitutive expression of Foxp3.[143] Tregs act to suppress the immune system via multiple strategies. One mechanism is by cell-cell contact via multiple surface receptors that modulate T cell activation or the stimulatory capacity of antigen-presenting cells. Tregs can also induce cytolysis of reactive immune cells by releasing perforin and granzyme B. And they can suppress effector T cell metabolism by delivery of cAMP and expression of ectoenzymes. Lastly, Tregs can secrete immunomodulatory cytokines such as IL-10, IL-35 and TGF-beta.[48,144-151] Multiple studies have demonstrated the involvement of Tregs in immune tolerance after exposure to fVIII. For example, knowing that IL-2 is important for Treg development, one recent study has shown that mice administered an analog of IL-2 fused to an Fc had a dramatic increase in activated Tregs that prevented inhibitor formation when delivered with fVIII gene therapy.[152] In fact, multiple types of engineered and expanded antigen-specific Tregs are being evaluated in *in vitro* and *in vivo* studies for their ability to induce tolerance to fVIII via suppression of fVIII-specific effector cell proliferation and/or anti-fVIII antibody production, including nTregs that express a fVIII TCR, a fVIII chimeric antigen receptor (CAR) Treg that is not MHC/HLA restricted, and B cell antibody receptor (BAR) Tregs that target the BCR.[143,153]

Yet another key factor to remember is the role of antigen exposure kinetics. If the antigen exposure and humoral inflammation are not intense enough to cause deletion of the antigen-specific cells, it appears that a source of continuous and sustained antigen exposure (i.e., very

prolonged antigen exposure at levels that do not fluctuate) is essential for establishing and maintaining tolerance. Whether the antigen exposure is maintained at extremely low levels to protect immune cell ignorance or induce anergy, or whether they are quite high in order to induce T/B cell exhaustion or regulatory cell formation after a humoral response, it appears that the antigen exposure kinetics and maintenance are key variables in determining the type of immune response that unfolds. This concept also appears to play an important role in current protocols used for immune tolerance induction to fVIII in patients with inhibitors.

1.4.2 FVIII immune tolerance induction (ITI)

Since the 1970s, a method called immune tolerance induction (ITI) has been used in attempt to eradicate inhibitors. The treatment was originally described as the "Bonn Protocol" by Brackmann and Gormsen in 1977 and consists of a frequent, high dose fVIII infusion regimen designed to force the immune system to reestablish tolerance to that fVIII protein.[154] Current protocols administer fVIII doses ranging from 40 – 300 IU/kg at frequencies of every 1 – 3 days. ITI is the only clinically employed method shown to eradicate inhibitors and induce sustained fVIII tolerance. However, the success rate is approximately 60 – 80% and correlates with the magnitude of the inhibitor titers prior to ITI. These protocols also come with a large increase in an already high treatment cost and quality of life burden, with costs as high as $75,000/month plus the cost of any fVIII bypassing agents used and possible therapy durations of >1 year. Additionally, ITI these protocols are still off-label and experimental in nature, and the immunological mechanisms of action are not well understood. Depending upon the patient's inhibitor titer and the exact ITI protocol employed, the patient could (1) reattain tolerance, wherein the therapeutic fVIII protein returns to normal pharmacokinetics, (2) reach partial tolerance, wherein fVIII does not trigger a memory response when delivered at normal doses, or (3) ITI can fail, making further use

of fVIII protein replacement to maintain hemostasis impossible.[155] While some patients can maintain whatever level of tolerance is achieved after ITI, some spontaneously relapse after cessation of ITI or in conjunction with a secondary, independent immune system perturbation. In patients with inhibitor titers >5 BU/mL who have failed ITI, the only strategy to prevent or treat bleeding is treatment with fVIII bypassing agents or mimetics, like rfVIIa, aPCC, or Hemlibra. However, no biologic will act as properly in the maintenance of normal hemostasis or be as tunable within the coagulation cascade mechanisms as fVIII. Therefore, one of the highest priorities in fVIII drug development is creating therapeutic products that avoid inhibitor induction and/or have a high inhibitor eradication success rate at the lowest possible cost to the patient.

Some fVIII products are being investigated not only for efficacy but also for their ability to induce immune tolerance to fVIII and avoid inhibitor formation and/or treat existing inhibitors. One such product is a fVIII-Fc fusion protein. The longer half-life could, depending on dose intervals, help avoid major fluctuations in antigen exposure levels to the immune system, but the main interest lies in the potential tolerogenic properties of the IgG Fc domain. It is unclear exactly how the Fc domain promotes immune tolerance. One mechanism is dependent upon the FcR (Fc receptor), particularly FcγIIb, which has been shown to inhibit B cells when engaged by IgG Fc and trigger B cell apoptosis when triggered by antigen-antibody ICs. The FcγR is also present on innate immune cells and on liver sinusoidal endothelial cells (the location of fVIII production). Another mechanism appears to be dependent upon the glycosylation status of the Fc domain, wherein carbohydrate modifications at asparagine 297 have been shown to modulate activities and effector functions of antibodies possibly via regulating FcγR expression on APCs and the way that antigen is presented by APCs. These IgG Fc fusion proteins may also engage FcRn, which can divert the fusion protein away from degradation and antigen presentation. FcRn has also been

linked to induction of Tregs in neonatal murine models. Finally, it has also been proposed that the IgG Fc domain of fusion proteins may generally make the process of uptake and presentation by APCs less efficient and that presentation of certain Fc domain epitopes on MHCII molecules can promote Treg activation. Preclinical research using fVIII-Fc fusion proteins has demonstrated induction of immune tolerance pathways and less robust inhibitor responses when they are induced.[133]

1.5 FVIII Gene Therapy

1.5.1 The development of gene therapy for hemophilia A

There are multiple reasons why hemophilia A is an ideal candidate for treatment with gene therapy and, indeed, this disease has one of the longest histories of gene therapy product development. Being a monogenic disorder, the gene therapy needs to transfer only one functional gene producing one functional protein in order to treat or functionally cure the disease. Taking together the fact that even small increases in fVIII production levels can lead to a significant improvement in disease phenotype and that gene therapy has the potential to require only one treatment event, gene therapy delivery of fVIII could mean a functional cure and a major improvement in quality of life, particularly for severe hemophilia A patients. Additionally, the ideal gene therapy would induce continuous, sustained therapeutic levels of fVIII production for the lifetime of the patient. This has led to great interest in the ability of gene therapy to establish immune tolerance and either avoid inhibitor induction or eradicate existing inhibitors.

Many different types of vectors have been investigated for application in therapeutic gene transfer, including retroviral, lentiviral (LV), adenoviral, adeno-associated viral (AAV), and non-

viral vectors. Overall, there are two main routes of gene therapy vector administration. The *in vivo* route involves infusion of the gene therapy vector directly into circulation, after which the vector migrates to the target tissue cell type(s) based on the inherent cell tropism determined by the structure of the packaging vector, and then the therapeutic transgene is expressed based on the cell-type specificity of the transcription regulation elements employed. The cells transduced *in vivo* are then responsible for producing and secreting the therapeutic protein product. The second approach occurs by treating cells with the gene therapy vector *ex vivo* and then delivering the genetically modified cells to the patient. These cells need to be able to take up residence and persist in the patient in order to continue providing the therapeutic protein, for example, via a bone marrow transplantation.

Development of recombinant viral vector gene transfer of a human fVIII transgene initially began right alongside the development of recombinant fVIII protein replacement products, with the first *in vitro* retroviral transfer of fVIII occurring in 1990.[156] Excitement over the technology and its curative potential led to rapid initiation of fVIII gene therapy clinical trials employing both *in vivo* and *ex vivo* approaches by the late 1990s. However, the efficacy and durability of these initial gene therapies was poor, resulting in fVIII activity levels still in the severe-to-moderate range, and adverse events were reported after delivery of one of the adenoviral vectors.[157,158] The gene therapy field in general was observing multiple failures in efficacy as well as adverse effects, including insertional mutagenesis and liver toxicity, that led to a mass retreat from commercial gene therapy product development. Academic research institutions took up the gene therapy mantle and what followed was a period of intense investigation into the mechanisms of gene transfer by different vectors, their efficacy and safety, as well as advancement of genetic and protein engineering approaches. This progress launched a rebirth of fVIII gene therapy product

candidate development (both commercial and academic) and the second wave of clinical trials.[159] Currently, there are two major types of product candidates at the forefront of gene therapy approaches for hemophilia A. One is *in vivo* delivery of a liver tropic AAV vector encoding the fVIII transgene with a synthetic hepatocyte-directed promoter (herein referred to as AAV-fVIII). This is the most common approach to gene therapy for hemophilia A in clinical trials (Table 1.2).[44,160]

AAV Gene Therapy Products in Clinical Trials for Hemophilia A

Sponsor	Product	Capsid	Promoter	Transgene	Dose (vg/kg)	Phase
Bayer	BAY2599023 (DTX201)	hu37	liver-specific	BDD-fVIII	not disclosed	1, 2
BioMarin	valoctocogene roxaparvovec (BMN 270)	AAV5	HLP	BDD-fVIII	6E12 – 6E13	1, 2, 3
	valoctocogene roxaparvovec (BMN 270) in patients with AAV5 antibodies	AAV5	HLP	BDD-fVIII	6E13	1, 2
Sangamo	SB-525	AAV2/6	liver-specific	BDD-fVIII	9E11 – 3E13	1, 2
Takeda	TAK-755 (formerly BAX 888/SHP654)	AAV8	TTR	BDD-fVIII	not disclosed	1, 2
Spark	SPK-8011	Spark200	liver-specific	BDD-fVIII	5E11 – 2E12	1, 2
	SPK-8016 in patients with fVIII inhibitors	Spark200	not disclosed	BDD-fVIII	dose-finding	1, 2
UCL/St. Jude	AAV2/8-HLP-FVIII-V3	AAV2/8	HLP	fVIII-V3	6E11 – 6E12	1

Table 1.2 AAV gene therapy products in clinical trials for hemophilia A.
This table outlines the various AAV gene therapy product candidates currently in or initiating clinical trials. BDD-fVIII refers to B-domain-deleted human factor VIII. HLP: hybrid liver-specific promoter, TTR: transthyretin, UCL: University College London. [Adapted from: Butterfield JSS, Hege KM, Herzog RW, Kaczmarek R. A Molecular Revolution in the Treatment of Hemophilia. Mol Ther. 2020 Apr 8;28(4):997-1015. doi: 10.1016/j.ymthe.2019.11.006. Epub 2019 Nov 13. PMID: 31843450; PMCID: PMC7132613.]

While studies suggest that any cell type that can secrete protein directly into the bloodstream is a viable target for fVIII biosynthesis after gene transfer, since LSECs are the native site of fVIII protein production and the liver is highly adept at protein production for secretion into the bloodstream, the most advanced AAV gene therapy product candidates target hepatocytes. The second major gene therapy approach for hemophilia A is autologous delivery of CD34+

hematopoietic stem and progenitor cells (HSPC) after *ex vivo* transduction with a LV vector encoding the fVIII transgene targeted for hematopoietic lineage-restricted expression (herein referred to as LV-fVIII).[161] While *in vivo* LV vector products have not progressed due to concerns regarding high transduction of antigen presenting cells and the resulting inflammatory response risk,[162-166] *ex vivo* LV-fVIII HSPC transduction avoids these complications as it allows for direct application of the gene therapy to the target cells. This approach has the added benefit of allowing for cellular product validation and quality control before delivery to the patient. Additionally, as LV vectors integrate into the cell genome, this results in a permanent source of fVIII production as long as the LV-fVIII HSPC transplantation is successful.

Recombinant AAV vectors became attractive for gene therapy product development mostly due to their safety as non-pathogenic and non-integrating episomal vectors, their selective tissue tropism that is useful for disease-specific product development, and because the route of administration via peripheral vein infusion is fast and simple (Fig. 1.7).

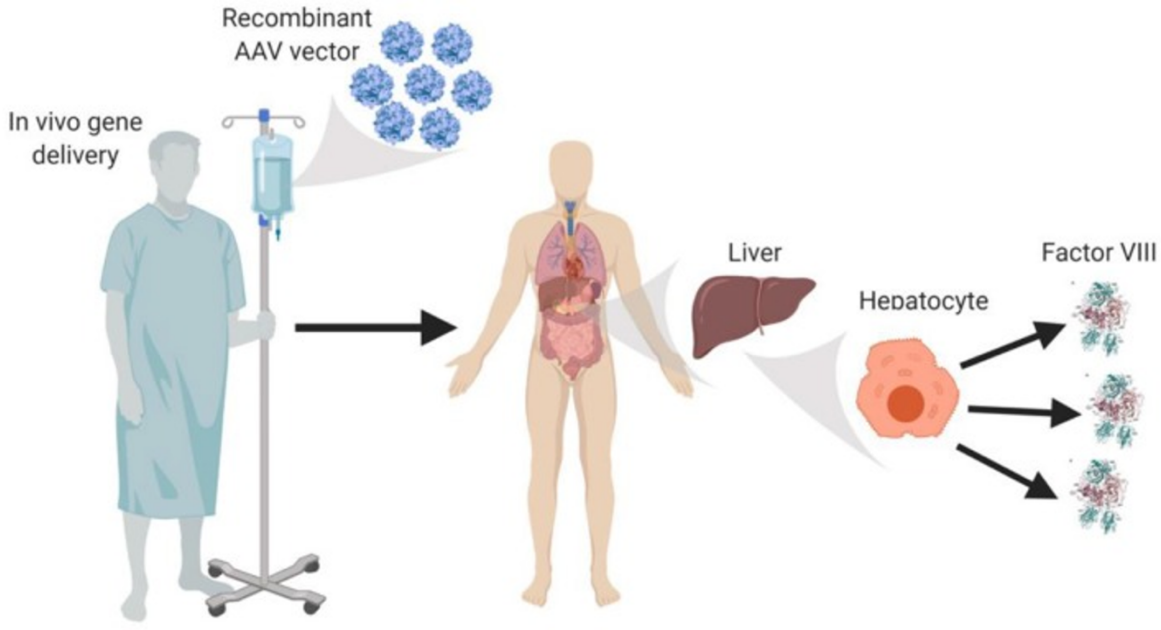

Figure 1.7 Delivery of an AAV-fVIII gene therapy vector.

Large scale *in vitro* preparations of AAV-fVIII gene therapy vector are delivered in one treatment session directly to the patient via peripheral vein infusion. The vector migrates to the target organ (the liver) where it transduces the target cells (hepatocytes). The hepatocytes take up one or more copies of the vector and begin to transcribe the encoded fVIII transgene and produce fVIII protein. This then endogenously produced fVIII protein is secreted directly into circulation, much like in the case of normal fVIII production. [This figure was reprinted from Frontiers: Patel SR, Lundgren TS, Spencer HT, Doering CB. The Immune Response to the fVIII Gene Therapy in Preclinical Models. Front Immunol. 2020 Apr 15;11:494. doi: 10.3389/fimmu.2020.00494. PMID: 32351497; PMCID: PMC7174743.]

Although wild-type AAV is known to integrate into the AAV integration site 1 (*AAVS1*) locus,[167] engineered recombinant AAV vectors used in gene therapy products are thought to lack this integration ability and instead exist entirely episomally or integrate non-specifically at very low levels. In general, the engineered single-stranded genome consists of two inverted terminal repeats (ITRs) that are serotype-specific and encompass a transgene cassette encoding a transcriptional promoter/enhancer element, followed by the therapeutic protein sequence, and a polyadenylation signal. The AAV protein capsids that package this genetic material have different serotypes depending upon the capsid structure. These capsid serotypes also display cell type tropism for transduction. The AAV capsid also imposes size constraints on the transgene, which must be limited to approximately 4.5 – 4.7 kilobases from end-to-end of the ITRs to ensure efficient packaging.[168,169] As F8 is such a large gene, the discovery that BDD-fVIII cDNAs generate fully functional proteins, along with advances in engineering of shorter, synthetic transcription regulation elements, have allowed fVIII cDNA to be packaged just within the limits of an AAV vector. Therefore, from this point forward, all fVIII proteins encoded by AAV vector transgenes can be assumed to lack the B domain (i.e., they are B-domain-deleted, BDD) regardless of species sequence origin.

1.5.2 AAV-fVIII product design and its effects on efficacy and immunogenicity

Beyond these general design parameters, AAV gene therapy product candidates have reached a point in drug development where each individual candidate contains customized sequence designs for each genetic component. The effects of these different design elements on AAV-fVIII efficacy (especially shorter-term efficacy) have been thoroughly investigated; however, the concomitant anti-fVIII immune response effects have not been interrogated nearly as thoroughly. The AAV serotypes used in current AAV-fVIII gene therapy product candidates are AAV 3, 5, 6, 7, 8 and hu37, as well as engineered versions thereof. The future could also bring with it engineered chimeric and/or completely novel AAV capsid structures. The serotype (i.e., capsid structure) will determine the cell type tropism profile and thus could influence the biodistribution of vector transduction and transgene expression. This in turn could influence how the immune system perceives the newly expressed fVIII protein. Different AAV serotypes have also been shown to differentially engage the unfolded protein response.[170] Some vectors may contain stimulatory hypomethylated CpG motifs, which have been suggested to be proinflammatory elements,[168,171,172] but no data exist confirming whether they have any impact on the fVIII inhibitor response. While recombinant fVIII proteins seem to be uniformly immunogenic, the bioengineered fVIII proteins encoded in AAV-fVIII vectors have shown variations in biosynthetic efficiency due to their transgene sequence, codon-optimization, and differential engagement of the unfolded protein response.[173-177] All of the vectors currently in clinical trials contain synthetic, high-expressing, liver-directed promoter/enhancer elements, and preclinical testing of multiple examples of such elements appears to support differential fVIII immunogenicity risk depending on their sequence design.[178] Further variations in vector design sequences could also be introduced via introns, the polyadenylation signals, and inverted terminal repeats.

The vector dose is another critical AAV-fVIII treatment parameter to consider given that all AAV particles are immunogenic in humans, as discussed below. But while a humoral response to the AAV capsid is expected, higher AAV capsid loads could also contribute to cellular stress from the high energetic burden of fVIII protein production in transduced cells and a proinflammatory microenvironment in the target organ that indirectly increase the immunogenicity risk of the transgene product.[179-183] Indeed, it is hypothesized that an additional benefit of targeting the liver for AAV-fVIII gene therapy is that this innately tolerogenic organ will in turn facilitate immune tolerance to the gene therapy fVIII protein product when secreted into circulation.[184-186] However, the other side of this coin is that the liver can be considered an immune organ, and immune responses are regulated by stimuli, their identity, strength, rate of change, and subsequent signaling mechanisms.[48,187] The liver contains resident immune cells, and its immunobiology and whether liver-directed AAV-fVIII gene therapy design and/or dose could induce a tunable shift in the organ between a tolerogenic and a proinflammatory state has not been investigated. It is possible that one of these factors could be dominant, or that multiple design elements could combine in synergy, to increase the risk of fVIII inhibitor incidence after gene therapy delivery (Fig. 1.8).

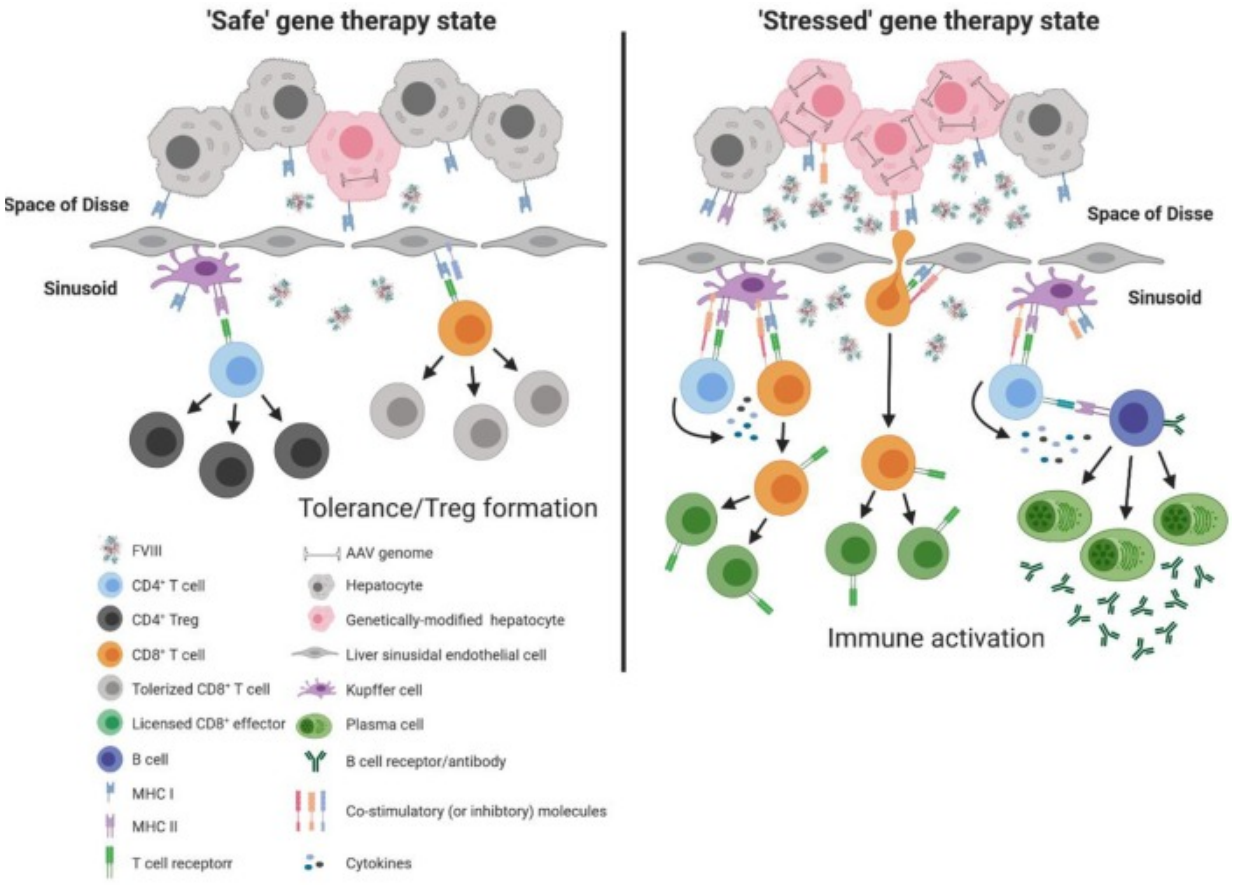

Figure 1.8 A model of possible immune response mechanisms to AAV-fVIII in the liver microenvironment.
The contribution of the liver to the immune response to fVIII produced after AAV-fVIII gene therapy remains unknown. However, the liver is an immunoprivileged organ with resident immune cells. This figure presents a schematic of possible cellular and molecular mechanisms that could promote a 'Safe' liver organ response and promotion of tolerance to the fVIII protein product vs. a scenario of a damaged or 'Stressed' liver organ in response to gene therapy that could lead to participation in activation of the immune system against the fVIII protein product. [This figure was reprinted from Frontiers: Patel SR, Lundgren TS, Spencer HT, Doering CB. The Immune Response to the fVIII Gene Therapy in Preclinical Models. Front Immunol. 2020 Apr 15;11:494. doi: 10.3389/fimmu.2020.00494. PMID: 32351497; PMCID: PMC7174743.]

As previously mentioned, many of the preclinical studies that directly inform human clinical trials of fVIII protein replacement product candidates are conducted in immunocompromised animals or in conjunction with immunomodulation of immunocompetent animals in order to assess efficacy without interference from the fVIII inhibitor response. This

extends to preclinical evaluation of fVIII gene therapy candidates.[188] Indeed, multiple confounding variables arise in the preclinical evaluation of fVIII protein product immunogenicity after AAV-fVIII gene therapy delivery, including not only the common use of immune-deficient or -suppressed animals, but also from differences in genetic backgrounds of the animals, limited early time point data, variations in study duration, differences in both vector design and the doses at which each vector is tested, variations in reagents and assays used to assess fVIII activity and inhibitors, and small sample sizes (particularly in large animal studies). For example, regarding strain variation in murine hemophilia A studies, results have demonstrated that delivery of liver-directed AAV-hAAT-hfVIII at $1x10^{11}$ vg/kg to BALB/c hemophilia A mice results in therapeutic fVIII levels plus low or undetectable inhibitor titers after recombinant hfVIII protein challenge with or without transient B cell depletion 1 week prior to gene therapy delivery.[74] However, repetition of the same study parameters in S129-C57BL/6 hemophilia A mice demonstrated attenuation of the inhibitor response after recombinant hfVIII protein challenge but not the same level of immune tolerance that was induced in the BALB/c background mice.[73] The genetic variations in these mouse models are akin to, but not as complex as, the variation one can expect in a human hemophilia A patient. Results of this nature highlight the potential impact that genetic polymorphisms of the recipient's immune system could have on both the sensitivity and the cellular/molecular mechanism(s) of anti-fVIII immunity after liver-directed AAV-fVIII delivery.

Cumulatively, results from murine preclinical studies on the immune response to recombinant fVIII suggest that inhibitor development is dependent upon CD4+ T cell help.[189-191] In the context of the BALB/c hemophilia A mice treated with AAV-hAAT-hfVIII, decreases in co-stimulatory molecules and immunomodulatory cytokines were observed after vector delivery, and adoptive transfer of CD4+ CD25+ cells from the gene therapy treated mice to naïve mice

resulted in some level of acquired immune tolerance to the transgene product upon *de novo* fVIII protein challenge.[74] In conjunction with other hepatotropic AAV gene therapy studies delivering not only fVIII, but other proteins such as fIX and ovalbumin (OVA) as well, Tregs are now thought to play a crucial role in establishing and maintaining tolerance to the gene therapy protein product produced by the transduced hepatocytes. This conclusion is mainly due to observed increases in antigen-specific CD4+ CD25+ Foxp3+ cells in the liver, the periphery, and even in the thymus that was accompanied by a lack of antigen-specific antibodies after gene therapy delivery that, in some cases, has also been reversed by *in vivo* depletion of Tregs using an anti-CD25 monoclonal antibody.[184-186,192] In addition, there are studies demonstrating that enhancement of Treg formation using strategies such as treatment with IL-2/IL-2R antibody complexes further promotes tolerance to fVIII after gene therapy, while other strategies such as rapamycin treatment have only been explored in conjunction with fVIII protein infusion.[193-200] Other studies using model antigens in hepatotropic AAV gene transfer have observed other immunoregulatory mechanisms for establishing tolerance, such as antigen specific CD4+ T cell anergy and/or deletion.[201] In spite of these immunological observations, the data are still conflicting regarding fVIII immunogenicity after AAV-fVIII delivery to immunocompetent hemophilia A mice.[74,173,175,178,202] There is no clear consensus about when fVIII immune tolerance vs. inhibitors develop and what the primary variables are that determine the outcome in the context of hepatotropic AAV-fVIII.

As a spectrum of mutations causing hemophilia A occur naturally in dogs, and these animals are not inbred to create genetically homogenous populations, canine models offer a clinically similar context for evaluating the immune response to AAV-fVIII gene therapy. There are two primary canine hemophilia A colonies that have been used to study liver-directed AAV-fVIII gene therapy.[203-208] The colony that resides at University of North Carolina (UNC) at Chapel

Hill possesses a mutation similar to the human intron 22 inversion and, also similar to humans, the canines also demonstrate variation in their tendency to form inhibitors after canine fVIII (cfVIII) treatment for reasons that are unclear. However, hepatotropic AAV-cfVIII treatment tends to both correct hemostasis and quite uniformly promote tolerance to the cfVIII protein product. In one study, one of the more "inhibitor prone" canines did develop a low (2.5 BU/mL) and transient inhibitor titer that resolved within 7 weeks after vector delivery, while the other eight canines in the study did not form a detectable inhibitor response. Immune tolerance to the therapeutic fVIII protein was maintained even after subsequent challenge with recombinant cfVIII.[209] However, it is worth noting that the doses used in the canine gene therapy studies were relatively low compared to what is currently being tested in both preclinical murine studies and human clinical trials.

Non-human primate (NHP) studies present an interesting case. Unlike preclinical studies in murine and canine models, naïve NHPs mount a robust immune response to the hfVIII protein derived from liver-directed AAV-hfVIII gene therapy, even at relatively low vector doses. One study administering vectors with different amino acid linkers in place of the B domain demonstrated that a high $2x10^{13}$ vg/kg dose of rAAV8-HLP-codop-hfVIII-N6 (N6: 226-amino acid linker) resulted in peak fVIII activity levels of approximately 65% and 105% of normal human fVIII activity in two animals. Lower doses of an identical vector excepting an alternative linker, rAAV-HLP-codop-hfVIII-V3 (V3: 17-amino acid linker), resulted in 138% and 43% of normal human fVIII activity levels when administered at $7x10^{12}$ or $2x10^{12}$ vg/kg, respectively. Three out of four of these animals developed inhibitor titers (3-15 BU/mL) within 6 weeks of vector delivery. However, the NHP that did not develop inhibitors was treated with the low dose of the vector with lower predicted potency, rAAV-HLP-codop-hfVIII-V3. The three inhibitor response animals were treated with rituximab and cyclophosphamide to eradicate the inhibitors.[210] During preclinical

evaluation of BMN 270, an AAV5-HLP-hfVIII vector referred to as valoctocogene roxaparvovec and currently in phase 3 clinical trials, three out of four NHPs developed inhibitors within 8 weeks after AAV-fVIII delivery. Two animals were administered $3.6x10^{13}$ vg/kg and the other two were administered $1x10^{13}$ vg/kg, and the one animal that did not mount an anti-fVIII antibody response was likewise in the lower dose cohort.[188] It is quite surprising that wild-type NHPs expressing fVIII protein that is 99% identical to human fVIII would so readily mount an inhibitor response to the transgene derived fVIII protein after delivery of hepatotropic AAV-hfVIII. As discussed in the immune response differences observed in different strains of hemophilia A mice, one possible explanation points back again to genetic variation in critical host immune system constituents. The 1% difference in the human fVIII peptide could result in an interaction with the NHP MHC that is just different enough from the interaction with human HLA that it triggers an antibody response after presentation of the recombinant human fVIII peptide complexed with MHC to the TCRs on NHP peripheral T cells. Nuances in the interaction of TCRs with slight variations in structure of the MHC/antigen peptide complex could result in a spectrum of downstream T cell responses.[211,212]

While many animal studies have demonstrated the preclinical efficacy and potential clinical efficacy of liver-directed AAV-fVIII gene therapy vectors, a clear and unifying understanding has not been established for this class of genetic medicine in general or for the individual vectors regarding long-term treatment efficacy, potential systemic and target organ toxicity and pathology, and especially fVIII immunogenicity risk and mechanisms of fVIII inhibitor development.[73,74,173,175,177,178,188,202,203,209,210,213-218] It appears clear that recipient genetics and the resulting foundational architecture of the immune system play a critical role in the nature of the immune response to the gene therapy transgene derived fVIII protein, but when and why

different species do or do not develop fVIII inhibitors after administration of certain doses of various hepatotropic AAV-fVIII vectors remains unclear.

1.5.3 Concerns and challenges facing AAV-fVIII gene therapy

There are several challenges, concerns and gaps in knowledge associated with AAV-fVIII gene therapy for hemophilia A beyond that of fVIII protein product immunogenicity. Firstly, while there are many positives associated with using an AAV vector, humans and NHP are the native hosts for wild-type AAV and most humans will have been infected by the end of adolescence. Neutralizing anti-AAV capsid antibodies (NABs) can develop as a result of the exposure, and this can significantly impede transduction of target cells if the patient harbors NABs against the serotype used to package the gene therapy transgene. Compounding the reduced transduction efficiency, AAV capsid memory cytolytic CD8+ T cells (CTLs) could trigger destruction of the gene therapy transduced cells and further decrease transgene expression. Additionally, if the patient does not harbor AAV NABs prior to gene therapy delivery, the AAV-fVIII gene therapy itself could cause a similar immune response to that of wild-type AAV. This means that if the initial AAV-fVIII administration does not produce efficacious fVIII levels, the fVIII expression does not last long-term, or the patient develops inhibitors, a repeat administration of the same AAV-fVIII gene therapy vector would not be a viable option. Indeed, after liver-directed AAV-fIX gene therapy clinical trials for hemophilia B resulted in patients with humoral anti-AAV8 capsid immune responses and possible T cell immunity to the AAV transduced cells, future AAV-fVIII and AAV-fIX clinical trials have been limited to patients who screen negative for NABs to the AAV vector serotype.[219,220] Strategies that circumvent AAV NABs and allow for repeat exposures are currently being investigated, such as engineered capsid variants, capsid shuffling, and decoy capsids to divert the immune response.[169,183,221-224] Also, the same vector design and

dosing parameters that could influence the immunogenicity of the fVIII protein product could potentially influence the AAV capsid immunogenicity and NAB generation, which has yet to be rigorously investigated.

Another important factor to consider is potential toxicity and pathology risk to the liver. For example, heterologous expression of fVIII has been shown to engage the unfolded protein response (UPR), the discovery of which was greatly aided by development of recombinant fVIII products. UPR is a highly coordinated cellular process in place to regulate accumulation of improperly folded proteins in the endoplasmic reticulum, but if this process is highly engaged during expression of normal to high levels of fVIII protein after gene therapy, it could lead to cytotoxicity, engagement of inflammatory pathways, and cell death.[174,225-232] One study revealed that fVIII can form reversible amyloid-like aggregates that greatly increase the protein's retention in the endoplasmic reticulum, especially when the burden of fVIII expression is constitutively high, as would be the case in gene therapy approaches.[3] Therefore, it is of interest to engineer fVIII transgenes whose protein products cause less cellular stress during production and secretion. Our group discovered that recombinant porcine fVIII and the human/porcine chimeric ET3i fVIII appear to avoid engagement of the UPR, leading to increased biosynthetic efficiency and secretion.[174,233,234] Use of such fVIII transgenes in gene therapy vectors could lead to more potent vectors that can be delivered at lower doses. This may be ideal from a liver toxicity/pathology and inflammation perspective. Preclinical studies have thus far failed to both recapitulate the liver inflammation seen in early stages of clinical gene therapy trials[188] and establish a direct relationship between engagement of the UPR and inhibitor development.[230,231] However, preclinical studies have demonstrated induction of an ER stress response following DNA delivery of fVIII and AAV-fVIII delivery, as well as that protein misfolding in the ER of hepatocytes

combined with chronic inflammation led to nonalcoholic steatohepatitis and hepatocellular carcinoma pathogenesis.[3] Additionally, elevation of liver enzymes observed in clinical trials approximately 6-20 weeks after treatment that seem to correlate with AAV vector dose. However, these are generally transient and respond to an extended course of high-dose steroids.[160] It has been suggested that an optional liver biopsy be incorporated into liver-directed gene therapy trials in order to ascertain the cause of this early liver transaminitis. Importantly, the long-term effects of AAV-fVIII on transduced hepatocytes and resulting effects on the organ remain unknown.

Targeting the liver also creates a complication for application of AAV-fVIII gene therapy across the entire hemophilia A patient population. Assuming that a hepatotropic AAV-fVIII vector is developed that maintains both curative fVIII levels and fVIII immune tolerance after a single administration over the span of an average human lifetime regardless of the causative F8 mutation, then ideally this gene therapy would be administered to any hemophilia A patient upon diagnosis. However, especially in developed nations, most patients with the moderate-to-severe phenotype are diagnosed in childhood, with more severe phenotypes being diagnosed earlier. The complication arises from the fact that the livers of pediatric patients are still growing and, as AAV vectors are non-integrating, the therapeutic benefit of liver-directed AAV-fVIII gene therapy could be severely attenuated. The situation is complicated further by the aforementioned challenges associated with repeat administration of AAV gene therapy vectors. The problem also then becomes a financial one, as the patient's family would have to consider the cost of first treating with fVIII protein replacement products and waiting to administer the gene therapy, while running the risk of inhibitor development, against the prospect of the cost of an ultimately failed initial gene therapy attempt plus the cost of a second gene therapy treatment and associated treatments that make that second administration possible. We also have yet to understand any adverse

outcomes that may be associated with repeat administration of such a hepatotropic AAV gene therapy vector at the dose ultimately required to achieve durable efficacy.

Circling back to the fVIII inhibitor problem, another gap in knowledge of great clinical interest regards the utility of liver-directed AAV-fVIII gene therapy for inhibitor eradication and immune tolerance induction. If a single AAV-fVIII administration could not only provide a functional disease cure but also be a reliably successful solution for treatment of inhibitor patients, it would be an ideal alternative to non-fVIII protein therapeutics and/or current immune tolerance induction protocols. Past attempts by our group to eradicate fVIII inhibitors in hemophilia A mice using liver-directed AAV-fVIII have been unsuccessful.[202] In contrast to murine models, delivery of AAV-cfVIII has been successful in eradicating preexisting inhibitors in three hemophilia A canines within 4-5 weeks.[217] While one of these animals did have an amnestic response following gene therapy delivery with a peak inhibitor titer of 216 BU/mL, this response resolved within 18 months, which is rapid compared to most attempts at immune tolerance induction protocols utilizing infused fVIII protein. Additionally, an increase in CD4+ CD25+ Foxp3+ Tregs was also observed prior to inhibitor eradication and all animals maintained cfVIII tolerance for over 5 years post vector delivery, including after further challenge with recombinant cfVIII protein. However, it is important to note that attempts at inhibitor eradication in canine studies used a species-matched fVIII transgene, unlike the murine studies that attempted inhibitor eradication with vectors encoding human fVIII or further bioengineered human fVIII transgenes. It is possible that expression of peptides identical to the endogenous protein induced central and peripheral tolerance mechanisms in a manner that bioengineered xenogeneic proteins could not. The possible benefit or requirement of a species-specific fVIII transgene for inhibitor eradication using hepatotropic AAV-fVIII necessitates further investigation.

This also brings to the forefront the concern over fVIII immunogenicity risk after AAV-fVIII delivery to previously untreated patients with little to no fVIII CRM remaining in circulation. As recombinant human fVIII, or further modified and/or chimeric versions of fVIII, could all be seen as fundamentally xenogeneic to a basically fVIII-naïve immune system, the need for a clearer and more complete understanding of the immune response mechanisms and primary immunogenicity risk factors after liver-directed AAV-fVIII is obvious. Again, given the amount of variation in preclinical study results between animal models, and between different AAV-fVIII vectors and study designs evaluated within the same species, it is imperative to investigate the effects that vector design and dosing have on these gaps in knowledge and points of concern regarding hepatotropic AAV-fVIII gene therapy. One or multiple of these independent variables in vector design and delivery could have multiple downstream effects, including but not limited to: (1) the regions of the liver that are transduced and the degree of transduction, (2) transduced hepatocyte intracellular processes, stress responses, and cell turnover, (3) the quality and consistency of the therapeutic fVIII protein that is produced and secreted into circulation, (4) the overall short- or long-term health of the liver organ, (5) the immunobiology of the liver microenvironment, which could influence the balance between tolerance and immunity, (6) the degree of any off-target AAV vector transduction, (7) the anti-AAV capsid immune response, (8) and the kinetics of therapeutic fVIII transgene expression and resulting antigen exposure. Together, these factors could most certainly influence occurrence of adverse outcomes, the durability of efficacious fVIII activity levels, and the immune response to the fVIII transgene protein product. The continued progression of AAV-fVIII gene therapy vectors in clinical trials makes thorough preclinical characterization of these relationships even more pressing.

1.6 Overarching Thesis Hypotheses and Objectives

There is much that remains unknown regarding mechanisms of the immune response to therapeutic proteins, why some patients develop antibodies to these proteins and others do not, and how to predict when such a response is likely to occur. Consequently, there are no reliable strategies for preventing or eradicating this response in patients. Considering the value of the biological drug class and the potential financial, morbidity and mortality consequences for the patient should these drugs fail, research into immune response mechanisms, methods of assessing immunogenicity risk and predicting antibody response incidence, optimal frameworks for evaluating biological medicine product candidates, and strategies for preventing and eradicating antibodies against therapeutic proteins is absolutely critical.

The goal of the current study is to identify the critical pharmacological determinant(s) of fVIII immunogenicity in the preclinical context of AAV gene therapy for hemophilia A and whether pharmacokinetic principles can then be applied to model incidence and risk of the fVIII inhibitor outcome. We hypothesize that there is one dominant, tunable factor in liver-directed AAV-fVIII vector design responsible for incidence of the anti-fVIII inhibitor response after AAV-fVIII delivery to previously untreated hemophilia A mice. Therefore, this study will also help characterize the potential immunogenicity risk of AAV-fVIII in previously untreated hemophilia A patients. Secondarily, the extensive AAV-fVIII dose response study and PK/PD modeling of resulting fVIII activity and inhibitor response data help to fill in the gaps in preclinical evaluation of these gene therapy vectors, as well as help establish a framework for drug development of genetic medicines such as AAV-fVIII to better support the transition into human clinical trials.

Chapter 2

Pilot studies investigating gene therapy design and the inhibitor response to bioengineered fVIII protein and AAV-fVIII vectors in hemophilia A mice

This work is unpublished.

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Taran Lundgren designed and performed experiments and analyzed data.

Chengyu Prince, Athena Russell, and Shanmuganathan Chandrakasan conceived of and/or assisted with experiments and data analysis for c-MPL studies.

Jordan S. Alexander and Kristopher Knight developed and taught experimental techniques.

H. Trent Spencer and Christopher B. Doering conceived and advised on experiments.

2.1 Abstract

Many fVIII gene therapy products are entering and progressing through clinical trials during what is a revolutionary time for gene therapy research. However, uncertainty still remains regarding the mechanisms of the immune response vs. tolerance to recombinant fVIII protein in hemophilia A patients as well as in the ability to predict inhibitor risk. This uncertainty only increases in the arena of gene therapy approaches for hemophilia A. For example, in the case of AAV-fVIII vectors, the understanding of how components such as AAV serotype, promoter design, the transgene sequence and resulting structure of the fVIII protein product, and vector biodistribution affect the fVIII immune response, target organ toxicity, and the durability of protein product expression remains incomplete. Additionally, given the well-established immunogenicity of fVIII in hemophilia A patients, AAV-fVIII gene therapy clinical trials isolate enrollment to patients with >150 prior fVIII exposures and no history of inhibitors. Therefore, there exists no human data on the fVIII immunogenicity risk after AAV-fVIII treatment in previously untreated patients or patients with a history of inhibitors, let alone whether AAV-fVIII can be used to eradicate an active inhibitor response. In the case of LV-fVIII transduction of hematopoietic stem and progenitor cells (HSPC) followed by delivery of these genetically modified cells via bone marrow transplant (BMT), the major concern remains the risk involved in currently established methods of conditioning for BMT with some secondary concern over insertional mutagenesis. One approach being intensely investigated for non-genotoxic BMT conditioning is the conjugation of an immunotoxin to a protein, be it a natural ligand, antibody, etc., that targets HSPCs in the bone marrow compartment with increased specificity. Development of such technology would allow for wide application of such gene therapy approaches to diseases for which the risks associated with current genotoxic BMT conditioning methods are unacceptable. This chapter presents a collection

of pilot studies pertaining to the major questions and concerns associated with fVIII gene therapy products in the arena of their initial stages of design and development.

2.2 Introduction

Two of the greatest challenges in the treatment of hemophilia A are development of a neutralizing antibody response (inhibitors) to the therapeutic fVIII replacement protein and the high patient burden from lifelong factor replacement, especially for patients with the severe phenotype and/or inhibitors. Being a monogenic bleeding disorder for which even small increases in circulating fVIII levels can have a significant clinical benefit, hemophilia A is a prime candidate for functional cure by gene therapy. Both of the currently primary approaches to gene therapy are being investigated for hemophilia A: delivery of genetically modified HSPCs expressing fVIII via lentiviral vector transduction and intravenous delivery of AAV vector expressing a fVIII transgene targeted for hepatocyte expression. The major roadblock in clinical evaluation and implementation of HSPCs stably expressing fVIII are the genotoxic conditioning protocols currently required for successful bone marrow transplant and engraftment of the therapeutic cells. So, while this approach likely has the added benefit of inducing central immune tolerance to the fVIII protein produced by these cells, unfortunately, this approach has been held back largely due to significant health risks associated with the delivery method. Liver-directed AAV vectors are the predominant approach to fVIII gene therapy currently advancing through clinical trials. Indeed, these vectors have been in clinical testing for treatment of both hemophilia A and B for years, with AAV-fVIII for hemophilia A trials yielding multi-year, therapeutic fVIII activity levels and reduced annualized bleeding rates for the first time.

While this is a transformative clinical achievement, the parameters governing the preclinical immune response to fVIII in that context remain unclear, as evidenced by existing preclinical and clinical findings that are somewhat paradoxical and extensive variation in the design of published preclinical AAV-fVIII studies.[48,202] Furthermore, AAV-fVIII products under clinical study demonstrate uniform immunogenicity in non-human primate (NHP) studies at clinical doses despite the high degree of identity between B-domain-deleted human fVIII and the circulating endogenous NHP fVIII.[188,210] Despite the perplexities in preclinical data, AAV gene therapy product candidates continue to advance rapidly through clinical testing, and AAV-fVIII product development progressed to the state where customization of individual candidates has become standard practice. Common design elements include vector packaging (e.g., lipid nanoparticle composition, AAV serotype, lentiviral vector pseudotype), enhancer/promoter sequence(s), transgene sequence (e.g., codon optimization, bioengineered protein products), introns, polyadenylation sequences, and terminal elements (inverted terminal repeats or long terminal repeats). Although characterization of the effects of design elements on efficacy parameters is standard practice, currently, there exists little understanding of the impact these factors have on the immune response to gene therapy products, i.e., their immunogenicity.

Multiple factors involved in the liver-directed AAV vector design and/or in characteristics of the treatment subject could affect the immune response to the transgene-derived therapeutic fVIII protein, including:

<u>Vector</u>

1. Vector serotype
2. Transcriptional regulatory elements (i.e., promoter and enhancer elements)
3. Transgene sequence

4. Vector dose
5. Biodistribution of the vector and protein product

Recipient

6. Genetics
7. Immune system status
8. Liver microenvironment

To elaborate, for example, bioengineered fVIII proteins have shown variations in biosynthetic efficiency due to their transgene sequence, codon-optimization, and differential engagement of proteins involved in fVIII protein production, folding, modification, trafficking, and the unfolded protein response.[3,173-177] Testing of various synthetic, liver-directed promoter/enhancer elements also appears to support differential immunogenicity risk depending on their sequence design.[178] A variable inherent to the structure of the AAV particle is its vector serotype with resulting cell type tropism. Vector dose is another important consideration given that all AAV particles are immunogenic in humans. While development of anti-AAV neutralizing antibodies (NABs) is expected, higher AAV capsid loads could contribute to cellular stress from high energetic burden in transduced cells and a proinflammatory microenvironment in the target organ that indirectly increase the immunogenicity risk of the transgene product.[179-183] The goal of initial phase of this dissertation was to characterize AAV-fVIII gene therapy candidates comparable to those currently under clinical investigation and investigate multiple parameters predicted to be complicit in fVIII immunogenicity after AAV-fVIII gene therapy, with the hypothesis being that one or more of these factors could be identified as the primary contributor(s) to fVIII immunogenicity risk in the context of liver-directed AAV gene therapy.

2.3 Materials and Methods

Mice. All studies were performed under the guidelines set by the Emory University Institutional Animal Care and Use Committee. Every AAV-fVIII and AAV-FLUC-P2A-GFP vector was evaluated in fVIII naïve, male exon 16-disrupted hemophilia A mice back-crossed onto a C57BL/6 background (30% S129 and 70% C57BL/6). The AAV-HCB-HSQ and AAV-E06.TTR-HSQ vectors were also evaluated in TKO mice, which are 100% C57BL/6 mice with the entire F8 coding region deleted. All mice were 8-12 weeks of age at study initiation. While AAV vectors are exclusively administered to male mice, both male and female E16 mice were used in the Advate vs. An53 recombinant protein immunization study.

AAV vectors. A pUC57 plasmid containing the 5' and 3' AAV2 inverted terminal repeats was modified to contain either the HCB or E06.TTR promoter along with the either the HSQ, ET3 or AN53 fVIII gene.[173,175,178,235] All fVIII vector sequences are liver codon-optimized using a liver-specific algorithm previously described[173] and do not contain any CpG dinucleotides. The bicistronic reporter vector contains a firefly luciferase (FLUC) and GFP with a P2A linker transgene substituted in place of the fVIII gene within the same plasmid. The reporter plasmids were evaluated by transfection in Expi293F and HUH-7 cells using TransIT transfection reagent (Mirus Bio). Transfections were performed in white 96-well plates (Corning) and bioluminescence was read using an ELISpot plate reader. The plasmids were provided to Vigene Biosciences for AAV8 vector production and titration. Upon receipt of the product, an SDS-PAGE gel was run for visualization of capsid proteins, and Cryo-EM for packaging efficiency was performed by the Emory University core facility. *In vivo* vector delivery was based on the genome copies/mL titer provided by Vigene Biosciences, which was quantified by qPCR targeting the ITR. All AAV vector administrations were delivered in a total volume of 100µl in PBS via retro-orbital sinus

injection. Blood was always collected via retro-orbital capillary with 3.8% sodium citrate. Plasma was harvested via centrifugation and stored at -80ºC.

An53 immunogenicity study. Male and female E16 mice were pre-randomized into group "A" or "B" for blinded administration of either ancestral fVIII 53 (An53) or human fVIII protein (n=19 mice per group). An53 was produced and purified in house, and Advate was used for the human fVIII comparator. The mice were administered 1ug of each fVIII protein in a total 100ul PBS delivered via retro-orbital sinus injection once a week for 5 weeks. Blood was collected after weeks 3 and 5 via retro-orbital capillary with 3.8% sodium citrate. Plasma was harvested via centrifugation and stored at -80ºC. Samples were assayed for anti-fVIII IgG by ELISA. At week 20 post-initiation of the immunization course, 9 male mice that had been immunized with Advate were treated with either AAV-HCB-ET3 or AAV-E06.TTR-ET3 at 2E13 vg/kg. The mice were divided into the two vector treatment groups based on the Bethesda titers such that each vector was delivered to animals with a similar range of inhibitor titers. Plasma samples were analyzed for fVIII inhibitors 1 month after AAV delivery.

Biodistribution studies. The AAV-HCB-AN53 vector was administered at 2E13 vg/kg to a male E16 mouse. At 5 months post-vector delivery the mouse was sacrificed and the following organs were removed, flash frozen in liquid nitrogen, and stored at -80ºC: liver, small intestine, large intestine, kidney, spleen, lymph nodes, gonads, brain, lungs, heart, bone marrow, blood. Frozen tissue was ground using a mortar and pestle over liquid nitrogen before DNA and RNA were extracted using Qiagen DNeasy and RNeasy kits. qPCR was performed on 600ng of DNA with primers specific to the AN53 gene and SYBR green master mix. A Superscript VILO (Invitrogen) kit was used for generation of cDNA, and 10ul of this first-strand reaction were used in the same qPCR protocol (equivalent to 500ng RNA from the original sample). The plasmid used to generate

the AAV-HCB-AN53 vector was used as a standard for the qPCR reaction. An equivalent amount of naïve E16 mouse liver genomic DNA was spiked into each qPCR sample, including the plasmid standard, but not into the no template water control. For *in vivo* assessment of biodistribution, 2 mice were administered either 6.67E12vg/kg or 6E13 vg/kg of either the AAV-HCB-FLUC-P2A-GFP or the AAV-E06.TTR-FLUC-P2A-GFP reporter. At 5 days post-vector delivery, mice were administered 150 µl/g of Luciferin via intraperitoneal injection and bioluminescence was imaged for 60 seconds using an IVIS Spectrum Imaging System.

AAV-fVIII mouse studies. Initial evaluation of vector efficacy was performed by delivering 10µl (approximately 1.6E13 vg/kg) of each vector (AAV-HCB-HSQ, AAV-E06.TTR-HSQ, AAV-HCB-ET3, AAV-E06.TTR-ET3) to one E16 mouse. The study was then expanded by delivering each vector at 1.6E13 vg/kg to n=3 E16 mice as well as the same dose of AAV-HCB-HSQ and AAV-E06.TTR-HSQ to n=3 TKO mice. Mice were followed for up to 48 weeks. FVIII activity was monitored using a chromogenic plate assay and inhibitors were monitored using an anti-fVIII IgG ELISA.

FVIII assays. FVIII activity in mouse plasma samples was measured via Chromogenix Coatest SP4 Factor VIII chromogenic assay following the manufacturer's instructions (Diapharma). Human Factor Assay Control Plasma (FACT) was used to create the linear standard curve for sample quantification (George King Bio-Medical, Inc.). Prior to anti-fVIII antibody titer assays, plasma was heat inactivated for 30 min – 1 hr at 56°C depending on aliquot volume, then maintained on ice. IgG titers were determined as previously described.[236] Briefly, high-binding, half-area 96-well plates (Corning) were coated overnight at 4°C with 1.5 µg/mL purified recombinant fVIII corresponding to the AAV vector transgene product (i.e., Advate for HSQ; ET3i purified in-house[161]), washed twice with an HBS+0.05% Tween-20 buffer and put in a 2%BSA

blocking buffer at 4°C for a 16 hr minimum or until use (within 2 weeks). After 2 washes, sample plasma was applied in 8 serial 1:2 dilutions to the plate and incubated at room temperature for 1 hr. Detection was performed using goat-anti-mouse IgG-alkaline phosphatase and Alkaline Phosphatase Substrate solution (BioRad). The plate was incubated in the dark for 20 min and read by spectrophotometer at 405nm. The absorbance values were plotted against the logarithm of the plasma dilution, and the titers were determined by the reciprocal of the dilution at which the optical density (OD) value was three times that of the background (naive E16 mouse plasma), OD=0.3. A mixture of anti-fVIII monoclonal antibodies was used as a positive control. FVIII inhibitor Bethesda assays were performed as previously described,[237] with the modification that the Bethesda titer was binned into a range based on dilutions that yielded a residual fVIII activity between 40 - 60%. Any plasma sample that had a readout below the limit of quantification by any antibody assay was given a working value of zero in any data analysis and/or graphical representation; however, the actual value could be somewhere between zero and the limit of quantification. If the data are presented graphically on a log scale, a zero value for fVIII activity or antibody titer was given an arbitrary value of 0.001.

MPL targeting. Full-length human TPO, the N-terminal 163 amino acids of human TPO, an anti-MPL scFV, and a 14 amino acid peptide termed A12505, were cloned into a pVB expression vector preceding the sequence for an IgG2a-Fc domain to create a fusion protein upon expression. The c-MPL targeting proteins were cloned in using gene blocks purchased from Integrated DNA Technologies. These constructs are under the control of a CMV promoter, followed by either a TPO or IL-2 signaling peptide, and with or without a 6x-His tag between the signaling peptide and the (GGGGS)3 linker that precedes the coding region for the c-MPL targeting protein. The two recombinant anti-MPL plasmid pcDNA3.4 expression vectors encoding either the anti-MPL heavy

chain or light chain (Mouse IgG1-kappa) were obtained from Creative Biolabs. The anti-MPL heavy and light chain sequences were then also cloned into the pVB IgG2aFc fusion protein vector construct. Plasmids were transfected into Expi293F cells using TransIT-X2 transfection reagent (Mirus Bio) in FreeStyle 293 culture medium (Gibco). If supernatant was concentrated before use in TPO blocking studies, this was performed in an Amicon filter with 1XHBS+0.05%Tween-20. HEL cells were cultured in complete RPMI-10 medium followed by stimulation with purified, recombinant human TPO at 200 ng/mL, 400 ng/mL or 1 mg/mL for 15, 30, or 60 minutes. The cells were stained for 1 hour at 4ºC with a PE-p-STAT5 antibody, fixed with 4%PFA, and run on a Cytek Aurora flow cytometer to assess c-MPL receptor activation. Following expression of the anti-MPL heavy and light chain plasmids in Expi293F cells, either 20 µl or 50 µl of the culture supernatant, or 1 µl or 4 µl of the concentrated culture supernatant were added to HEL cells in culture for either 30 or 60 minutes. The HEL cells were then washed and purified, recombinant human TPO was added at 400 ng/mL for 45 minutes. The cells were then harvested and stained for p-STAT5 flow cytometry.

2.4 Results

2.4.1 The comparative anti-fVIII IgG response to bioengineered fVIII proteins

It has long been the goal of recombinant fVIII protein engineering to develop fVIII proteins with improved pharmacological properties, most especially longer half-life, decreased immunogenicity, and increased manufacturing efficiency. Regarding immunogenicity, potential immunogenicity risk of a protein drug can be analyzed *in silico* via immunodominant B and T cell epitope and peptide:HLA haplotype binding predictions. While these methods can be valuable for

screening potential therapeutic proteins, and the databases being built from protein biochemistry and immunology data could be excellent resources to guide biopharmaceutical development, *in silico* results cannot be completely relied upon to accurately predict immunogenicity. This parameter should also be evaluated *in vivo* using animal models. Of course, this also cannot completely accurately predict the immunogenicity risk in a human subject, but it is an essential safety and efficacy evaluation step in the drug development pipeline prior to clinical trial initiation. As part of gene therapy design and preclinical evaluation, the recombinant fVIII proteins expressed by the transgenes are evaluated for immunogenicity via intravenous infusion in hemophilia A mice compared to a standard of care recombinant BDD human fVIII protein (Fig. 2.1A), which is also the only form of fVIII incorporated in AAV gene therapy vectors currently in clinical trials. If a bioengineered fVIII demonstrates significantly reduced immunogenicity when delivered intravenously as recombinant protein in preclinical models, it may be an ideal candidate for incorporation in a gene therapy vector and subsequent endogenous production. Members of our group and colleagues have developed a human-porcine chimeric BDD fVIII protein called ET3i, in which the A1, a3-A3 domains are substituted with the porcine sequence (Fig. 2.1B). [175,234,235,238] Taking incorporation of fVIII sequences from other species a step further, members of our group and collaborators at Georgia Institute of Technology have engaged the technique of ancestral sequence reconstruction (ASR) to engineer forms of fVIII that takes advantage of the evolutionary pressures experienced by all species to mine for sequence variations that confer improved pharmacological properties. It is possible that somewhere in evolutionary history or along the phylogenetic path of another species, a fVIII sequence existed that is superior to the extant human fVIII, or that domains of other fVIII sequences could be substituted into the human fVIII sequence to confer superior properties. One example of such a fVIII highlighted by this study is a common

ancestor of the primate and rodent lineages designated Ancestor 53 (An53, Fig. 2.1C). An53 demonstrated reduced cross-reactivity with human patient plasmas containing inhibitors to human fVIII. Another positive aspect is that both ET3i and An53 show enhanced biosynthesis *in vitro*, which could also translate into a more ideal gene therapy transgene candidate.[177,234]

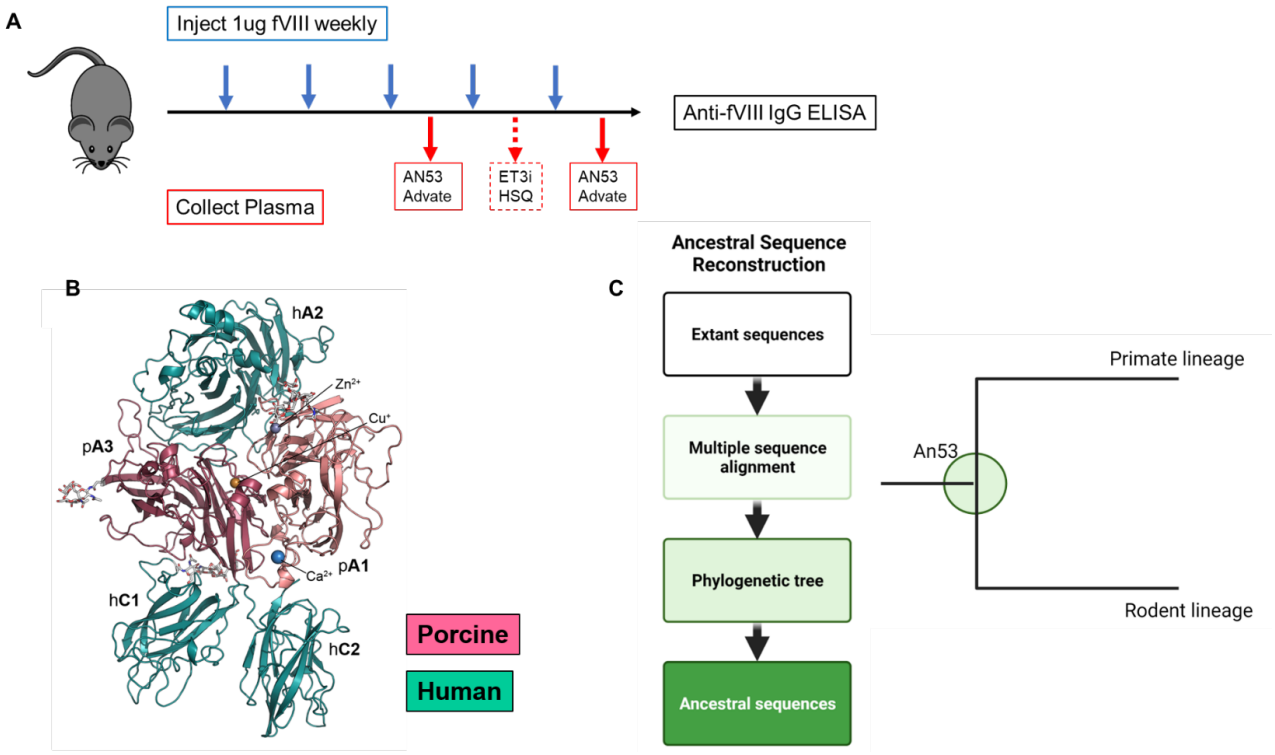


Figure 2.1. The comparative immunogenicity of An53, ET3i and human fVIII proteins.
(A) Hemophilia A (E16) mice were administered weekly 1 µg injections of either Advate or An53 protein for 5 weeks. Plasma was collected 3 days after immunization during weeks 3 and 5. The plasma was then tested for anti-fVIII IgG by ELISA. The ET3i and HSQ immunization comparison was published previously.[161] **(B)** The ET3i protein structure with porcine regions in pink and the human regions in green [ET3i structure: Smith IW, d'Aquino AE, Coyle CW, Fedanov A, Parker ET, Denning G, Spencer HT, Lollar P, Doering CB, Spiegel PC Jr. The 3.2 Å structure of a bioengineered variant of blood coagulation factor VIII indicates two conformations of the C2 domain. J Thromb Haemost. 2020 Jan;18(1):57-69. doi: 10.1111/jth.14621. Epub 2019 Sep 8. PMID: 31454152; PMCID: PMC6940532.] **(C)** This schematic outlines the general steps followed in the process of ancestral sequence reconstruction, which identified ancestral fVIII sequence 53 (An53) as a common fVIII protein ancestor along the primate and rodent lineages that may have superior pharmacological properties [This figure was created using Biorender.com].

A previous study compared the immunogenicity of ET3i to recombinant human BDD fVIII (HSQ) in hemophilia A mice. In that study, mice were administered four weekly 1ug infusions of either ET3i or HSQ, followed by evaluation of fVIII inhibitor production by anti-fVIII IgG ELISA (Fig. 2.1A); however, there was no significant difference observed in the immunogenicity of these two proteins (Fig. 2.2A).[161] ET3i proved just as immunogenic as HSQ. After identification of An53, another *in vivo* immunogenicity study was conducted evaluating An53 wherein hemophilia A mice were given five weekly 1ug infusions of either recombinant An53 or Advate, blood samples were taken three days after the third and fifth infusions, and an anti-fVIII IgG ELISA was used to compare immunogenicity (Fig. 2.1A). An SDS-PAGE gel containing Advate and An53 +/- thrombin from pre-prepared and banked injections prior to unblinding is shown in Figure S2.1. At week 3, there was no significant difference in anti-fVIII IgG titer between Advate and An53 immunized mice (Fig. 2.2B). Interestingly, at week 5 the An53 mice actually had a significantly higher median anti-fVIII IgG titer compared to animals immunized with Advate (Fig. 2.2C).

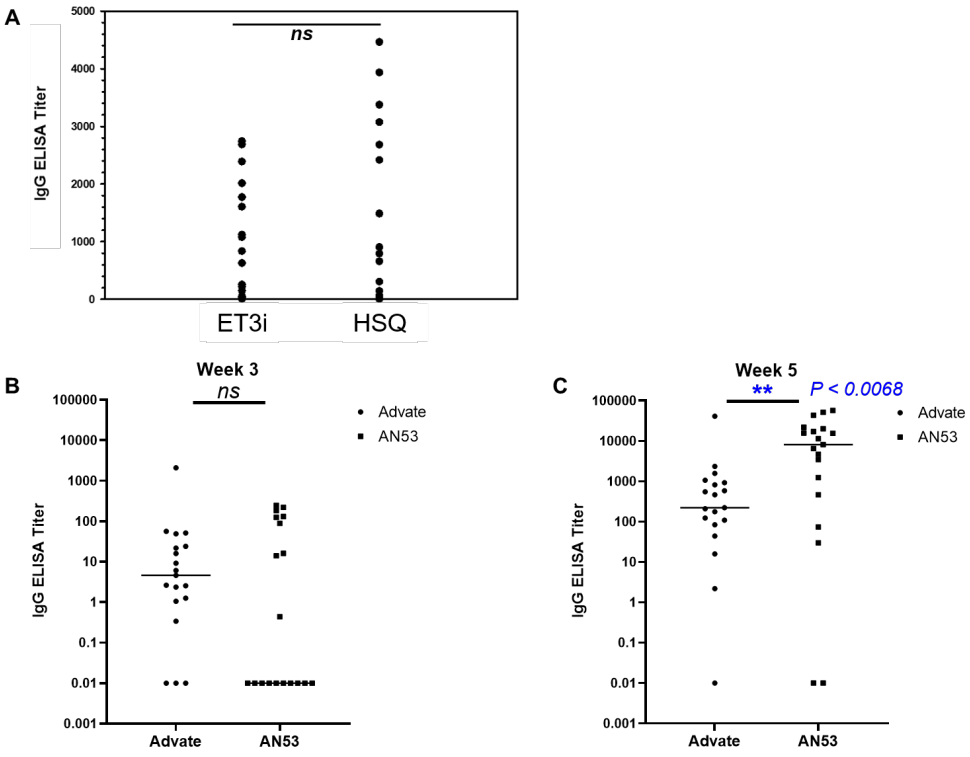


Figure 2.2 The anti-fVIII IgG titers following immunization with ET3i vs. HSQ, or Advate vs. An53. **(A)** The previously published immunization study comparing ET3i and HSQ showed now significant difference anti-fVIII IgG titers. **(B)** At week 3, there was no significant difference in anti-fVIII IgG titers between Advate and An53 immunized mice. **(C)** While most mice immunized with either Advate or An53 had quantifiable anti-fVIII IgG titers by week 5, the median titer was higher in the An53 immunized mice. **(B-C)** Statistical comparisons were assessed by Mann-Whitney U test.

Although An53 immunized mice displayed higher titer values after five immunizations, after week 3 only 47.4% of An53 immunized mice had detectable anti-fVIII IgG compared to 84.2% of Advate immunized mice, which can be visualized in Figure 2.3, panels A and B. The An53 immunized mice displayed a greater increase in anti-fVIII IgG titer from week 3 to week 5, as comparison of linear regressions delineating the change in IgG titers for Advate and An53 immunized mice cannot be represented by the same slope (Fig. 2.3C). However, after week 5 approximately 90% of An53 immunized mice had detectable anti-fVIII IgG titers (17 of 19 mice) compared approximately 95% of Advate immunized mice (18 of 19 mice). Despite differences in the progression of the inhibitor response, most of the mice developed inhibitors to both Advate and An53 proteins, demonstrating that both versions of fVIII are immunogenic in this animal model of hemophilia A, much like ET3i.

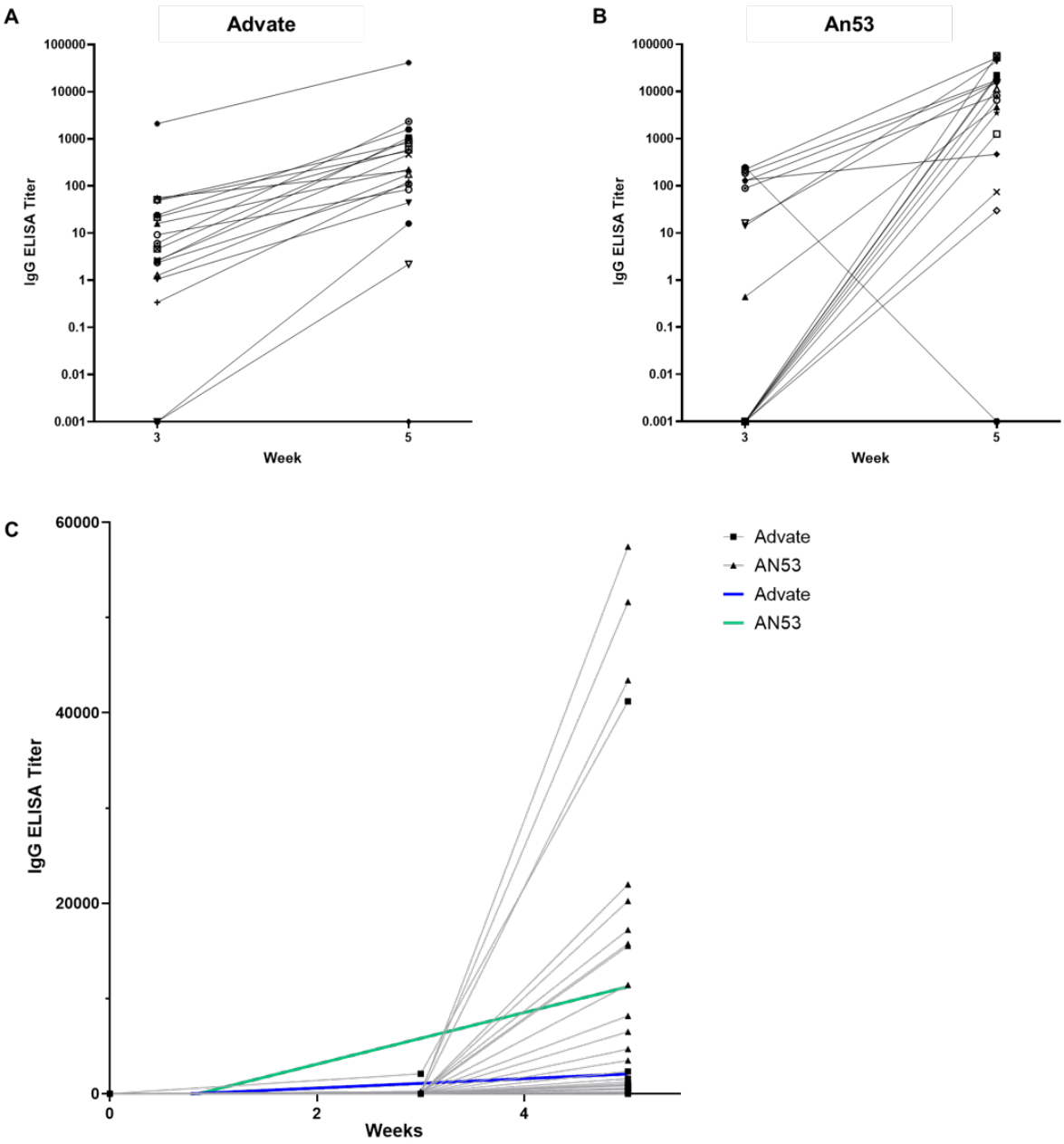


Figure 2.3. The anti-fVIII IgG titers over time following immunization with Advate vs. An53.
(A) This plot demonstrates the change in anti-fVIII IgG ELISA titer from week 3 to week 5 for each mouse immunized with Advate. **(B)** This plot demonstrates the change in anti-fVIII IgG ELISA titer from week 3 to week 5 for each mouse immunized with An53. **(C)** The progression of anti-fVIII IgG titer magnitudes for the Advate and An53 mice were each fit with a linear regression with shared parameters (Advate in blue; An53 in green). Comparison of the slopes of the linear regressions determined that each group cannot be described by the same line, with the An53 immunized mice showing a larger increase in IgG ELISA titer over time.

2.4.2 The characterization of four AAV2/8-fVIII vectors

While thus far the fVIII protein appears to be uniformly immunogenic despite variations in sequence engineering, another component to AAV vector design that could contribute to the fVIII protein product immunogenicity is biodistribution. What cell types the AAV-fVIII vector transduces are dependent upon the serotype of the AAV capsid, and beyond that, the transcription regulatory elements will determine what cell types can express the protein product after transduction. This in turn will affect where the therapeutic fVIII protein is produced and the localization and timing with which the immune system detects that fVIII. As the goal is fVIII production by the liver, all of the vectors evaluated in this project are packaged in AAV8 capsids, as serotype 8 has liver tropism and has demonstrated reduced immunogenicity compared to some other AAV capsids. Additionally, only liver-directed synthetic promoters will be used in order to ensure hepatocyte production of fVIII (Fig. 2.4A). However, despite careful engineering to direct transcription and translation of the AAV-fVIII transgene to hepatocytes, it is important to evaluate *in vivo* expression of the vectors based on the design components that direct expression (i.e., AAV capsid and promoter/enhancer elements). Indeed, evidence exists that supports differential immunogenicity based on promoter/enhancer sequence design. The liver-directed promoter designated E06.TTR has been shown by Grieg, et. al. to result in more rapid increase to therapeutic fVIII activity levels as well as increased inhibitor production within an AAV8 vector compared to other liver-directed promoter/enhancer designs. This study also completed analysis of vector biodistribution and found that human fVIII transcript levels under the E06.TTR promoter were expressed at an average of 1,000-fold lower in tissues other than the liver.[178] The HCB promoter, previously designed and characterized by our group,[173] is shorter and less potent than the E06.TTR promoter. AAV-fVIII vectors employing the HCB promoter have not resulted in inhibitor

formation at doses and with transgenes tested prior to this project. After delivery of an AAV8-HCB-AN53 vector to a male hemophilia A mouse, multiple organs were harvested and processed for both DNA and RNA extraction. (RT)-qPCR results indicate that both fVIII DNA and RNA are expressed in the liver with negligible expression in other tissues after delivery of an AAV-fVIII vector encoding the HCB promoter within an AAV8 capsid (Fig. 2.4B and C).

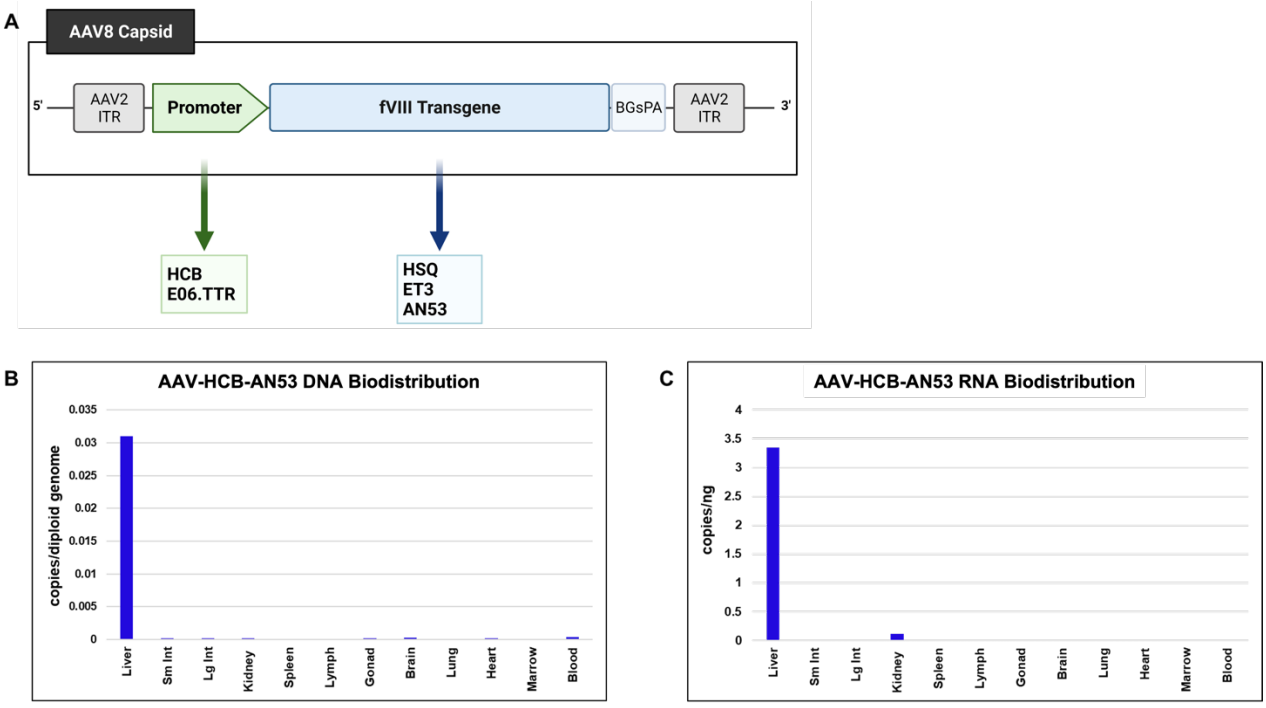


Figure 2.4. The biodistribution of an AAV8 vector encoding the HCB promoter.
(A) This schematic outlines the structure of all AAV-fVIII vectors used in this project [This figure was created using Biorender.com]. **(B)** AAV8-HCB-AN53 DNA distribution assessed by qPCR demonstrates that An53 DNA is almost exclusively detectable in the liver. **(C)** AAV8-HCB-AN53 RNA distribution assessed by RT-qPCR demonstrates that An53 RNA is expressed almost exclusively in the liver with some low-level expression detected in the kidney.

To further assess the *in vivo* biodistribution and expression of AAV8 vectors encoding either the HCB or E06.TTR promoter, a bicistronic AAV8 reporter vector was designed. This vector encodes a transgene containing a firefly luciferase (FLUC) and green fluorescent protein (GFP) gene connected by a P2A (ribosomal skipping) sequence (Fig. 2.5A). *In vitro* evaluation of

the reporter vectors in Expi293F and Huh-7 cells is shown in Figure S1.2. The AAV8-HCB-FLUC-P2A-GFP and AAV8-E06.TTR-FLUC-P2A-GFP vectors were delivered to mice at doses of 6.67E12 vg/kg and 6E13 vg/kg. At day 5 post vector delivery, the mice were administered luciferin and imaged for bioluminescence. Despite differences in the predicted potency of the two promoters, there was no significant difference in bioluminescence signal between the two promoters at each dose (Fig. 2.5B). However, it is clear from the images that *in vivo* expression is localized in the area of the liver (Fig. 2.5C).

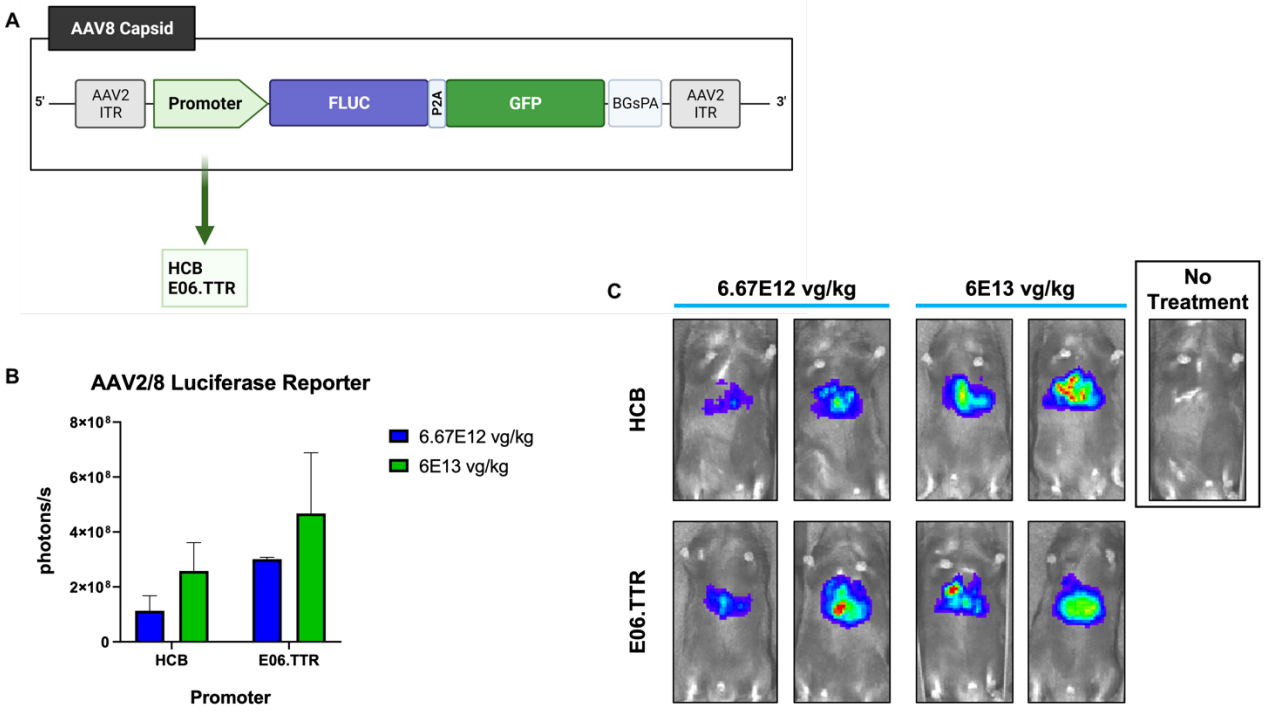


Figure 2.5. The biodistribution of AAV8 vectors encoding the HCB or E06.TTR promoter.
(A) This schematic outlines the structure of the bicistronic AAV8-FLUC-P2A-GFP reporter vector designed to assess expression driven by either the HCB or E06.TTR promoter [This figure was created using Biorender.com]. **(B)** Expression of either the HCB or E06.TTR reporter vectors at either 6.67E12 vg/kg or 6E13 vg/kg does not show a significant difference in bioluminescence signal between each vector at each dose 5 days after vector delivery (Two-way ANOVA, p values between 0.1 – 0.17). **(C)** Representative images of mice from all treatment groups show that bioluminescence signal is centered on the liver with no signal observed in the no treatment control.

Evaluation of individual components of these AAV-fVIII vectors confirms liver expression without any clear indication of what components could be the main cause of differential fVIII protein immunogenicity following AAV-fVIII delivery. Therefore, a pilot study was initiated using four different vector designs. As E06.TTR is a promoter that has demonstrated high fVIII production and higher immunogenicity signals, and HCB is a promoter that has yet to yield an anti-fVIII immune response when incorporated in an AAV-fVIII vector, both of these promoters were chosen to be incorporated in the comparison. The human BDD fVIII protein is the only version of recombinant fVIII incorporated in AAV vectors currently in clinical testing and offers a baseline comparison to standard of care treatment as far as the fVIII protein sequence is concerned. Hence, human BDD fVIII (herein referred to as "HSQ") was chosen for one of the fVIII transgenes. As a comparator to HSQ, ET3 was chosen as the other fVIII transgene sequence. There is no difference in the recombinant protein's immunogenicity, but there exists an abundance of data on the efficacy and biosynthesis of ET3. Indeed, ET3 has been shown to display higher biosynthetic efficiency than HSQ due to decreased engagement of the unfolded protein response (UPR), leading to enhanced secretion.[174] ET3 is also being utilized in multiple open investigational new drug (IND) gene therapy programs. Table 2.1 lists the components of the vectors evaluated *in vivo* for comparative efficacy and immunogenicity. Representative cryo-EM images of each vector and the calculated packaging efficiencies are shown in Figure S2.3.

FVIII transgene	**Promoter**	**B domain deletion**	**Codon optimization**	**Sequence alteration**
LCO human fVIII (HSQ)	HCB	Yes	Liver	14 amino acid SQ linker replacing B domain
LCO human fVIII (HSQ)	EnTTR-En34-TTR (E06.TTR)	Yes	Liver	14 amino acid SQ linker replacing B domain
LCO ET3 (ET3)	HCB	Yes	Liver	Porcine sequences in A1, activation peptide, A3
LCO ET3 (ET3)	EnTTR-En34-TTR (E06.TTR)	Yes	Liver	Porcine sequences in A1, activation peptide, A3

Table 2.1. Design components of the AAV8-fVIII vectors.

For initial evaluation of the four vectors, an n-of-1 pilot study was initiated in male E16 hemophilia A mice. Each mouse was administered 1.6E13 vg/kg of one of the vectors (Fig. 2.6). Plasma samples were taken every 2 weeks for the first 8 weeks, and again at weeks 12, 38 and 60 post-AAV-fVIII delivery. The vectors encoding ET3 showed the highest initial peaks in fVIII activity, approximately 4 IU/mL by week 4, while the vectors encoding HSQ showed week 4 activities between 1.4 – 1.5 IU/mL. The mouse administered E06.TTR-ET3 died sometime between weeks 38 and 60. The mice administered HCB-HSQ and HCB-ET3 maintained therapeutic levels of fVIII through week 60, approximately 0.6 IU/mL. Interestingly, the mouse administered E06.TTR-HSQ lost activity by week 38 yet anti-fVIII IgG was undetectable in the plasma.

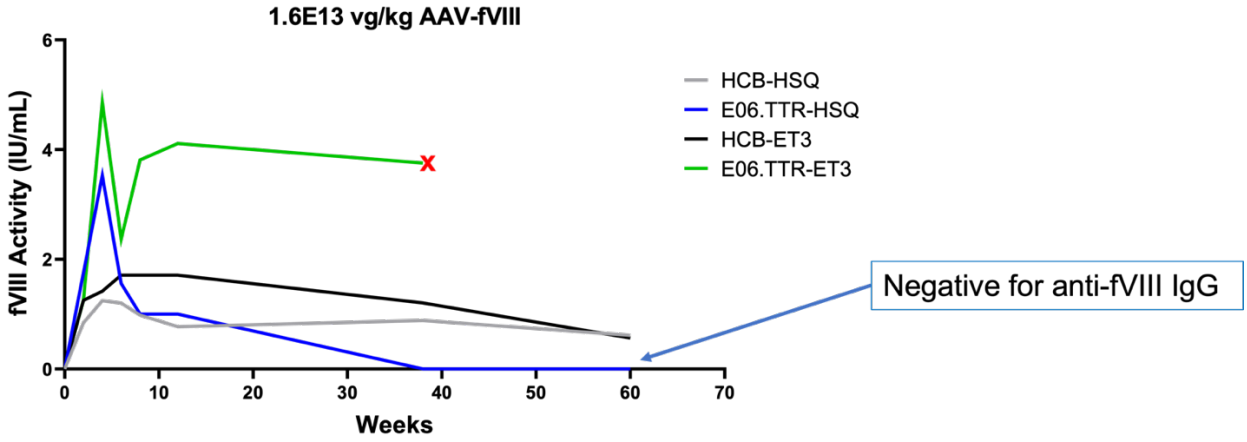


Figure 2.6. FVIII activity over time after delivery of four different AAV-fVIII vectors in E16 mice. One hemophilia A mouse was treated with 1.6E13 vg/kg of one of the four AAV-fVIII vectors evaluated in this study, and the plasma fVIII activity was monitored over a period of 60 weeks.

The pilot *in vivo* screening of the four vectors demonstrated their efficacy but provided no preliminary conclusions regarding fVIII immunogenicity. The study was expanded to treat an additional n=3 mice with 1.6E13 vg/kg of each vector (Fig. 2.7). Plasma samples were taken every 2 weeks for the first 6 weeks, and again at weeks 9, 17, 20, 25 and 48. The mice administered

AAV8-HCB-HSQ showed gradual initial increases in fVIII activity that plateaued at therapeutic levels between 0.6 – 1 IU/mL. Mice administered either AAV8-E06.TTR-HSQ or AAV8-HCB-ET3 showed the highest initial peaks in fVIII activity at 2 weeks, and one out of three mice in each of those groups developed an anti-fVIII IgG titer by week 48. The mouse administered E06.TTR-HSQ lost fVIII activity earlier (by week 17) than the mouse administered HCB-ET3 (by week 48). The third mouse in the HCB-ET3 group died under anesthesia just before the first plasma collection. The mice administered E06.TTR-ET3 had much lower fVIII activity levels at week 2, with one mouse showing no detectable fVIII activity. By week 6, two of these mice had detectable anti-fVIII IgG titers and the third mouse had a detectable titer by week 9. The anti-fVIII IgG titers for all three mice persisted through time of sacrifice at week 48.

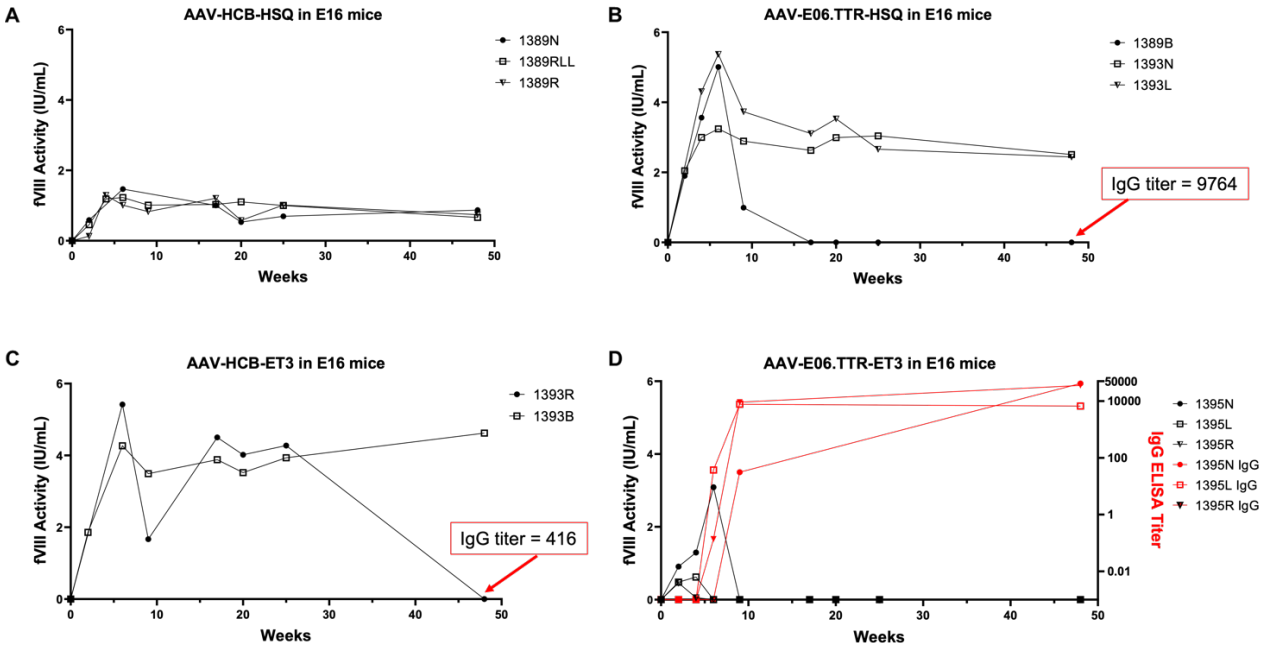


Figure 2.7. Expanded evaluation of fVIII activity and inhibitors after treatment with four different AAV-fVIII vectors in E16 mice.
N=3 E16 mice were administered 1.6E13 vg/kg of either **(A)** AAV-HCB-HSQ, **(B)** AAV-E06.TTR-HSQ, **(C)** AAV-HCB-ET3, or **(D)** AAV-E06.TTR-ET3, and both fVIII and anti-fVIII IgG were monitored for up to 48 weeks post-AAV delivery. Anti-fVIII IgG ELISA titers are indicated with either red arrows **(B-C)** or red curves **(D)**. The third mouse in **(C)** died under anesthesia just prior to the first plasma collection.

Interestingly, although the AAV-HCB-HSQ mice maintained fVIII activity levels through week 48 and were negative for inhibitors, mouse 1389R had a large growth on the liver at time of sacrifice. The histopathology report on the excised growth from University of Georgia determined that it was "Hepatocellular hyperplasia with hepatocellular vacuolar degeneration and cholangiolar hyperplasia." This indicates a noncancerous hyperplasia in the nodule that was replacing and compressing sinusoids and adjacent liver tissue, which would usually result from hepatic injury (Fig. S2.4).

Beyond vector design, another variable hypothesized to have great importance regarding fVIII immunogenicity, whether delivered as a recombinant protein drug or produced endogenously after gene therapy delivery, is the genetics of the host and the type of *F8* mutation. For comparison based on *F8* mutation, n=3 total F8 knock-out mice (TKO) were also administered 1.6E13 vg/kg of either AAV-HCB-HSQ or AAV-E06.TTR-HSQ (Fig. 2.8). The TKO mice administered HCB-HSQ showed a similar sustained fVIII activity profile to the E16 mice, except that they had much higher initial peak fVIII activity levels at 2 weeks, approximately 1.4 IU/mL in the TKO mice compared to around 0.4 IU/mL in the E16 mice. Additionally, one of these mice had very low activity levels at 48 weeks (0.122 IU/mL) but no inhibitors. The other two TKO mice administered AAV-HCB-HSQ had higher sustained therapeutic fVIII activity levels of approximately 2 IU/mL compared to their E16 counterparts. Interestingly, the TKO mice administered AAV-E06.TTR-HSQ did not demonstrate the high fVIII activity levels that were sustained for two of the three E16 mice. Instead, while one TKO mice had 2 IU/mL fVIII activity at week 2, the other two mice had no fVIII activity. All three TKO mice had undetectable fVIII levels by week 4 and showed signs of anti-fVIII IgG titer development as early as week 2. Anti-fVIII IgG titers fluctuated for the first

6 weeks, but were sustained by week 9, with one mouse (139N) dying after week 9. It should also be noted that the genetic background of the TKO mice is 100% C57BL/6, while the genetic background of the E16 mice is approximately 70% C57BL/6 and 30% S129. Therefore, contributions of host genetics to the observed anti-fVIII immune response cannot be ruled out.

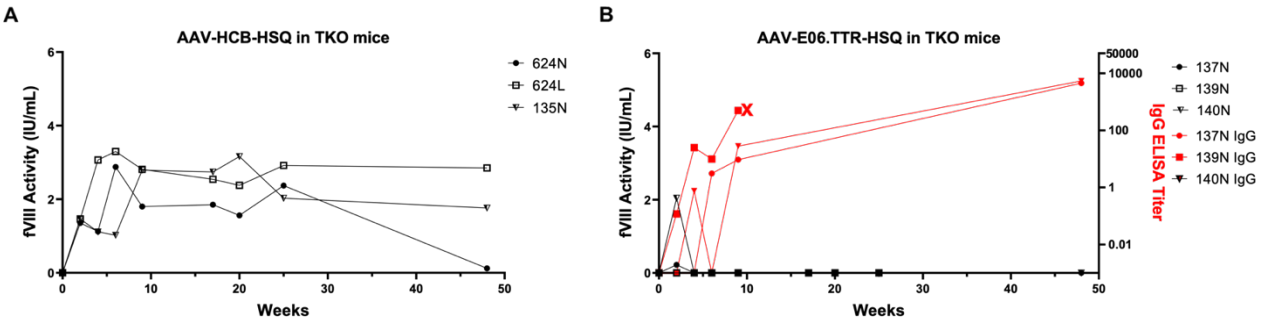
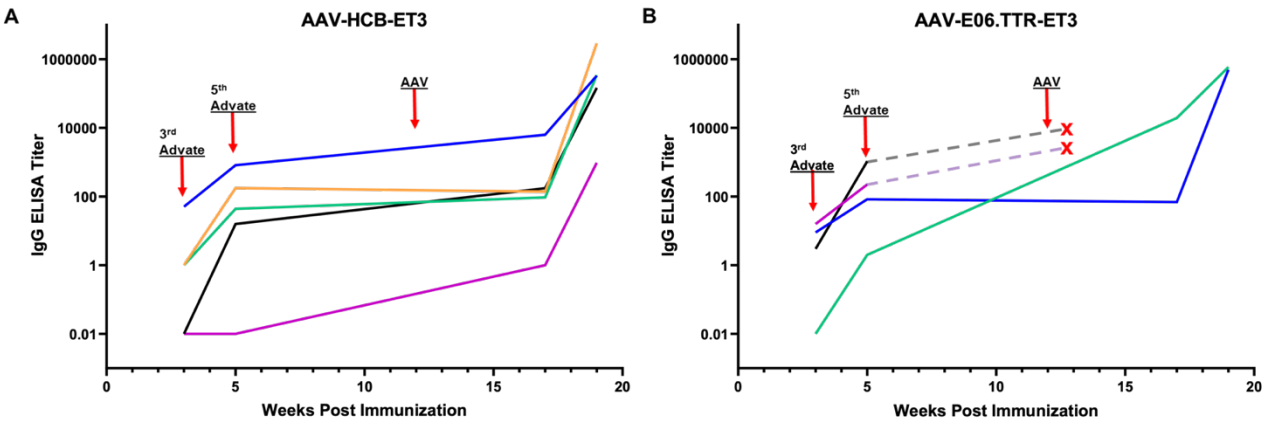


Figure 2.8. FVIII activity over time after treatment with AAV-HSQ vectors in TKO mice.
N=3 TKO mice were administered 1.6E13 vg/kg of either **(A)** AAV-HCB-HSQ or **(B)** AAV-E06.TTR-HSQ, and both fVIII and anti-fVIII IgG were monitored for up to 48 weeks post-AAV delivery. The anti-fVIII IgG ELISA titers are indicated by red curves in **(B)**.

One area of great interest in gene therapy research that is related to the immune response to fVIII is whether or not in can be used to establish and maintain tolerance to the fVIII protein and induce tolerance in patients with inhibitors. Preclinical studies have had more success in this area with AAV-fIX gene therapy than with AAV-fVIII; however, some canine studies have yielded data supporting the prospect of immune tolerance induction with AAV-fVIII. Despite this, our group has yet to successfully eradicate inhibitors in hemophilia A mice using AAV gene therapy. As an additional, preliminary way to add insight to this question, the 9 male E16 mice that were immunized with Advate in the An53 vs. Advate immunization study (Figs. 2.1 – 2.3) were treated with either AAV-HCB-ET3 or AAV-E06.TTR-ET3 to assess whether liver-directed AAV gene therapy expressing a fVIII with some superior pharmacological properties could reduce or ameliorate inhibitor titers. At 12 weeks post initiation of Advate immunization, plasma samples were assessed for functional inhibitor titers by Bethesda assay. The mice, designated by letters A

through I, were ranked based on Bethesda titer and randomly divided between the two treatment groups such that each group contained mice with a complete range of Bethesda titers (Fig. 2.9). At 3 days post-Bethesda titer determination, mice A, B, D, G and H were administered 1E13 vg/kg AAV-HCB-ET3 (Fig. 2.9A) and mice C, E, F and I were administered 1E13 vg/kg AAV-E06.TTR-ET3 (Fig. 2.9B). Plasma was collected at weeks 4 and 6 post-AAV delivery (approximately weeks 17 and 19 post-initiation of Advate immunization) and assessed for anti-fVIII IgG by ELISA, and Bethesda titers were quantified once again on the week 19 samples. Two mice (C and E) died between AAV-E06.TTR-ET3 delivery and the first plasma collection at 17 weeks. Interestingly, these two mice had higher IgG titers at time of AAV-E06.TTR-ET3 delivery but lower Bethesda titers. None of the mice administered AAV-HCB-ET3 died within 6 weeks post-vector delivery (study week 19). However, all Advate immunized mice treated with either of the AAV-ET3 vectors displayed both an increase in anti-fVIII IgG and Bethesda titer within the study timeframe. In fact, inhibitors increased to extremely high levels with IgG titers around 10^6 and Bethesda titers around 500 BU/mL for the mice surviving out to week 19.



Bethesda titer over time (Weeks)				
	3	5	12	19
H	--	--	79	523
G	--	--	47	>>500
D	--	--	21	582
B	--	--	13	430
A	--	--	--	6

Bethesda titer over time (Weeks)				
	3	5	12	19
F	--	--	40	>>500
I	--	--	50<BU<500	>>500
E	--	--	25	DEAD
C	--	--	20<B<50	DEAD

Figure 2.9. FVIII inhibitors increase after delivery of AAV-ET3 to Advate immunized E16 mice. The 9 male Advate immunized mice from the comparative immunogenicity study of Advate and An53 protein were administered 1E13 vg/kg of either **(A)** AAV-HCB-ET3 or **(B)** AAV-E06.TTR-ET3 at 12 weeks post-initiation of the immunization course. Mice were divided between AAV vector treatment groups based on week 12 Bethesda titers. Plasma was collected at weeks 17 and 19 post-immunization (4 and 6 weeks post-AAV vector delivery) and assayed for anti-fVIII IgG. The week 19 plasma was also assayed for Bethesda titer.

Mouse A in the AAV-HCB-ET3 treatment group was the only mouse to still test negative for anti-fVIII IgG at time of vector delivery and was also the only mouse to show transient, detectable fVIII activity levels at week 17 (0.015 IU/mL, data not shown). Mouse A did maintain low anti-fVIII IgG and Bethesda titers through week 19; however, the titers followed the same trend of increasing after AAV-ET3 delivery. None of the other AAV-ET3 treated mice had detectable fVIII activity at weeks 17 or 19.

2.4.3 A potential conditioning strategy for transplantation of LV-fVIII transduced HSPCs

Should preclinical and clinical studies show that liver-directed AAV-fVIII gene therapy has limited ability to establish immune tolerance to the therapeutic fVIII protein and/or is unable to induce tolerance in patients with inhibitors, another avenue of gene therapy investigation that has great promise in this area is bone marrow transplant with HSPCs transduced with lentiviral fVIII (LV-fVIII) gene therapy. However, as previously mentioned, the major barrier to this treatment option is the high-risk genotoxic conditioning regiments required for depletion of endogenous HSPCs that allows for engraftment of the gene therapy modified HSPCs. Members of our group have pioneered preclinical studies establishing a non-genotoxic conditioning protocol that employs an immunotoxin targeting CD117 (c-kit). This protocol uses an ani-CD117 monoclonal antibody conjugated to saporin, which is a protein that irreversibly inactivates ribosomes leading to cell death. This immunotoxin was used to successfully administer HSPCs

transduced with a lentiviral vector expressing ET3 in hemophilia A mice. One major advantage of this approach is the hypothesis that endogenous HSPC depletion followed by delivery of HSPCs expressing the therapeutic fVIII into the bone marrow will lead to establishment of central immune tolerance and bolster maintenance of peripheral immune tolerance to fVIII. A critical component of drug development for this approach is identification of a suitable target for the immunotoxin, one that effectively depletes endogenous HSPCs with limited off-target effects. For example, CD117 is expressed in other tissues, including the central nervous system. If an alternative target with high expression on HSPCs but limited expression in other tissues could be found, this would be more pharmacologically ideal. Therefore, in parallel with investigations into AAV-fVIII therapeutic development and the immune response to fVIII following gene therapy, our group has also begun investigating alternative targets for HSPC depletion using immunotoxins. One alternative target of interest is the c-MPL receptor, whose native ligand is thrombopoietin (TPO). C-MPL has robust expression in the hematopoietic cell compartment with a more limited expression profile in other tissues compared to CD117. Native c-MPL and its ligand, TPO, play important roles in megakaryocyte differentiation and maturation, promoting platelet production, and in CD34+ HSPC cell functions such as survival, DNA repair, and quiescence (Fig. 2.10).

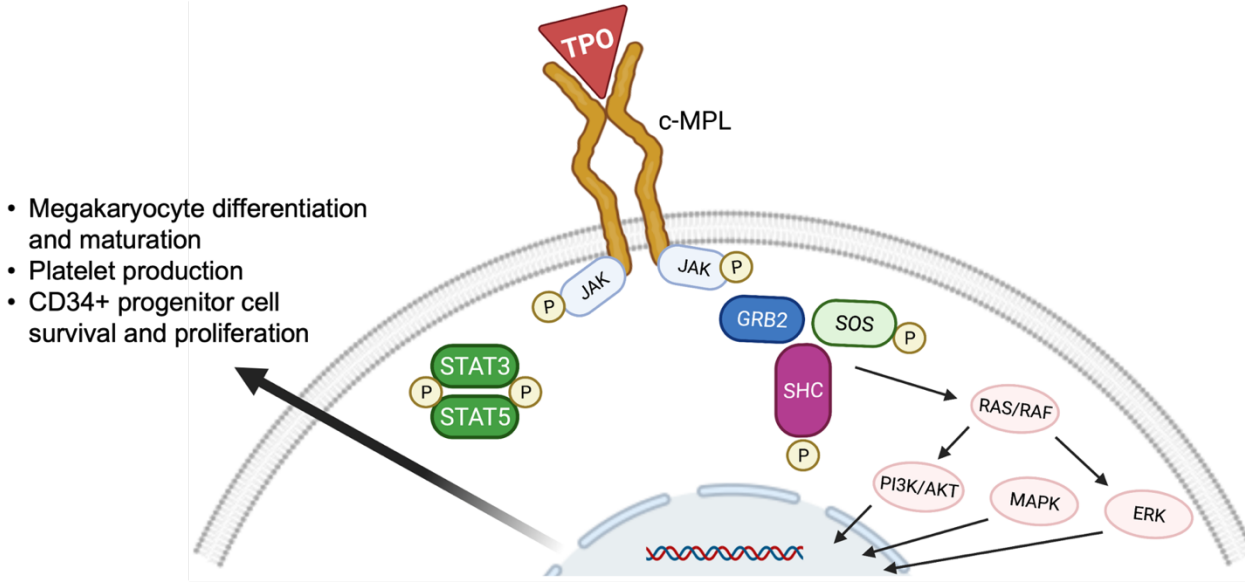


Figure 2.10. C-MPL receptor signaling.
This figure demonstrates the main steps in intracellular signaling following c-MPL receptor activation by its native ligand, TPO. [This figure was created using Biorender.com]

Preliminary data incorporated in an NIH U54 project grant with our group and multiple collaborators from three institutions demonstrates that c-MPL expression is enriched in hematopoietic stem cells (HSC) compared to hematopoietic progenitor cells (HPC) in both humans and mice with minimal expression outside of the hematopoietic compartment, which would be ideal for HSC targeted depletion prior to delivery of LV-fVIII HSPCs (Fig. 2.11).

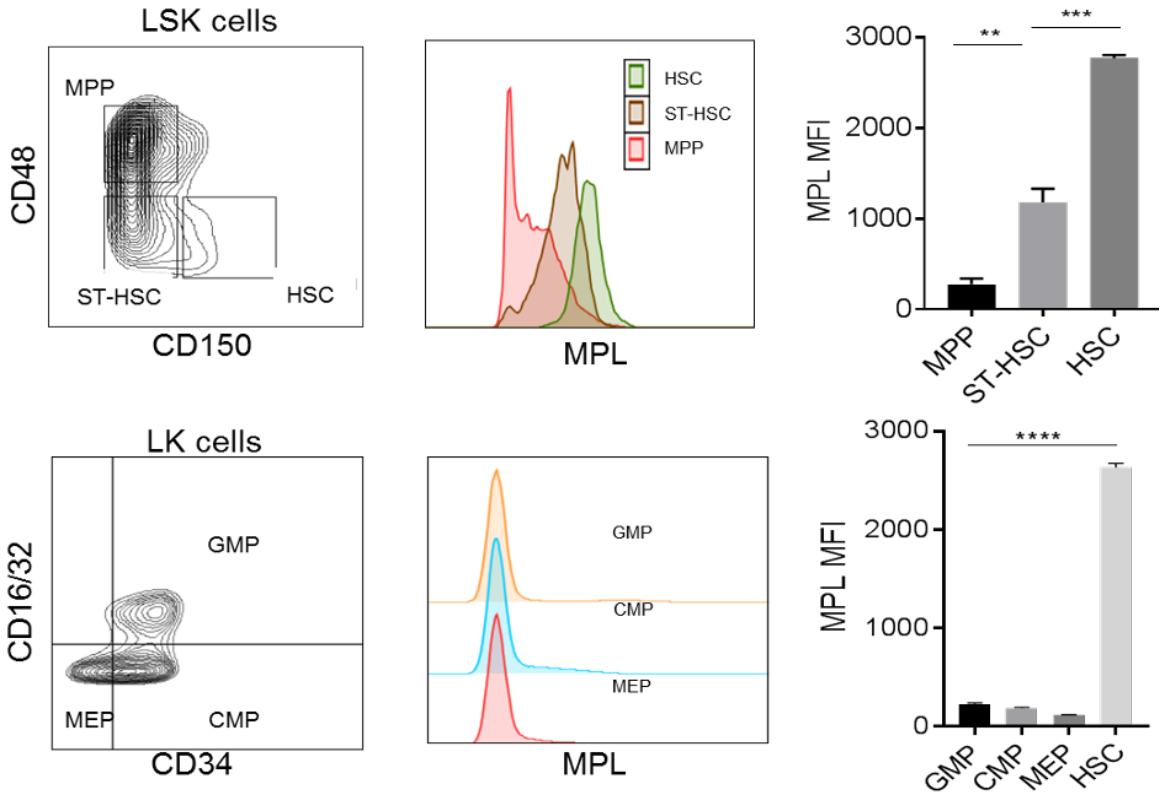
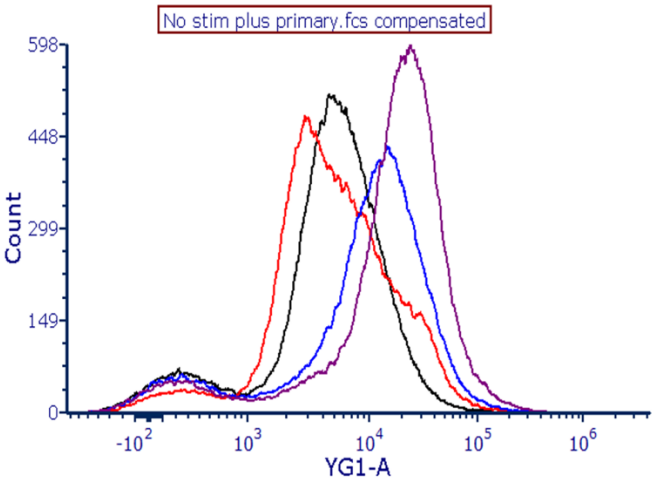


Figure 2.11. c-MPL expression in the hematopoietic compartments of mice.
These data profile c-MPL expression in LSK and LK cell populations in n=12 mice by flow cytometry, demonstrating highly enriched expression on HSCs.

In order to evaluate candidate proteins to target the c-MPL receptor once conjugated to saporin or another toxin, an assay must be developed that reports successful engagement of the c-MPL receptor. For translational relevance, the cell-based assay developed here employs a human erythroleukemia cell line (HEL) that is stimulated *in vitro* with TPO followed by fluorescent antibody staining of the cells for phosphorylated STAT5 (p-STAT5), which is a downstream signaling component following c-MPL receptor activation. Successful stimulation of c-MPL was confirmed via detection of p-STAT5 after treatment with 200 ng/mL, 400 ng/mL, and 1 mg/mL of TPO for 15, 30 or 60 minutes. Flow cytometry data from a 400 ng/mL treatment course are shown here, and histogram overlays display the %p-STAT5 positive cells compared to HEL cells without stimulation (Fig. 2.12).



Histogram #	Filename	Parameter	% Positive
1	No stim plus primary.fcs compensated	YG1-A	0.00
2	2ug TPO 15 min.fcs compensated	YG1-A	7.24
3	2ug TPO 30 min.fcs compensated	YG1-A	35.85
4	2ug TPO 60 min.fcs compensated	YG1-A	54.68

Figure 2.12. TPO stimulation of HEL cells shows increased p-STAT5
HEL cells were stimulated in culture with purified, recombinant human TPO and assessed for c-MPL receptor activation by flow cytometry for p-STAT5 (PE antibody on the YG1 channel). %Positive cells indicates the percent increase in p-STAT5 positive cells compared to the no-TPO stimulation control HEL cells.

This TPO stimulation of HEL cells could be used to screen potential c-MPL targeting candidates via their ability to block TPO stimulation leading to a reduction or absence of detectable

p-STAT5. Five candidates were designed and cloned for future evaluation of their ability to bind c-MPL and block TPO activation (Fig. 2.13A):

1. Recombinant anti-MPL heavy chain and light chain sequences intended for co-transfection
2. Full-length human TPO fused to an IgG2aFc with a TPO signaling protein
3. N-terminal (c-MPL-binding domain) human TPO fused to an IgG2aFc with a TPO signaling protein
4. An anti-MPL scFv fused to an IgG2aFc with an IL-2 signaling protein
5. A 14 amino acid peptide referred to as AF12505 with high affinity for c-MPL (identical to the peptides linked to IgG1 heavy chain forming romiplostim, which is used to treat idiopathic thrombocytopenic purpura) fused to an IgG2aFc with an IL-2 signaling protein.

The human TPO, anti-MPL scFv, and AF12505 constructs were cloned with and without a 6x His tag. Initial *in vitro* transfection and expression studies were undertaken with the anti-MPL heavy and light chain plasmids in Expi293F cells and compared to an identical plasmid purchased from Creative Biolabs that was used for preliminary testing alongside an anti-MPL antibody (Fig. 2.13B). Troubleshooting remains to be done to achieve ideal expression of the anti-MPL heavy and light chain polypeptides.

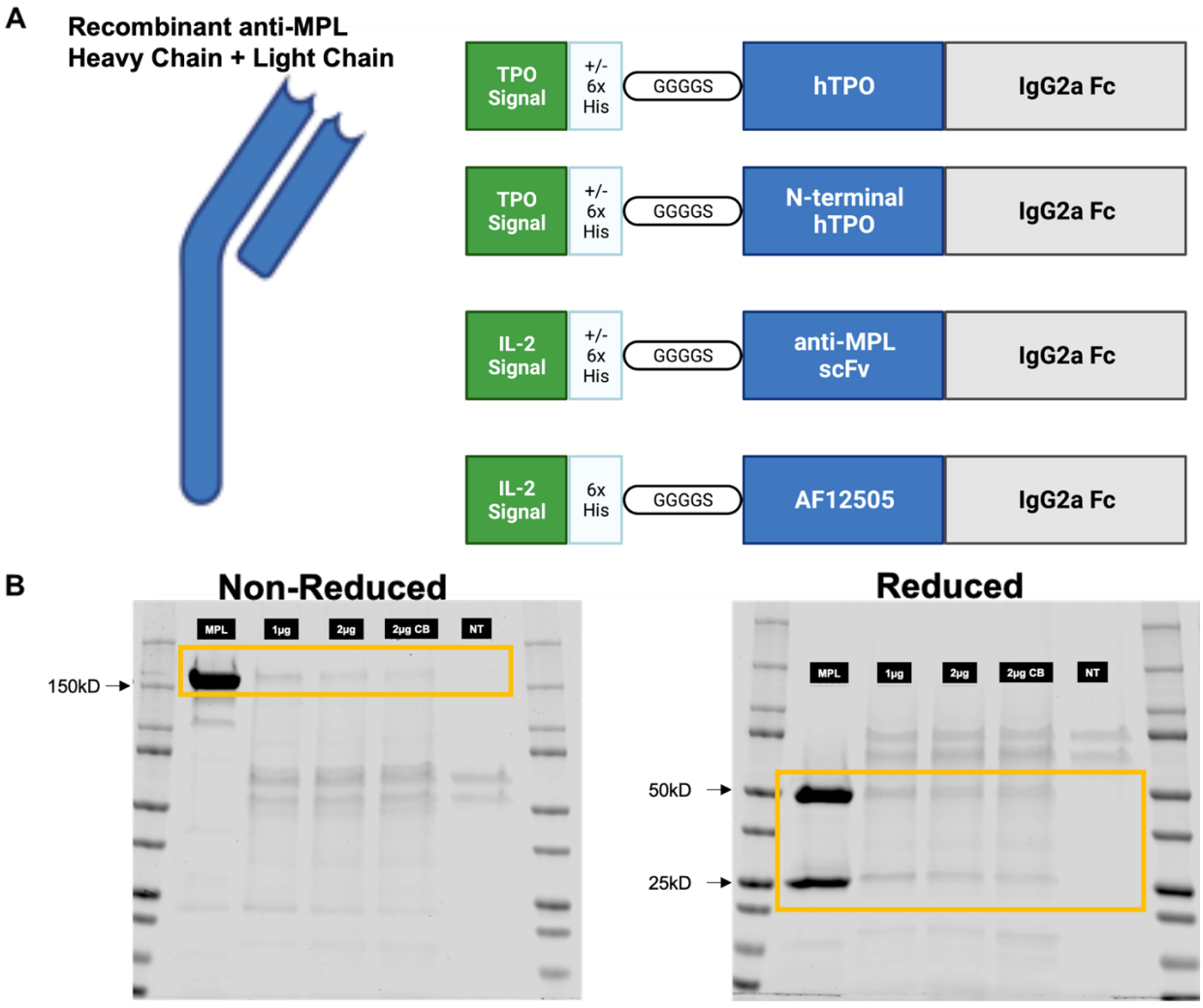
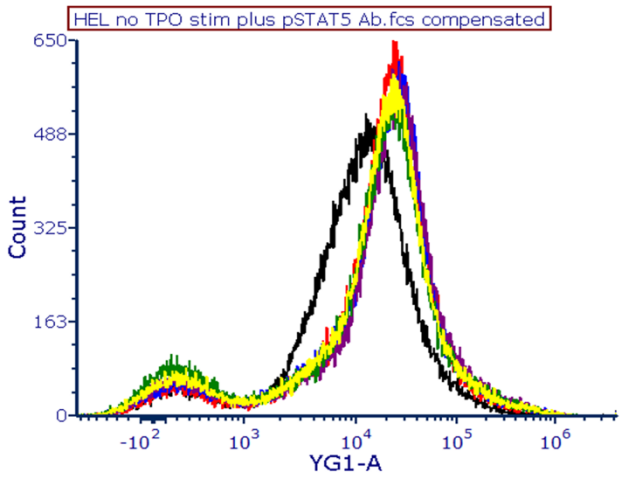


Figure 2.13. Construct design for recombinant IgG2aFc proteins targeting c-MPL
(A) Five candidates were chosen for evaluation of their ability to target c-MPL, with four of these being designed as IgG2aFc fusion proteins. [This figure was created using Biorender.com]. **(B)** This SDS-PAGE gel shows initial anti-MPL heavy chain and light chain plasmid co-transfection results in both non-reduced and reduced conditions. (MPL = a commercial anti-MPL antibody, "1 µg" = a co-transfection of 1 µg/mL of each plasmid, "2 µg" = a co-transfection of 2 µg/mL of each plasmid, "2 µg CB" = a co-transfection of 2 µg/mL of each Creative Biolabs commercial plasmid, and "NT" = no transfection control)

Although expression was not robust, supernatant was collected from the initial anti-MPL heavy and light chain co-transfections and tested for its ability to block TPO stimulation of HEL cells. While the neat co-transfection supernatant was unable to reduce p-STAT5 signaling, a concentrated preparation of the supernatant did show some reduction in p-STAT5 positive cells

(Fig. 2.14). Optimization of the expression and protein collection protocols will likely improve c-MPL targeting and the TPO blocking results.



HEL no TPO stim plus pSTAT5 Ab.fcs compensated
HEL plus TPO stim.fcs compensated
20ul HCLC 30 min.fcs compensated
50ul HCLC 30 min.fcs compensated
1ul Conc HCLC 30 min.fcs compensated
4ul Conc HCLC 30 min.fcs compensated

Histogram #	Filename	Parameter	% Positive
1	HEL no TPO stim plus pSTAT5 Ab.fcs compensated	YG1-A	0.00
2	HEL plus TPO stim.fcs compensated	YG1-A	24.34
3	20ul HCLC 30 min.fcs compensated	YG1-A	24.14
4	50ul HCLC 30 min.fcs compensated	YG1-A	26.15
5	1ul Conc HCLC 30 min.fcs compensated	YG1-A	18.78
6	4ul Conc HCLC 30 min.fcs compensated	YG1-A	21.13

Figure 2.14. TPO stimulation of HEL cells after blocking with anti-MPL heavy chain + light chain
After co-transfection of the anti-MPL heavy and light chain expression plasmids, the neat or concentrated culture supernatant was transferred to HEL cells in culture. After blocking for 30 minutes, the cells were washed and stimulated with purified, recombinant human TPO for 45 minutes. The HEL cells were then assessed for c-MPL receptor activation by flow cytometry for p-STAT5 (PE antibody on the YG1 channel). %Positive cells indicates the percent increase in p-STAT5 positive cells compared to the no-TPO stimulation control HEL cells.

2.5 Discussion

Similar to the body of preclinical work on fVIII protein immunogenicity and the immune response to fVIII following AAV-fVIII gene therapy, the evaluations of the factors predicted to contribute to fVIII immunogenicity after AAV gene therapy performed here do not reach a definite conclusion as to the primary parameter responsible for inhibitor development. In line with preclinical and clinical observations thus far, the fVIII protein is uniformly immunogenic. Despite the novelty and the potential value for drug development provided by ancestral sequence reconstruction as a means for informing protein bioengineering, both the standard of care Advate protein and the An53 protein generate fVIII inhibitors when delivered intravenously to hemophilia A mice. Thus far, it seems unlikely that the key to avoiding an inhibitor response to fVIII after

AAV-fVIII gene therapy lies in the specific sequence engineering of the fVIII protein product. While AAV-fVIII biodistribution could be an important factor for both safety and immune system recognition of the fVIII protein, the AAV gene therapy field remains centered on hepatocyte-directed expression and the vectors being designed appear to target the liver specifically as intended. However, an abnormal liver growth was observed in one of the AAV-fVIII treated E16 mice. Even though the growth was not cancerous, it could still be expected to impair liver function and the potential for malignant growths fueled by stress on the liver resulting from long-term AAV-fVIII vector expression cannot be ruled out. Indeed, several adverse events observed in clinical trials of other liver-directed AAV gene therapy products, as well as the initial increases in liver enzymes and long-term decreases in fVIII activity following AAV-fVIII treatment in current clinical trials, could all be indicators that AAV gene therapy has some negative effects on liver function. Furthermore, there is currently an effort to incorporate optional liver biopsies in AAV gene therapy clinical trials. More preclinical studies centered on the liver after AAV gene therapy focusing on molecular mechanisms of AAV transduction and protein product production, the immune response to the protein product, hepatocyte cell stress, and the short- and long-term effects on liver function need to be conducted.

After initial comparison of a 1.6E13 vg/kg dose of four different AAV8-fVIII vectors in E16 mice, it does not appear that any one promoter or fVIII transgene element is more immunogenic than another. However, it does seem that the AAV-HCB-HSQ vector resulted in the lowest initial peak fVIII activity, had the steadiest fVIII levels over the course of the study, and was the only group to show no inhibitor development in any of the mice over 48 weeks. This indicates that an AAV-fVIII vector that generates supraphysiological fVIII activity levels early after vector delivery could carry an increased risk of inhibitor development. Although all of the

AAV-E06.TTR-ET3 treated mice developed inhibitors even though they did not demonstrate higher initial peak levels than the AAV-HCB-ET3 and AAV-E06.TTR-HSQ treated mice, that could simply indicate that they reached supraphysiological fVIII levels earlier than the initial week 2 plasma collection. This points to the necessity for plasma fVIII activity and inhibitor evaluations at earlier time points, which is something that is missing from many preclinical AAV-fVIII studies. The two AAV-HSQ vectors were also evaluated in TKO mice. These mice have a total F8 gene deletion, which means they will harbor no fVIII cross-reactive material (CRM) compared to the possibility for some residual fVIII fragments circulating in the E16 mice. In keeping with the hypothesis that the severity of the F8 mutation, leading to less CRM, reduces the threshold to fVIII inhibitor development, all three of the TKO mice administered AAV-E06.TTR-HSQ developed inhibitors compared to the single E16 mouse. The TKO mice also developed inhibitors at earlier time points. Like the E16 mice administered AAV-HCB-HSQ, none of the TKO mice treated with this vector developed inhibitors within the study timeframe. However, the TKO mice administered AAV-HCB-HSQ did have higher fVIII activity levels, and it is possible that the one mouse demonstrating greatly reduced fVIII activity by week 48 could have been in the process of developing inhibitors. This last observation also highlights the importance of conducting longer-term preclinical studies of liver-directed AAV-fVIII activity to assess the durability of fVIII activity levels and whether inhibitors could form long after initial AAV-fVIII treatment.

Another aspect of great interest in the development of AAV-fVIII therapeutics is their utility in inducing immune tolerance for patients who already have inhibitors from recombinant fVIII replacement therapy. Our group has yet to have success in eradicating inhibitors from immunized mice using liver-directed AAV-fVIII, and the additional evaluation presented herein was also unsuccessful. Disconcertingly, the severity of the inhibitor response drastically increased

after AAV-fVIII treatment, and two of the mice administered the AAV-E06.TTR-ET3 vector died within 4 weeks after vector delivery. Given this result, as well as the fact that all of the naïve E16 mice in the n=3 study developed inhibitors after AAV-E06.ET3 delivery, it is possible that this vector is extremely potent and that this is problematic for liver function. However, it remains unknown whether the pre-immunized AAV-fVIII treated mice would have eventually attained immune tolerance to fVIII after their initial anamnestic response flare up. It is reasonable to suspect that intense stimulation of immune cells in this manner could eventually have led to activation induced cell death and/or exhaustion of T and B cells, which could have led to establishment of a state of peripheral immune tolerance. Considering the importance of the questions surrounding the ability of liver-directed AAV-fVIII gene therapy to induce immune tolerance, and the pace with which AAV-fVIII products are advancing through clinical trials, intelligently designed and properly powered murine studies of immune tolerance induction and inhibitor eradication with AAV-fVIII are warranted. Should liver-directed AAV-fVIII prove unreliable or unsafe for establishing fVIII immune tolerance or inhibitor eradication, potentially adding in the establishment of central tolerance via bone marrow transplant of HSPCs transduced with LV-fVIII could prove to be the ideal gene therapy approach. However, genotoxicity resulting from endogenous HSPC depletion protocols remains a steadfast impediment. Discovery of a safe and effective non-genotoxic bone marrow conditioning regimen for transplant is a holy grail for many areas of research, including gene therapy, cancer, infectious disease, and autoimmunity.

2.6 Acknowledgments

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2.7 Supplemental Information

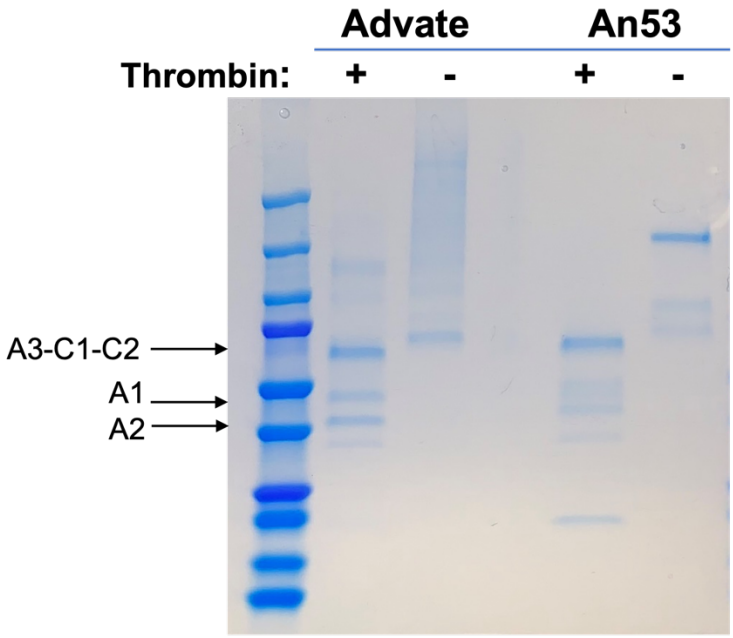
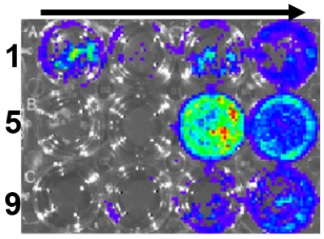


Figure S2.1. Advate and An53 recombinant protein on an SDS-PAGE gel.
Advate and An53 protein injections were pre-made, aliquoted, blinded, and then stored at -80ºC until use. Some of these injections were thawed and run on an SDS-PAGE gel with or without thrombin to compare protein amounts actually received by the mice. After thrombin treatment, the heavy and light chain fVIII components are comparable. However, An53 is B domain deleted and Advate is not. Therefore, this could create variability in how the immune system perceives the exogenous recombinant fVIII protein.

A

Well	Promoter	ug Plasmid	TransIT Ratio	Cell type
1	HCB	1	3:1	293F
2	HCB	1	6:1	293F
3	E06.TTR	1	3:1	293F
4	E06.TTR	1	6:1	293F
5	NT			293F
6	HCB	1	6:1	HUH7
7	E06.TTR	1	3:1	HUH7
8	E06.TTR	1	6:1	HUH7
9	NT			HUH7
10	HCB	1	3:1	HUH7
11	HCB	2	3:1	HUH7
12	E06.TTR	2	3:1	HUH7

B



C

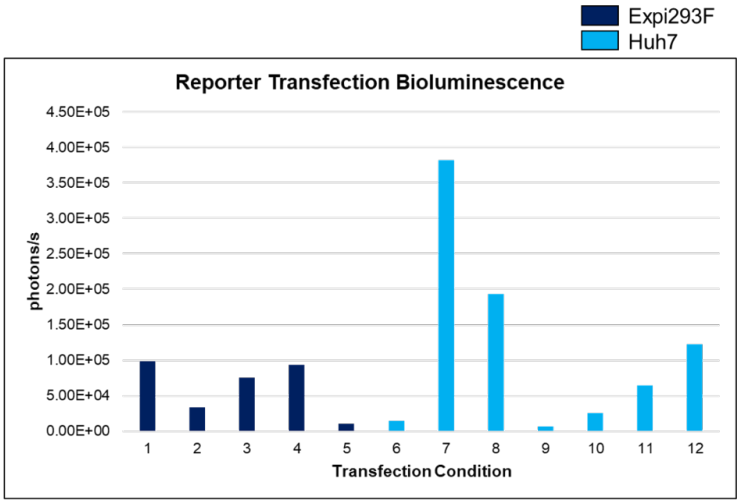


Figure S2.2. *In vitro* evaluation of the FLUC-P2A-GFP reporter plasmids.
Prior to manufacture of the AAV8 reporter vectors, the HCB/E06.TTR-FLUC-P2A-GFP plasmids were evaluated by transfection in Expi293F (HEK) and Huh-7 cells. As expected given that the promoters are liver-directed, higher bioluminescence signal was observed in Huh-7 cells under the most ideal transfection conditions using TransIT-X2 reagent (Mirus Bio).

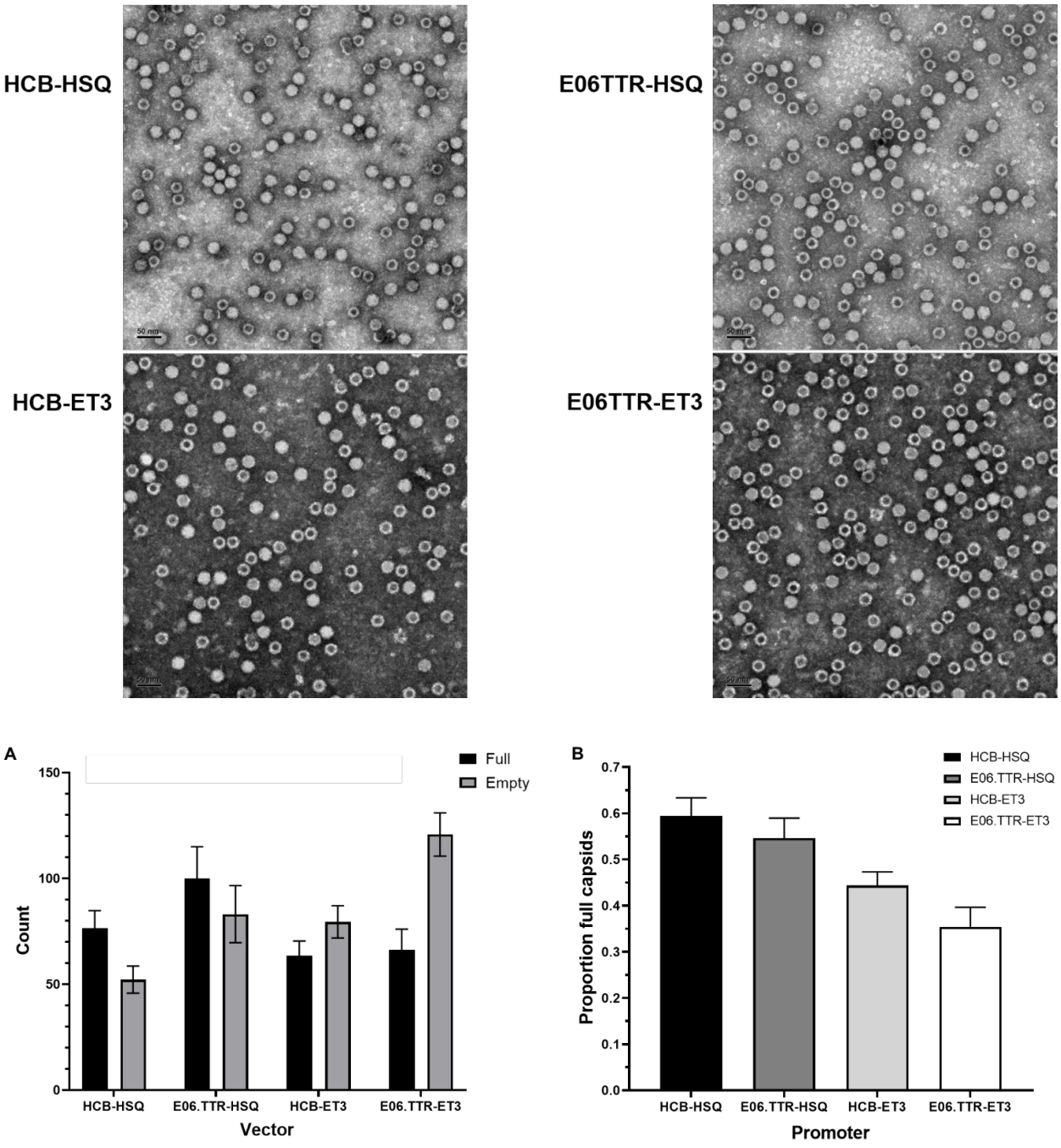


Figure S2.3. Packaging efficiency of the AAV8-fVIII vectors.

Cryo-EM images were used to assess the packaging efficiency of the AA8-fVIII vectors, and representative images of the AAV-HCB-HSQ, AAV-E06.TTR-HSQ, AAV-HCB-ET3, and AAV-E06.TTR-ET3 vectors used in this project are shown here. **(A)** 10 images were counted twice for empty and full capsids for each vector. **(B)** The full and empty capsid counts were used to calculate a % full capsids (or packaging efficiency) for each vector.

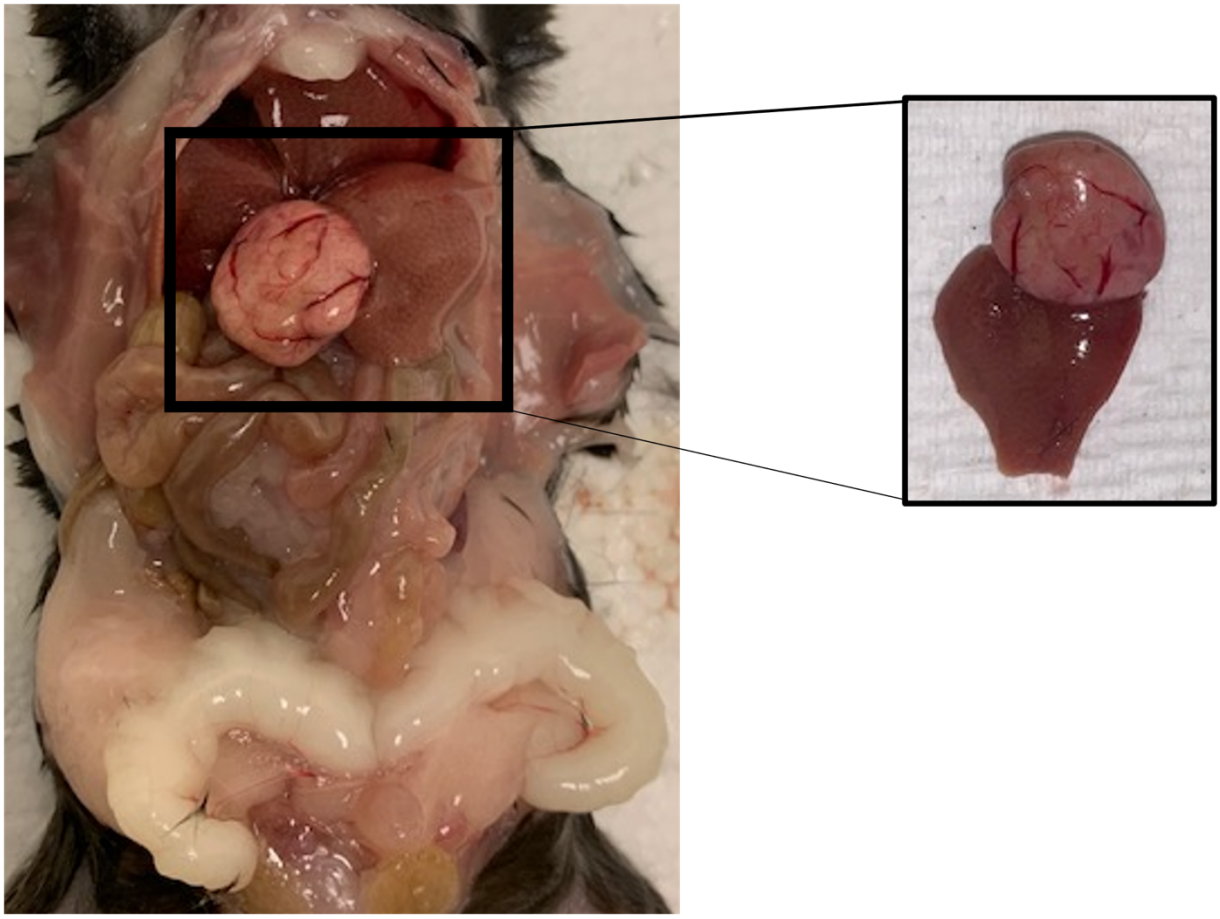


Figure S2.4. Liver growth histopathology for animal 1389R.
This abnormal liver growth was found upon necropsy of animal 1389R. The growth was excised and delivered to Emory University Division of Animal Resources, which sent the tissue to University of Georgia for a veterinary pathology report. The result determined the growth to be a "hepatocellular hyperplasia with hepatocellular vacuolar degeneration and cholangiolar hyperplasia."

Chapter 3

Pharmacokinetic analysis of fVIII exposure identifies an immunogenicity threshold after AAV-fVIII gene therapy in a murine hemophilia A model

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Taran Lundgren designed and performed experiments, analyzed data, and wrote and edited the manuscript.

Gabriela Denning performed the vector qPCR assays.

H. Trent Spencer advised on experiments and data analysis and reviewed the manuscript.

Christopher B. Doering assisted on conception of experiments, advised on data analysis, and assisted in writing and reviewing the manuscript.

3.1 Abstract

Immunogenicity is a key safety parameter of biologic drugs, including genetic medicines, that is also extremely challenging to assess and predict. Despite this fact, many genetic medicines have made it through clinical testing and onto the market with revolutionary effects on 21st century medicine. This is made evident, for example, by the recent approval of several gene therapy products, development of gene-modified cellular therapies like chimeric antigen receptor (CAR) T cells, and the long history of vaccine development, including the mRNA vaccines at the forefront of the COVID-19 pandemic. However, this class of drug differs fundamentally from classical biotherapeutics since the therapeutic agent is typically manufactured inside the patient by genetically modified cells, providing a continuous supply of the transgene protein product (i.e., antigen) for presentation to the immune system as opposed to the episodic intravenous delivery of most protein drugs. This challenges the existing framework for drug development and necessitates new approaches that enable preclinical and clinical studies to successfully inform drug dosing, safety, and efficacy predictions. For the first time, the current study presents a detailed, longitudinal dose-response evaluation of liver-directed AAV vectors expressing factor VIII (fVIII) in a preclinical murine model of hemophilia A. A pharmacokinetic/pharmacodynamic model was generated from these data that identifies the primary factor predictive of anti-fVIII antibody incidence, quantifies the fVIII immunogenicity risk, and suggests both therapeutic efficacy and immunological safety windows for AAV-fVIII treatment. This approach has the potential to inform drug development guidelines for other genetic medicine candidates and help avoid adverse clinical outcomes.

3.2 Introduction

Hemophilia A, a severe X-linked bleeding disorder resulting from defective or deficient levels of coagulation factor VIII (fVIII), is a monogenic disorder with one of the longest histories of gene therapy and transgene product development.[4,42,44,239-242] Liver-directed adeno-associated viral (AAV) vector delivery is the most common and clinically advanced gene therapy approach for hemophilia A. While tremendous progress has been made, there continue to be significant challenges and points of uncertainty for AAV-fVIII gene therapy, including:

1. The durability of AAV transduction and fVIII protein expression.
2. AAV inhibitors and development of AAV neutralizing antibodies (NABs) post vector delivery.
3. Short- and long-term target organ toxicity and pathology.
4. The gap in knowledge regarding the utility of AAV-fVIII for fVIII immune tolerance induction.
5. The immunogenicity of the fVIII protein product in the context of AAV-fVIII therapy.
6. As clinical trial enrollment thus far requires immune tolerance of many prior fVIII protein exposures, the utility and risk of AAV-fVIII in previously untreated patients remains unknown. There also exists no official preclinical model of fVIII immune tolerance for use in drug development.

As listed, the parameters governing the preclinical immune response to fVIII in the context of liver-directed AAV-fVIII gene therapy remain unclear, as evidenced by existing preclinical and clinical findings that are somewhat paradoxical and extensive variation in the design of published preclinical AAV-fVIII studies.[48,202] Furthermore, AAV-fVIII products under clinical study demonstrate uniform immunogenicity in non-human primate (NHP) studies at clinical doses despite the high degree of identity between B-domain-deleted human fVIII and the circulating

endogenous NHP fVIII.[188,210] Immunogenicity also remains a major challenge in standard fVIII replacement therapy, as approximately 30% of severe hemophilia A patients develop neutralizing antibodies, or "inhibitors", to the therapeutic fVIII.[51] Treatment in the context of inhibitors with bypassing agents, fVIII mimetics, or tolerance induction protocols comes with higher financial burden and without guaranteed success.[52] Therefore, all clinical trials have been limited to patients who have received extensive fVIII replacement therapy without a history of inhibitors. To date, clinically significant inhibitors have not been observed despite utilization of doses as high as 6E13 vector genomes (vg)/kg. However, the translatability of these findings to the previously untreated patient population along with the critical immunogenicity determinant(s) of these and other gene therapies remain unknown. Several independent variables in gene therapy design could influence transgene product immunogenicity, i.e., the ability of the transgene protein product to illicit a pathogenic immune response. For example, bioengineered fVIII proteins have shown variations in biosynthetic efficiency due to their transgene sequence, codon-optimization, and differential engagement of the unfolded protein response.[173-177] Testing of various synthetic, liver-directed promoter/enhancer elements also appears to support differential immunogenicity risk depending on their sequence design.[178] A variable inherent to the structure of the AAV particle is its vector serotype with resulting cell type tropism. Vector dose is another important consideration given that all AAV particles are immunogenic in humans. While development of anti-AAV neutralizing antibodies (NABs) is expected, higher AAV capsid loads could contribute to cellular stress from high energetic burden in transduced cells and a proinflammatory microenvironment in the target organ that indirectly increase the immunogenicity risk of the transgene product.[179-183]

Indeed, preliminary investigations described in Chapter 2 of this dissertation were also unable to form conclusions about the primary variable(s) responsible for anti-fVIII immunogenicity

following AAV gene therapy in hemophilia A mice. Protein drugs are typically evaluated in animal models and by *in silico* analysis of predicted peptide:HLA haplotype binding. However, relevant differences exist regarding immunogenicity in the gene therapy setting, wherein a continuous supply of the transgene product protein is presented to the immune system from endogenous sources in contrast to the conventional episodic delivery of exogenous protein drugs. Transgene protein product immunogenicity is a parameter that is particularly challenging to evaluate and frequently neglected in preclinical gene therapy studies. Since the therapeutic agent is typically manufactured inside the patient by genetically modified cells, genetic medicines differ fundamentally from classical biotherapeutics and challenges the existing framework for drug development. This necessitates new approaches that enable preclinical and clinical studies to successfully inform drug dosing, safety, and efficacy predictions. Therefore, although this class of therapeutics are already revolutionizing 21st century medicine, the primary determinants of an immune response to the gene therapy transgene product remain largely unknown, as do the preclinical methods that can best predict their safety.

The goal of the current study is to identify the critical pharmacological determinant(s) of fVIII immunogenicity in the preclinical context of AAV gene therapy for hemophilia A and whether pharmacokinetic principles can then be applied to model incidence and risk of the fVIII inhibitor outcome. Exact pharmacokinetic and pharmacodynamic (PK/PD) analysis of a biologic like AAV gene therapy is very complex (Fig. 3.1). There are multiple exposure and elimination rate constants, beginning with vector administration and clearance, followed by many intermediate intracellular processing steps, and ending with protein product production, protein clearance and transduced cell turnover.[243,244] Additionally, there are two drug doses to consider: that of the administered AAV vector and that of the bioavailable therapeutic protein product.

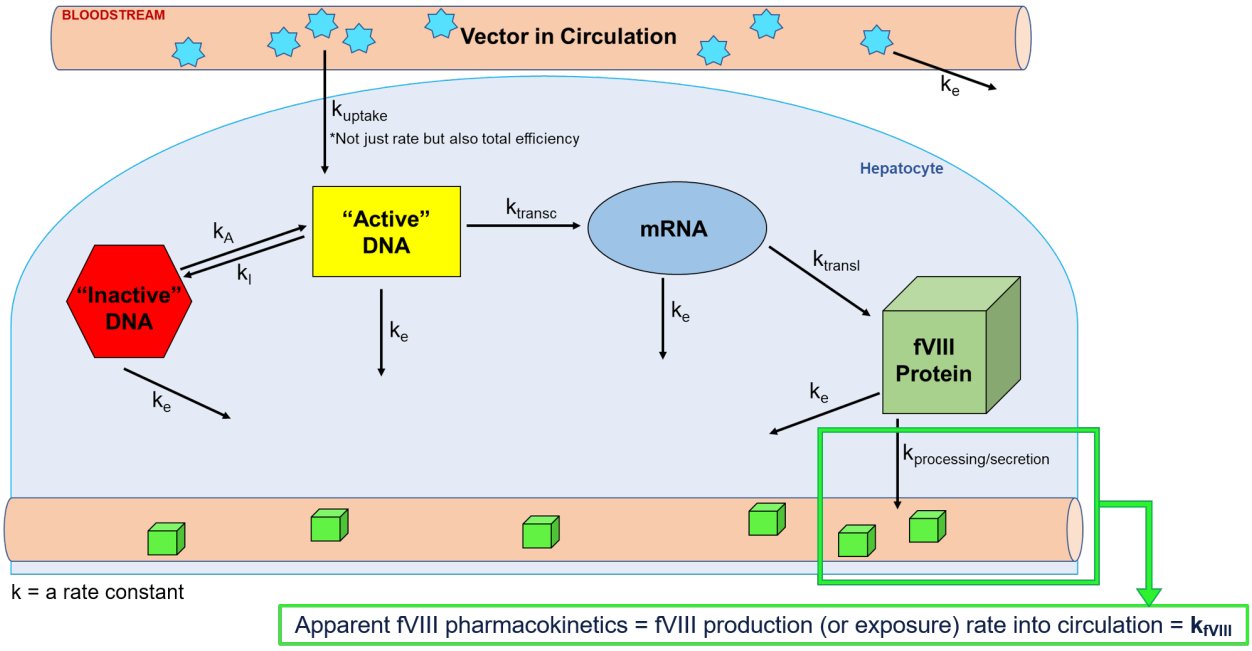


Figure 3.1. This schematic proposes the complexity if AAV-fVIII gene therapy pharmacokinetics. This schematic represents all of the proposed steps between AAV-fVIII delivery and secretion of the fVIII protein product from the transduced hepatocyte into the bloodstream. Each of these steps is associated with its own rate, k. While the final concentration of fVIII in the plasma is simple to measure in the absence of an inhibitor response, all of the molecular steps occurring in the individual hepatocytes from vector uptake through protein processing are extremely difficult to quantify.

Clearly, this overall process does not obey standard pharmacokinetic models and individual steps in this process would be challenging or even impossible to accurately measure. However, for proteins secreted into circulation, the process can be reduced to apparent pharmacokinetics of the transgene product detectable in the plasma (Fig. 3.1 and 3.2). This allows for use of a one-compartment model, that compartment being the circulation (or plasma) with first-order elimination kinetics (Fig. 3.2A). The transgene product production is then expressed as a rate (e.g., fVIII IU/day at a given time point for a given vector dose) that operates at a pseudo-steady state modeled as a continuous intravenous infusion, i.e., continuous secretion from the liver (Fig. 3.2D). This method of delivery from AAV-fVIII transduced hepatocytes should then bypass the cyclic

peaks and troughs in plasma fVIII concentration that are characteristic of periodic intravenous infusions of the recombinant fVIII protein used in prophylaxis. The expectation is that the resulting findings may be translatable through similar modeling to a larger spectrum of products in this rapidly advancing class of genetic medicine.

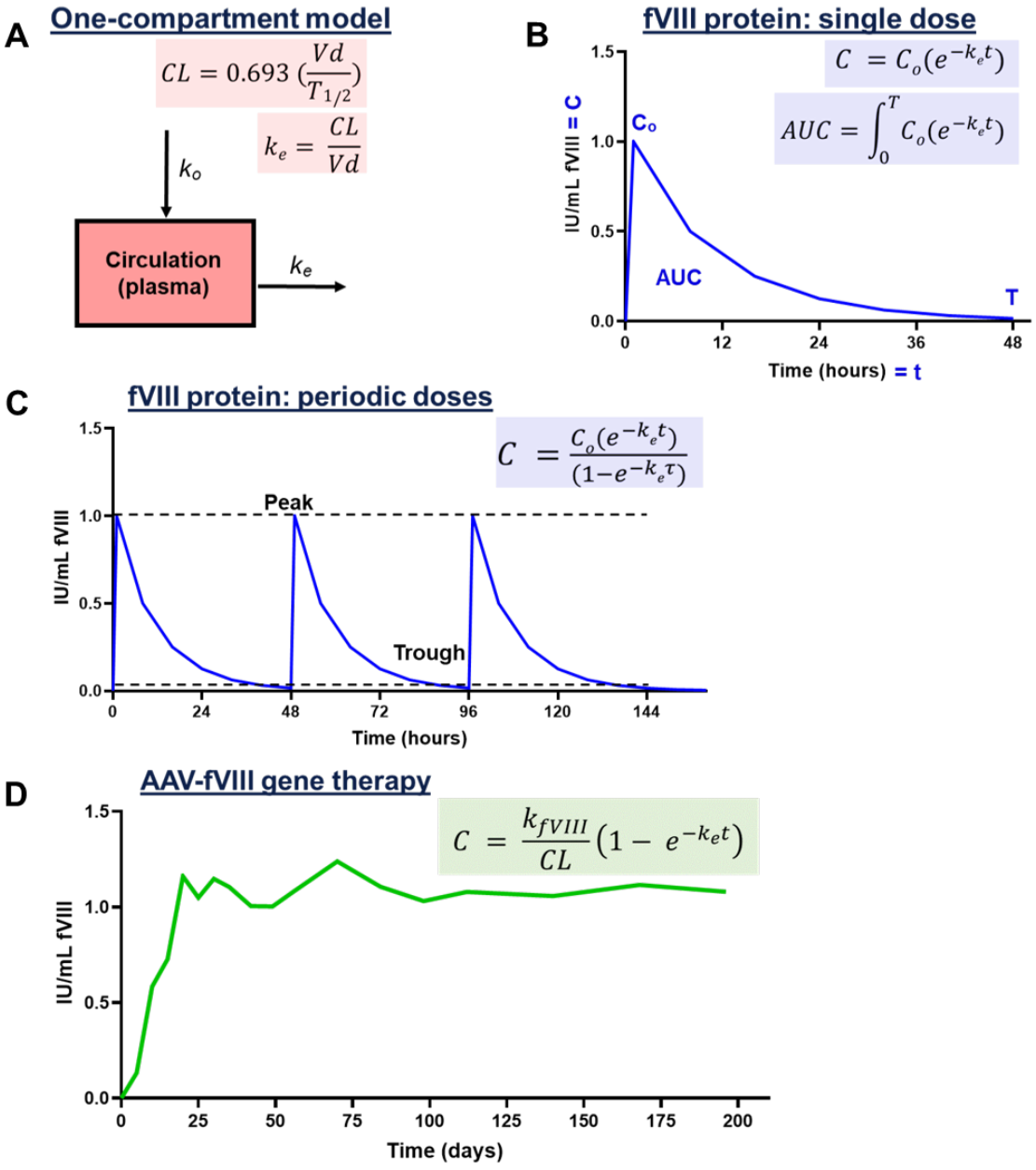


Figure 3.2. The pharmacokinetics of intravenous protein infusion(s) vs. the proposed apparent pharmacokinetics of fVIII protein exposure after AAV-fVIII gene therapy delivery.

Drugs delivered or released directly into the circulation follow a one-compartment model with that compartment being the circulation **(A)**. This allows for the use of first-order elimination kinetics, which provides equations for calculating clearance, CL, and the elimination rate constant, k_e, based on the known half-life of the drug (be it a protein or small molecule drug), $T_{1/2}$, and volume of distribution of the recipient, V_d. Panel **(B)** demonstrates the initial peak in plasma fVIII concentration, C, followed by its clearance after one infusion, with the AUC being the total fVIII protein exposure. Panel **(C)** demonstrates the cyclic peaks and troughs of periodic fVIII protein infusions characteristic of standard fVIII protein replacement prophylaxis. Panel **(D)** displays the equation for a constant rate infusion to represent constant fVIII secretion from the liver into the bloodstream after AAV-fVIII delivery. In this study, the fVIII activity levels measured from the plasma of experimental mice substitutes for the fVIII plasma concentration, C.

3.3 Materials and Methods

Mice. All studies were performed under the guidelines set by the Emory University Institutional Animal Care and Use Committee. Every AAV-fVIII and AAV-FLUC-P2A-GFP vector was evaluated in fVIII naïve, male exon 16-disrupted hemophilia A mice back-crossed onto a C57BL/6 background (30% S129 and 70% C57BL/6). The AAV-HCB-HSQ and AAV-E06.TTR-HSQ vectors were also evaluated in TKO mice, which are 100% C57BL/6 mice with the entire F8 coding region deleted. All mice were 8-12 weeks of age at study initiation. While AAV vectors are exclusively administered to male mice, both male and female E16 mice were used in the Advate vs. An53 recombinant protein immunization study.

AAV vectors. A pUC57 plasmid containing the 5' and 3' AAV2 inverted terminal repeats was modified to contain either the HCB or E06.TTR promoter along with the either the HSQ, ET3 or AN53 fVIII gene.[173,175,178,235] All fVIII vector sequences are liver codon-optimized using a liver-specific algorithm previously described[173] and do not contain any CpG dinucleotides. The plasmids were provided to Vigene Biosciences for AAV8 vector production and titration. Upon receipt of the product, an SPS-PAGE gel was run for visualization of capsid proteins, and Cryo-EM for packaging efficiency was performed by the Emory University core facility. *In vivo* vector delivery was based on the genome copies/mL titer provided by Vigene Biosciences, which was quantified

by qPCR targeting the ITR. All AAV vector administrations were delivered in a total volume of 100µl in PBS via retro-orbital sinus injection. Blood was always collected via retro-orbital capillary with 3.8% sodium citrate. Plasma was harvested via centrifugation and stored at -80ºC.

In vivo mouse studies. All AAV-fVIII vector and ET3i protein administrations were made up in sterile PBS and delivered via a 100µl retro-orbital sinus injection. During the study, blood was collected via retro-orbital application of a 50µl or 100µl capillary with 5µl or 10µl 3.8% sodium citrate, respectively. Plasma was immediately isolated at 4°C, aliquoted on ice, and stored at -80°C. At sacrifice, blood was collected by cardiac puncture with 3.8% sodium citrate and liver tissue was flash frozen in liquid nitrogen for DNA extraction. In the AAV-fVIII dose-response study, mice were administered either AAV2/8-E06.TTR-ET3, AAV2/8-HCB-ET3, AAV2/8-E06.TTR-HSQ, or AAV2/8-HCB-HSQ at the doses indicated. All required animals were pre-randomized into the vector/dose groups (n=4) and entered the study via rolling enrollment in cohorts of 14-20 as they came of age. Blood was collected every 5 days for the first 35 days, then weekly for weeks 6 to 8, every 2 weeks through week 16, and every 4 weeks thereafter. The first cohort of mice enrolled are missing day 35 and week 7 plasma samples, as these time points were added after observation of fVIII activity profiles in the first cohort. During the ET3i protein challenge study, mice administered AAV vectors encoding ET3 that did not develop inhibitors after a minimum of 37 weeks and a maximum of 42 weeks were enrolled in a recombinant ET3 protein (ET3i) challenge study. Animals were grouped pre-challenge according to steady state fVIII activity levels, with one no-challenge control per group. As a positive control for IgG induction, 4 fVIII-naïve animals were challenged alongside the AAV-ET3 treated challenge animals. Animals were administered 1 µg ET3i per week for 5 weeks, and blood was collected 3 days after each injection. Two additional blood samples were taken at weeks 6 and 7 post-challenge

initiation. Animals received a 1 µg ET3i booster injection 5 weeks after conclusion of the challenge course and were sacrificed 4 days later.

FVIII activity. FVIII activity in mouse plasma samples was measured via Chromogenix Coatest SP4 Factor VIII chromogenic assay following the manufacturer's instructions (Diapharma). Human Factor Assay Control Plasma (FACT) was used to create the linear standard curve for sample quantification (George King Bio-Medical, Inc.).

Anti-fVIII antibody assays. Prior to anti-fVIII antibody titer assays, plasma was heat inactivated for 30 min – 1 hr at 56°C depending on aliquot volume, then maintained on ice. IgG titers were determined as previously described.[236] Briefly, high-binding, half-area 96-well plates (Corning) were coated overnight at 4°C with 1.5 µg/mL purified recombinant fVIII corresponding to the AAV vector transgene product (i.e., Advate for HSQ; ET3i purified in-house[161]), washed twice with an HBS+0.05% Tween-20 buffer and put in a 2%BSA blocking buffer at 4°C for a 16 hr minimum or until use (within 2 weeks). After 2 washes, sample plasma was applied in 8 serial 1:2 dilutions to the plate and incubated at room temperature for 1 hr. Detection was performed using goat-anti-mouse IgG-alkaline phosphatase and Alkaline Phosphatase Substrate solution (BioRad). The plate was incubated in the dark for 20 min and read by spectrophotometer at 405nm absorbance. The absorbance values were plotted against the logarithm of the plasma dilution, and the titers were determined by the reciprocal of the dilution at which the optical density (OD) value was three times that of the background (naive E16 mouse plasma), OD=0.3. A mixture of anti-fVIII monoclonal antibodies was used as a positive control. FVIII inhibitor Bethesda assays were performed as previously described,[237] with the modification that the Bethesda titers were binned into a range based on dilutions that yielded a residual fVIII activity between 40 - 60%. Any plasma sample that had a readout below the limit of quantification by any antibody assay was given a

working value of zero in any data analysis and/or graphical representation; however, the actual value could be somewhere between zero and the limit of quantification (20 for IgG ELISA titer and 5 for BU/mL titer). If the data are presented graphically on a log scale, a zero value for fVIII activity or antibody titer was given an arbitrary value of 0.005.

ET3i antigen ELISA. Multiple capture and detection antibody combinations were tested using anti-human fVIII antibodies targeting human sequence regions present in ET3i. The antibodies were purchased from Green Mountain Antibodies (GMA). Antibodies included in the test combinations: GMA-8016 (target A2 domain), GMA-8015 (target A2 domain), GMA-012 (discontinuous epitope targeting the A2 domain), GMA-8011 (target C1 domain), and GMA-8003 (target C2 domain). The combination used to measure ET3 antigen levels in this study was GMA-8016 capture and combined GMA-8011 plus GMA-8003 detection. Detection antibodies were buffer exchanged to PBS and concentrated using a 4mL Amicon Ultra filter. They were then biotinylated using an EZ-Link Biotin kit (Thermo Scientific) at a 1:1 mass ratio. The biotinylated antibodies were then buffer exchanged into 1XHBS+0.05%NaN_3. Antibody concentration was measured using a NanoDrop spectrophotometer. The biotinylated antibodies were stored at 4°C until use (within 2 weeks). All biotinylated antibody preps were tested using an ET3i standard curve and negative controls prior to use on sample plates. Half-area high-binding 96-well plates (Corning) were coated with 25 µl of 6 µg/mL capture antibody in 1XPBS+0.05NaN_3 overnight at 4°C. Plates were washed twice with wash buffer (1XHBS+0.05%Tween-20+0.05%NaN_3) and placed in blocking buffer (wash buffer+2%BSA) at 4°C overnight or until use (within 2 weeks). Purified ET3i protein was spiked into naïve E16-/- hemophilia A mouse plasma to a concentration of 1 µg/mL, incubated at 37°C for 15 minutes, and then diluted further to make the ELISA standard curve at 1:2 dilutions from 64 ng/mL to 1 ng/mL. Plasma samples were tested at dilutions of 1:5,

1:10, and 1:30 depending upon fVIII activity levels and inhibitor titers. The ET3i standard curve dilutions and plasma dilutions were made in dilution buffer (1XHBS+0.01%Tween-80+100mM 2-Mercaptoethanol) and incubated at room temperature for 30 min before addition to the assay plate. Prior to sample addition, the coated plate was washed 3 times with wash buffer. Then 25ul of the standards, samples, and a buffer blank were added to the plate. Samples incubated on the plate for 2 hr at room temperature with gentle rocking. The plate was washed 3 times with wash buffer. Then 25 µl of 1 µg/mL biotinylated detection antibodies in blocking buffer were added and incubated for 1 hr at room temperature with gentle rocking. The plate was washed 3 times with wash buffer. Then 25 µl of 1:5000 Streptavidin-AP (Jackson Immune Research) in blocking buffer was added and incubated for 1 hr at room temperature with gentle rocking. The plate was washed again 3 times with wash buffer. Then 40 µl of Alkaline Phosphatase Substrate solution (BioRad) was added and the plate was incubated in the dark. The plates were read at 10, 15 and 20 minutes using a spectrophotometer set at 405 nm absorbance. The 20-minute incubation was used for all antigen quantification using the linear range of the standard curve with an R-squared ≥0.988. The lower limit of quantification was 2-3 ng/mL.

ET3i antigen ELISA in human inhibitor plasma samples. Half-area high-binding 96-well plates (Corning) were coated with 25 µl of anti-ET3i specific antibody 15B9.F5.B8 (Green Mountain Antibodies) at 2 µg/mL in 1X PBS overnight at 4ºC. The plates were then washed 2 times with 100 µl of wash buffer (1XPBS+0.05%Tween-20). Blocking buffer without detergent (1% w/v Casein in 1XPBS from ThermoFisher Scientific: 37582 or 37528) was added at 80 µl/well and incubated overnight at 4ºC. The human plasma samples, FACT, and fVIII deficient human plasma were heat inactivated for 1 hour at 60ºC. ET3i stock (0.104 mg/mL) was diluted down to 200 ng/mL in fVIII deficient plasma and the standard curve was created in fVIII deficient plasma by

1:2 serial dilution. ET3i stock was then also diluted in the assay buffer (MatrixGuard Diluent from Surmodics: SM02-0050 or SM02-1000) and spiked into the human inhibitor plasma samples or FACT at 300 ng/mL, 100 ng/mL, 10 ng/mL, or 0 ng/mL (neat human inhibitor plasma sample). The samples and standard curve were mixed by pipette action and placed at 37ºC for 30 minutes to allow for any anti-fVIII inhibitor antibodies to bind to ET3i. The assay plate was then washed once with wash buffer and 100 µl per well of blocking buffer with detergent (1% w/v Casein in 1XPBS+0.05%Tween-20) was added back to the plate. The assay plate was then also incubated for 30 min at 37ºC. The samples (ET3i standard curve, ET3i spiked in inhibitor patient plasma at 3 concentrations, neat inhibitor patient plasma, ET3i spiked into FACT at 3 concentrations, neat FACT) were added at a 1:4 dilution in MatrixGuard in a mixing plate (40 µl of each standard or sample to 120 µl of MatrixGuard). FVIII deficient plasma in MatrixGuard served as the plate blank. Then the assay plate was washed 3 times in wash buffer and 40 µl of the diluted standards or samples were added to the assay plate. The plate incubated for 1 hour with gentle rotation at room temperature. The plate was washed 3 times in wash buffer. Then the biotinylated detection antibodies, which were a mixture of GMA-8015 and GMA-8016, were added each at a concentration of 0.5 µg/mL for a final detection antibody concentration of 1 µg/mL added to the plate at 40 µl/well. The plate was incubated for 30 minutes with gentle rocking at room temperature. The plate was washed again 3 times with wash buffer. Then 40 µl of Alkaline Phosphatase Substrate solution (BioRad) was added and the plate was incubated in the dark. The plates were read at 10, 15 and 20 minutes using a spectrophotometer set at 405 nm absorbance. The standard curve could be reliably quantified between approximately 1 ng/mL to 50 ng/mL ET3i. The data were analyzed and presented as |%Accuracy| of ET3i detected compared to the

known added concentration of ET3i. This was calculated by (%Accuracy = 100 – Error Rate), where

$$Error\ Rate = \left(\frac{|Observed\ Value - Actual\ Value|}{Actual\ Value}\right) X\ 100$$

Vector copy number qPCR. Vector copy number qPCR was performed on DNA isolated from flash frozen liver samples using a Blood and Tissue DNeasy Kit (Qiagen). For unknown samples, a 1X SYBR green master mix containing 250 nM final concentration of ET3-LCO primers (Forward primer: 5'-AGG TGG CCT ACT GGT ATA TC-3' and Reverse primer: 5'-GGC TGT CAT TCC TCT ATT CC-3') or HSQ-LCO primers (Forward primer: 5'- ATG CTG TGG GAG TCT CCT AC -3' and Reverse primer: 5'- CCT CCA GGG AAC ACC TTA TC -3') was prepared and added to 50 ng total genomic DNA. For standards, a 1X SYBR Green Master Mix containing 250 nM ET3-LCO primers and 50 ng of HEK293T/17 genomic DNA was prepared and mixed with serial dilutions of linearized AAV2/8-E06.TTR-ET3-LCO-NCG-PUC57 or AAV2/8-E06.TTR-HSQ-LCO-NCG-PUC57 plasmid ranging from 5 x 10^6 to 500 copies. All samples were analyzed in triplicate using the PrimePro48 PCR System (TECHNE) with the following thermocycling parameters: Stage 1, 50 °C for 1 min for 1 cycle, Stage 2 (Hot-Start), 95 °C for 10 min for 1 cycle, Stage 3 (Amplification), 95 °C for 15 s followed by 60 °C for 1 min for 40 cycles, and Stage 4 (Dissociation Step), 95 °C for 15 s, 60 °C for 30 s, and 95 °C for 15 s for 1 cycle. Total copies were determined by comparing unknown cycle threshold (Ct) values to known Ct values of the plasmid standard curve (5 x 10^6 to 500 copies). The Ct represents the PCR cycle at which the fluorescence signal in the PCR reaction reaches is an arbitrary level. For these studies the threshold was set automatically by the instrument. Copies/cell was calculated using the equation below, were

8333 represents the total number of diploid human genome equivalents present in 50 ng of genomic DNA:

$$\frac{Copies}{cell} = \frac{Total\ Copies}{8333}$$

Statistics. Where the data sets do not pass a normality test, data are presented as the median with interquartile range error bars and non-parametric comparisons are used. Data sets that pass a normality test are presented as the mean with standard deviation error bars. Significance level for all statistical analyses was set at $p \leq 0.05$. Data were analyzed using GraphPad Prism and Microsoft Excel software.

Pharmacokinetic/pharmacodynamic (PK/PD) analyses. Analyses included fVIII activity data from 66 animals through day 168. This represents the longest time point after which any AAV-fVIII treated animal developed inhibitors but before initiation of the ET3i protein challenge in the youngest AAV-ET3 treated animals (week 37 post AAV-ET3 delivery). Animal 1457B was excluded from PK/PD analyses due to loss of fVIII activity in the absence of any detectable anti-fVIII antibody response. All equations and parameters used in the k_{fVIII} (IU/day fVIII) and AUC_{fVIII} (IU fVIII) calculations for the single-compartment, first-order elimination kinetics with constant rate infusion pharmacokinetic model are outlined in the Results and Supplemental Table 1. Linear regression analysis with shared parameters between AAV-fVIII treated animals that developed inhibitors and those that did not was used to outline the fVIII exposure immunogenicity threshold. Simple logistic regression analysis of the k_{fVIII} and AUC_{fVIII} values at days 5 and 10 was also performed to calculate the inhibitor incidence odds for each value. Therefore, as the k_{fVIII} or AUC_{fVIII} value at a given time point increases by a value of 1, the odds of inhibitor occurrence increase by the multiplicative factor indicated by the β1 Odds. Likelihood Ratio Test for

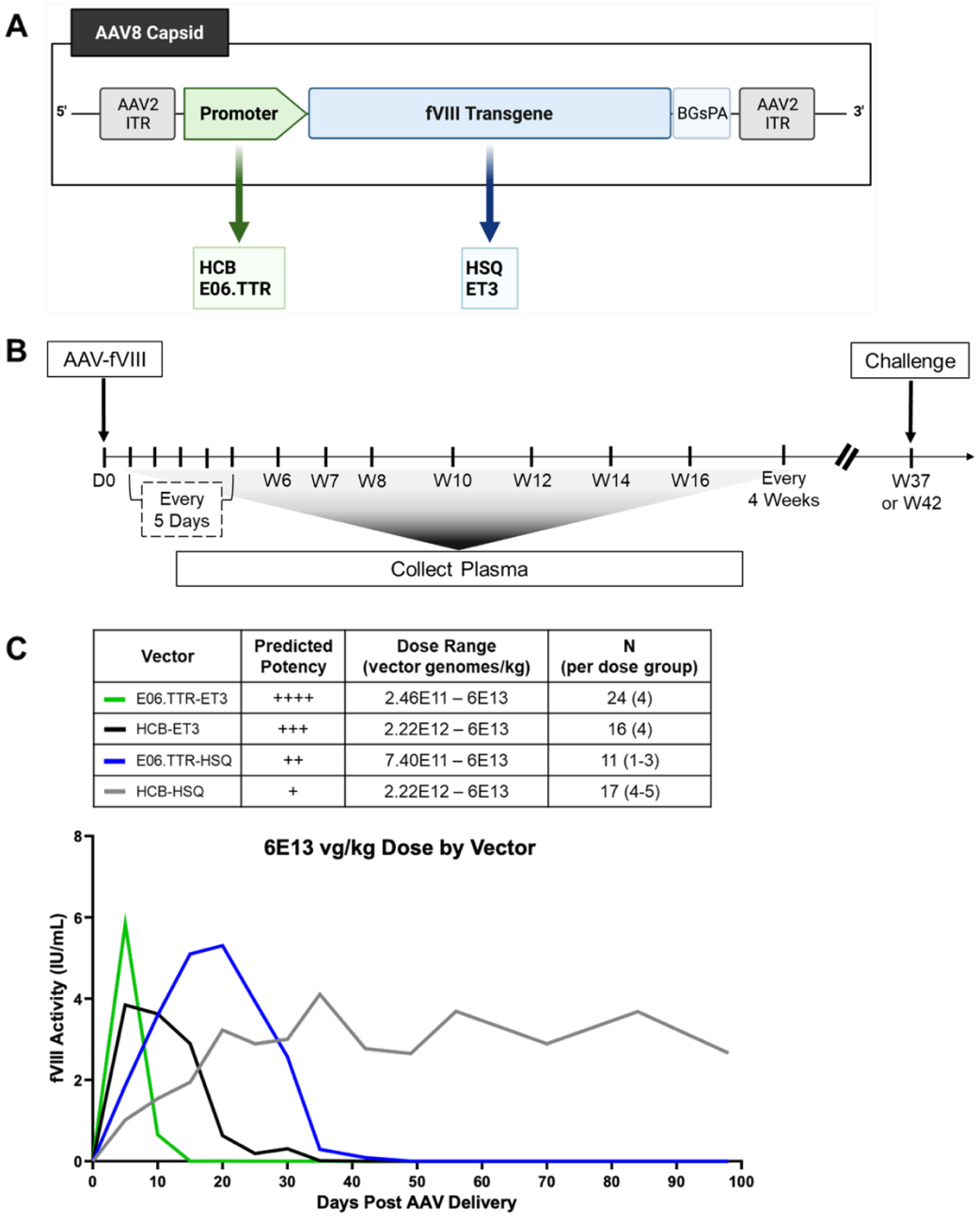
significance of the β1 variable in the logistic regression resulted in $p<0.0001$ and the area under ROC curves range from 0.9705 to 0.9826 with $p<0.0001$ for all four parameters. Additionally, the logistic regression analysis calculated values of k_{fVIII} and AUC_{fVIII} on days 5 and 10 at which 50% of AAV-fVIII treated animals would be expected to develop inhibitors (ID_{50}). A two-sided Fisher's exact test was used to calculate attributable risk increase (ARI) and a risk ratio (RR) of inhibitor incidence for animals that met the k_{fVIII} threshold at day 5 or day 10. Survival analysis was performed comparing animals with day 5 k_{fVIII} values at or above the ID_{50} to animals with day 5 k_{fVIII} values below the ID_{50} (survival = no inhibitors). The survival curve comparison was significant by both Mantel-Cox (Chi-square = 66.95, $p<0.0001$) and Gehan-Breslow-Wilcoxon test (Chi-square = 62.44, $p<0.0001$). Correlation analysis between the day 5 k_{fVIII} and the time to IgG titer incidence was performed using Spearman r, as the time to IgG titer data set does not pass a normality test and Spearman r is more robust against outliers.

3.4 Results

Despite numerous observations of transgene product immunogenicity in preclinical gene therapy studies, immunogenicity risk assessment remains an underdeveloped component of pharmacology/toxicology studies. The main objective of the current study was to simultaneously interrogate the effect that promoter sequence, fVIII transgene sequence, and vector dose have on fVIII inhibitor incidence after AAV-fVIII gene therapy and identify the dominant risk factor(s). To accomplish this, an extensive longitudinal AAV-fVIII dose-response study in male, fVIII-naïve, exon 16 disrupted (E16), immunocompetent hemophilia A mice between 8-12 weeks of age was conducted. Four different AAV vectors were evaluated, pairing two synthetic liver-directed promoters with two fVIII transgenes in a modular fashion within an AAV2 ITR/AAV8 capsid

vector (Fig. 3.3A and B). The HCB promoter, previously designed and characterized by our group,[173] is shorter and less potent than the E06.TTR promoter, which has shown immunogenicity signals in a study comparing multiple promoter/enhancer sequences in AAV-fVIII vectors.[178] The transgene sequences are: 1) human B-domain-deleted (BDD) fVIII with the SQ linker (herein referred to as HSQ), which is the fVIII encoded in the AAV gene therapy products currently in clinical trials, and 2) ET3, which is a human-porcine chimeric BDD fVIII previously described by our group and colleagues and encoded by transgenes utilized in multiple open investigational new drug (IND) gene therapy clinical programs.[175,234,235] All sequences are liver codon-optimized using a liver-specific algorithm described previously.[173] ET3 has been shown to display higher biosynthetic efficiency than HSQ due to decreased engagement of the unfolded protein response (UPR) leading to enhanced secretion.[174] The result is four vectors with varying potencies: AAV-E06.TTR-ET3 > AAV-HCB-ET3 > AAV-E06.TTR-HSQ > AAV-HCB-HSQ. These vectors were delivered at one of six doses, starting at 6E13 vg/kg with 3-fold dilutions to 2.46E11 vg/kg. Animals were pre-randomized to both vector and dose and entered the study in cohorts (n=14-20) via rolling enrollment.

Uncertainty associated with AAV-fVIII immunogenicity results from a shortage of preclinical study designs with both frequent, early sampling and long-term follow-up after vector delivery. These data are essential for robust PK/PD analyses, evaluating immunogenicity risk in the preclinical model, and observing the durability of fVIII expression, which is currently a major concern in clinical trials. Therefore, animals were followed for up to 43-53 weeks with a higher frequency of plasma sampling within the first 16 weeks after AAV-fVIII delivery. At the highest dose of 6E13 vg/kg, relative vector potency followed the expected ranking with respect to the time to peak fVIII activity levels (Fig. 3.3C).



Vector	Predicted Potency	Dose Range (vector genomes/kg)	N (per dose group)
E06.TTR-ET3	++++	2.46E11 – 6E13	24 (4)
HCB-ET3	+++	2.22E12 – 6E13	16 (4)
E06.TTR-HSQ	++	7.40E11 – 6E13	11 (1-3)
HCB-HSQ	+	2.22E12 – 6E13	17 (4-5)

Figure 3.3. A longitudinal AAV-fVIII dose response study reveals predicted potency of four AAV-fVIII vectors based on initial fVIII exposure kinetics.
(A) This design schematic depicts the four vectors administered to male, exon 16-disrupted hemophilia A mice. **(B)** AAV-FVIII infusion was followed by longitudinal plasma collections and an ET3i protein challenge course for a subset of the AAV-ET3 treated animals. [Figure created using Biorender.com] **(C)** Vectors were administered at 3-fold dose intervals within the range indicated in the table and ranked based on relative predicted potency. The median FVIII activity over time is shown for the 6E13 vg/kg dose of each vector. IQR error bars were removed for visual clarity but are shown for all vectors in Supplemental Figure S1.

For each vector, there appeared to be a dose level below which fVIII activity levels achieved a pseudo-steady state that was maintained in most animals for the duration of the study. However, for the vector and dose combinations inducing the fastest increases to supraphysiological fVIII activity levels, fVIII activity declined to baseline after AAV-fVIII administration in a timeframe directly correlated with time to peak fVIII activity (Figs. 3.3 – 3.5). This indicates development of either a cytotoxic or humoral immune response to transduced cells or the transgene product, respectively. The longitudinal fVIII activity data for the 6E13 vg/kg dose of each vector displays the stepwise effect of their different potencies on fVIII levels as well as time to loss of fVIII activity. FVIII activity dropped at 15 days after administration of the most potent vector, E06.TTR-ET3 (Fig. 3.4A) and then 25 – 30 days after administration of HCB-ET3 (Fig. 3.4B). Moving on to the vectors encoding HSQ, fVIII activity dropped between 42 – 80 days after administration of 6E13 vg/kg E06.TTR-HSQ (Fig. 3.5A). Animals treated with the least potent HCB-HSQ vector did not lose fVIII activity for the duration of the study (Fig. 3.5B), which was 64-69 weeks for AAV-HSQ treated animals.

This stepwise effect is recapitulated in the dose response administration of a single vector. In the case of AAV-E06.TTR-ET3 (Fig. 3.4A), fVIII activity dropped by day 15 after the highest dose, by day 30 after the 2E13 vg/kg dose, at between 35 – 56 days after the 6.67E12 vg/kg dose, and then after 112 days after administration of the 2.22E12 vg/kg dose. Animals administered AAV-E06.TTR-ET3 at doses below 2.22E12 vg/kg did not lose fVIII activity during the study. In the case of AAV-HCB-ET3 (Fig. 3.4B), animals administered 6E13 vg/kg started to lose fVIII activity between 20 – 35 days after vector delivery and between 35 – 42 days after 2E13 vg/kg. Animals administered doses lower than 2E13 vg/kg of AAV-HCB-ET3 did not lose fVIII activity.

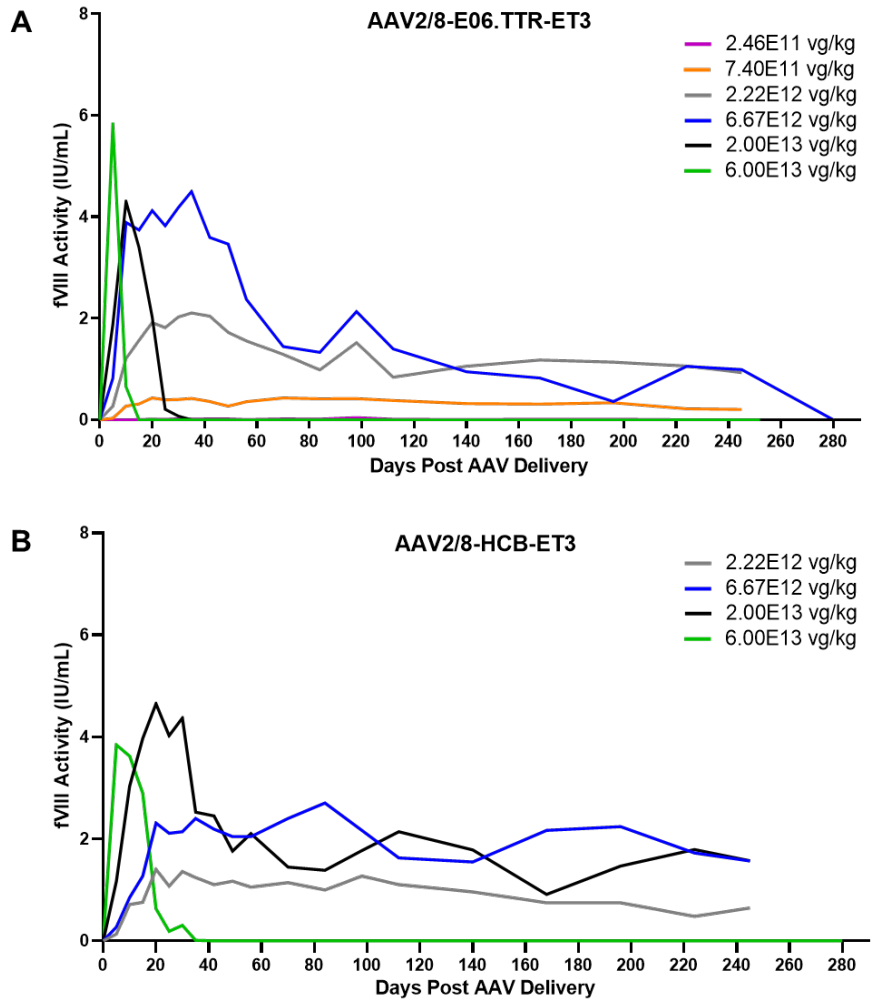


Figure 3.4. The longitudinal AAV-fVIII dose response profiles after administration of AAV-ET3 vectors.
These graphs profile the median FVIII activity (IU/mL) over time for each dose administered of **(A)** AAV-E06.TTR-ET3 and **(B)** AAV-HCB-ET3. FVIII activity (IU/mL) was measured by chromogenic plate assay for all samples assayed in this study. IQR error bars were removed for visual clarity but are shown for all vectors in Supplemental Figure S3.2.

Animals administered the AAV-E06.TTR-HSQ vector at 6E13 vg/kg lost fVIII activity between 25 – 70 days after delivery and no animals administered doses lower than this lost activity for the duration of the study (Fig. 3.5A). Again, no animals treated with the AAV-HCB-HSQ vector at any dose lost fVIII activity during the study after reaching therapeutic peak and steady state fVIII levels (Fig. 3.5B). Importantly, animals administered lower doses of AAV-HSQ vectors

(7.4E11 vg/kg of AAV-E06.TTR-HSQ and 2.22E12 vg/kg AAV-HCB-HSQ) did lose detectable fVIII activity by the end of the study (weeks 64 – 69) without development of anti-fVIII inhibitors.

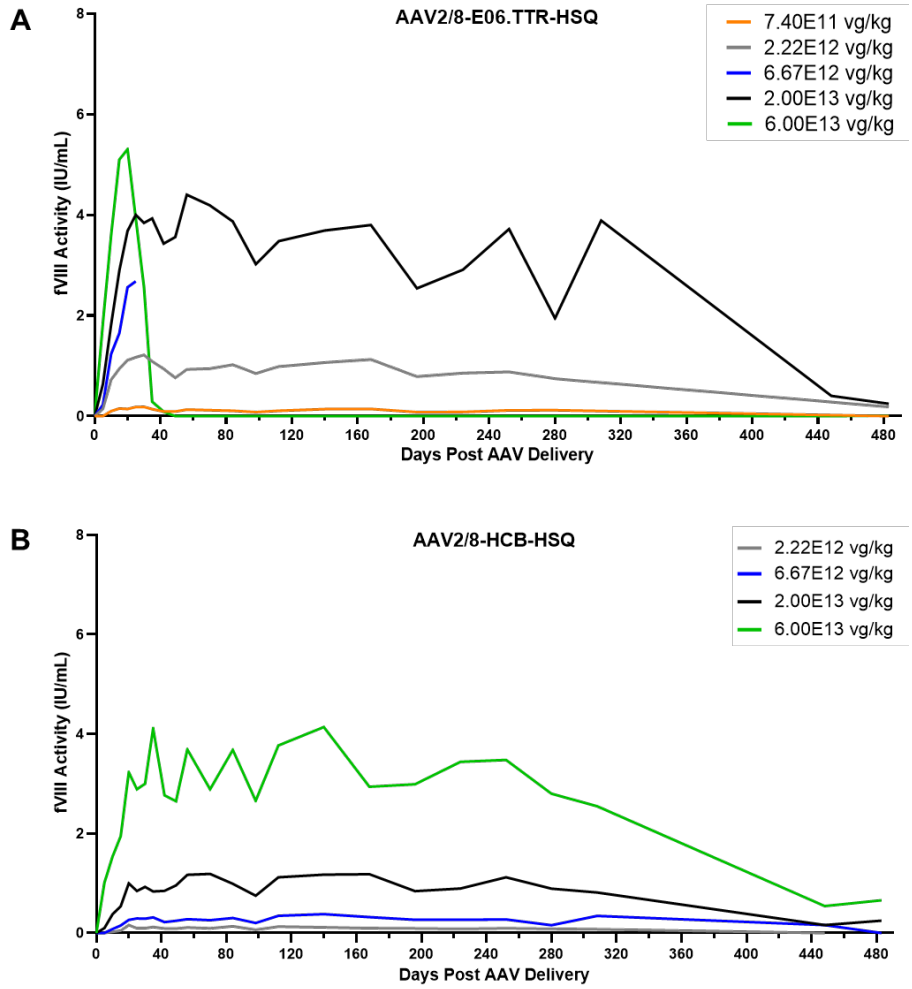


Figure 3.5. The longitudinal AAV-fVIII dose response profiles after administration of AAV-ET3 vectors.
These graphs profile the median FVIII activity (IU/mL) over time for each dose administered of **(A)** AAV-E06.TTR-HSQ and **(B)** AAV-HCB-HSQ. FVIII activity (IU/mL) was measured by chromogenic plate assay for all samples assayed in this study. IQR error bars were removed for visual clarity but are shown for all vectors in Supplemental Figure S3.2.

Dose-dependent AAV vector genome copy number (VCN) observed in liver DNA at the time of sacrifice in animals treated with ≥7.4E11 vg/kg suggests the latter to be the cause of fVIII activity decline. Furthermore, no significant difference in VCN was observed between the AAV-

fVIII vectors at all doses (Fig. 3.6). However, there is a trend toward the least potent AAV-HCB-HSQ vector demonstrating a higher terminal VCN compared to the other three vectors at each dose. The complete data set presented a clear trend toward correlation of combined vector potency and dose ranking with an increased risk of fVIII activity loss.

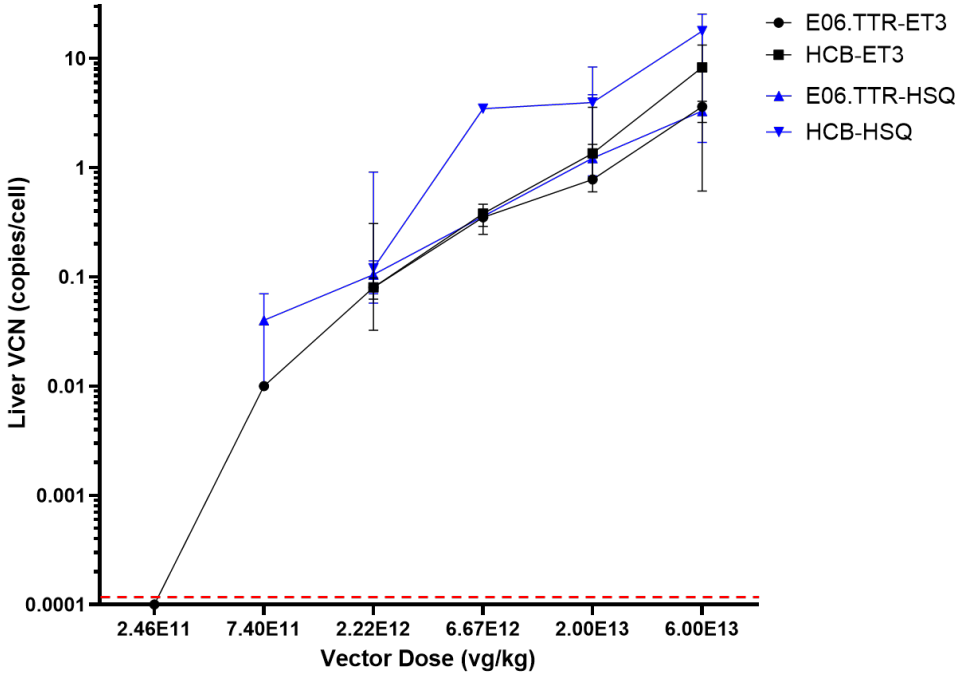


Figure 3.6. There is no significant difference in terminal liver VCN between vectors at each administered dose.
Terminal vector copy number (VCN) was quantified by qPCR on liver DNA. Data points represent the median VCN, and error bars indicate the interquartile range (IQR). Values below the red dashed line indicate that VCN was undetectable. The dose factor (p=0.0004) and vector design factor (p=0.0198) are significant, with only the E06.TTR-ET3 vs HCB-HSQ vector design comparison showing significance (p=0.0158) (main effects 2-way ANOVA with Tukey's multiple comparisons). There is no difference between the vectors when comparing median VCN values within each individual vg/kg dose group.

Normal human circulating fVIII levels are approximately 1 IU/mL (clinically defined as 0.5-1.5 IU/mL, equating to 50-300 ng/mL), and an ideal gene therapy would maintain fVIII levels between 0.5-1.5 IU/mL without major peaks and troughs over time. FVIII activity profiles and VCN for each animal administered the AAV-E06.TTR-ET3 vector are shown in Fig. 3.7.

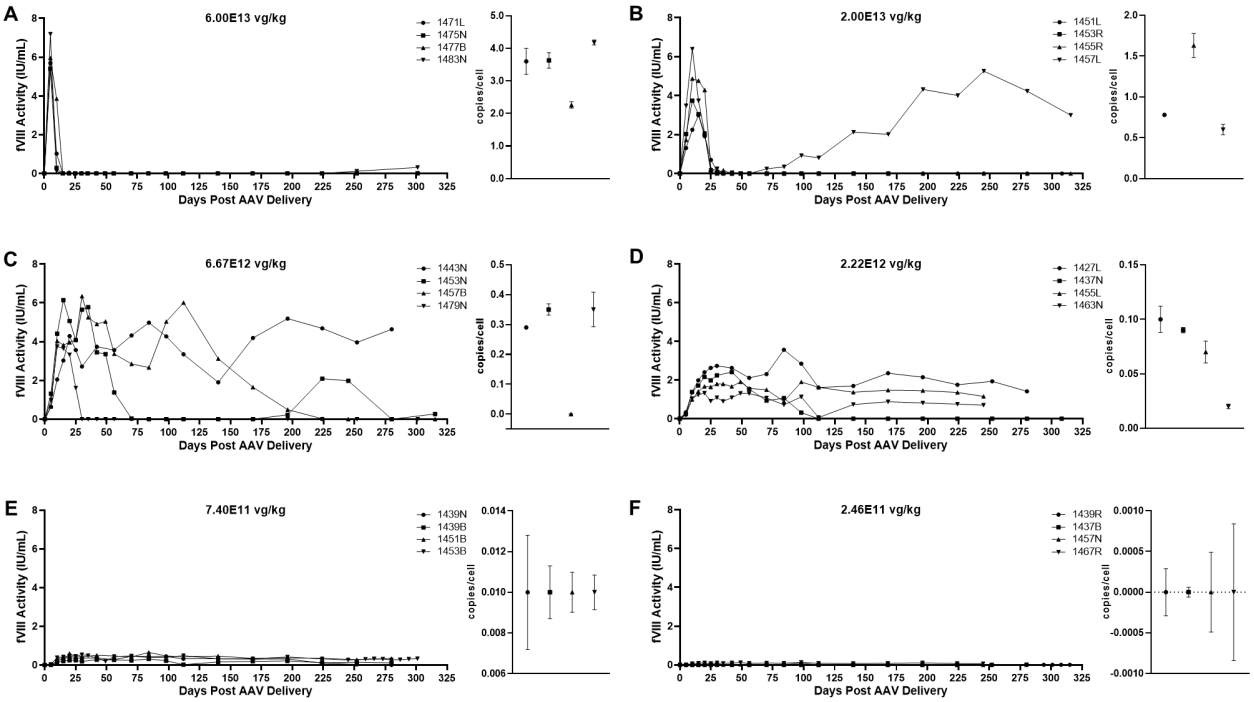


Figure 3.7. FVIII activity profiles and terminal VCN for individual animals administered AAV-E06.TTR-ET3.

These panels display FVIII activity over time and terminal VCN after administration of AAV-E06.TTR-ET3 at **(A)** 6E13 vg/kg, **(B)** 2E13 vg/kg, **(C)** 6.67E12 vg/kg, **(D)** 2.22E12 vg/kg, **(E)** 7.4E11 vg/kg, or **(F)** 2.46E11 vg/kg. Each panel represents one vector/dose group, and each curve represents a single animal. Terminal VCN was quantified by qPCR on liver DNA.

Animals administered 6E13 vg/kg reached peak, supraphysiological fVIII activity levels by day 5 (5.4-7.2 IU/mL) that then dropped to undetectable levels by day 15 (Fig. 3.7A). At 2E13 vg/kg, the same pattern was observed but over double the time frame. Peak fVIII levels were seen at day 10 (2.3-6.4 IU/mL) and became undetectable between days 30-35 (Fig. 3.7B). One animal in each of these dose groups spontaneously recovered fVIII activity, but this occurred much later for the 6E13 vg/kg dosed animal (>250 days) and earlier for the 2E13 vg/kg dosed animal (>70 days). The effects of vector potency and dose were more complex at the mid-range 6.67E12 and 2.22E12 vg/kg doses (Fig. 3.7C and D). Animals administered 6.67E12 vg/kg peaked at 3.8-6.1 IU/mL fVIII between days 10-20, and their fVIII activity began to drop by the next time point. However, all four animals proceeded to unique fVIII activity and immune response outcomes.

Animal 1457B (Animal ID) most likely lost AAV-ET3 genetically modified cells, as it had the highest initial fVIII activity in its dose group but was the only animal to lose both fVIII activity (week 32) and liver VCN. At 2.22E12 vg/kg, the fVIII levels peaked by day 30 (1.3-2.7 IU/mL) and held steady with fluctuations within ~1 IU/mL for three of the animals, while animal 1437N lost fVIII activity after 16 weeks. Most animals administered lower doses maintained fVIII activity levels in the severe to mild hemophilia range (<1 – 50% of normal, i.e., <0.01 – 0.5 IU/mL) (Fig. 3.7E and F).

The animals administered AAV-HCB-ET3 followed a similar dose response pattern to AAV-E06.TTR-ET3 animals, except that the fVIII activity profiles shifted in accordance with the lower AAV-HCB-ET3 vector potency ranking (Fig. 3.8).

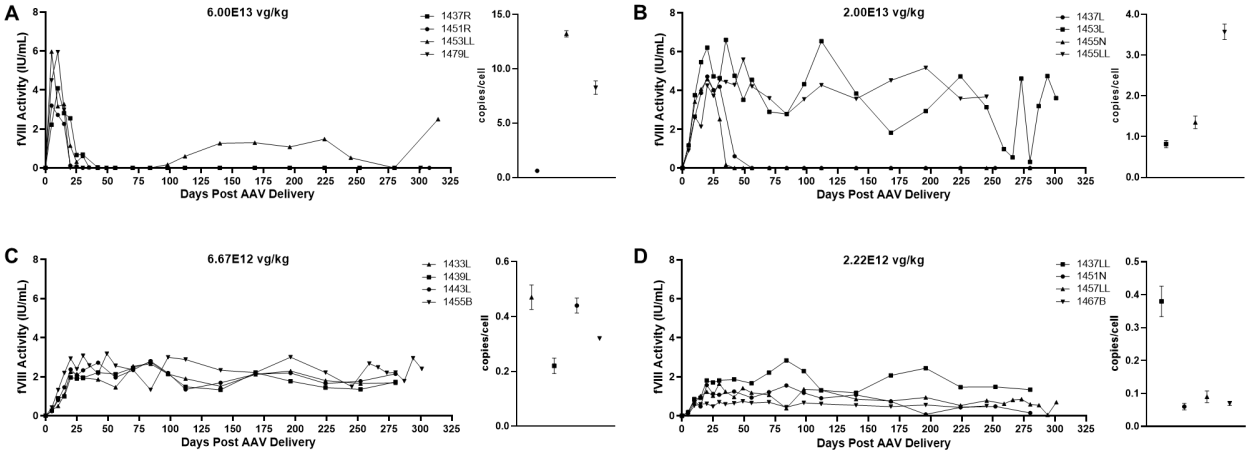


Figure 3.8. FVIII activity profiles and terminal VCN for individual animals administered AAV-HCB-ET3.
These panels display FVIII activity over time and terminal VCN after administration of AAV-HCB -ET3 at **(A)** 6E13 vg/kg, **(B)** 2E13 vg/kg, **(C)** 6.67E12 vg/kg, or **(D)** 2.22E12 vg/kg. Each panel represents one vector/dose group, and each curve represents a single animal. Terminal VCN was quantified by qPCR on liver DNA.

All animals administered 6E13 vg/kg of AAV-HCB-ET3 reached peak, supraphysiological fVIII activity levels (3.2 – 5.97 IU/mL) at 5 – 10 days after vector delivery and subsequently lost

fVIII activity between 20 – 35 days after delivery (Fig. 3.8A). Three animals in this dose group never regained fVIII activity during the course of the study, with animal 1437R dying of unknown causes after the week 40 plasma collection. Animal 1453LL displayed a multiphasic fVIII activity profile, losing activity initially after 35 days, regaining it at week 14, losing it again between weeks 35 – 40, and then it rose once more to 2.5 IU/mL at week 45. Upon sacrifice at week 48, 1453LL had a plasma fVIII activity level of 0.935 IU/mL. Animals administered the 2E13 vg/kg dose reached initial peak fVIII activity levels of 4.58 – 6.21 IU/mL on day 20 (Fig. 3.8B). Half of these animals (1453L and 1455LL) maintained fVIII activity that fluctuated between approximately 2 – 6.5 IU/mL through week 36. The other two animals (1437L and 1455N) lost fVIII activity between weeks 35 – 49, with animal 1437L dying at around the same time as its littermate 1437R. The complex outcomes and fVIII activity fluctuations observed at 2E13 vg/kg AAV-HCB-ET3 mirror those observed for the more potent AAV-E06.TTR-ET3 vector dosed 3-fold lower at 6.67E12 vg/kg. Animals administered 6.67E12 vg/kg AAV-HCB-ET3 displayed peak fVIII activity levels of around 2 – 3 IU/mL between days 20 – 30 and maintained therapeutic fVIII activity levels with shallower fluctuations in activity levels over time (Fig. 3.8C). None of these animals lost fVIII activity following their initial peak activity levels after vector delivery. The same holds true for the animals administered 2.22E12 vg/kg AAV-HCB-ET3; however, these animals had lower initial peak fVIII activity levels between 0.7 – 1.87 IU/mL between 30 – 42 days after vector delivery and maintained steady state fVIII levels between 0.5 – 1.5 IU/mL (Fig. 3.8D). Overall, terminal liver VCN data show levels that correlate with the dose delivered. Of note, the animal (1453LL) administered 6E13 vg/kg of AAV-HCB-ET3 that lost and subsequently spontaneously recovered fVIII activity is also the animal in that dose group with the highest terminal VCN. Animal 1455LL in the 2E13 vg/kg dose group had both the highest terminal fVIII activity level and liver VCN in

that group. The animals in the 6.67E12 vg/kg dose group maintained therapeutic fVIII activity levels with less fluctuation and also display liver VCN values that cluster more closely together. However, interestingly, the animals with the two highest VCN values in that group would later go on to show resistance to immunological challenge with ET3i protein in a subsequent experiment. While animals in the 2.22E12 vg/kg dose group mostly had liver VCN values at or below 0.1 copies/cell, the one animal (1437LL) had a VCN around 0.4 copies/cell, maintained higher steady state fVIII activity levels, and would also show resistance to immunological challenge in a subsequent experiment.

Again, the dose response pattern in fVIII activity profiles with an overall shift based on perceived vector potency continues to hold true with the AAV-E06.TTR-HSQ vector regardless of the change in fVIII transgene sequence (Fig. 3.9).

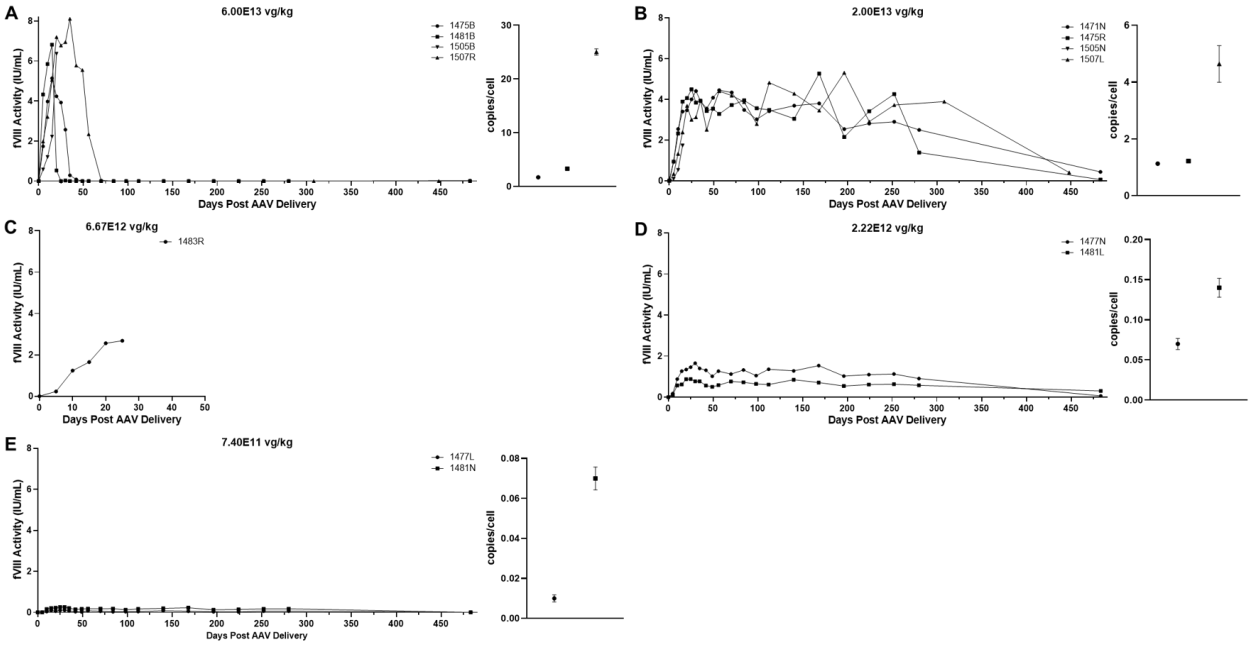


Figure 3.9. FVIII activity profiles and terminal VCN for individual animals administered AAV-E06.TTR-HSQ.
These panels display FVIII activity over time and terminal VCN after administration of AAV-E06.TTR-HSQ at **(A)** 6E13 vg/kg, **(B)** 2E13 vg/kg, **(C)** 6.67E12 vg/kg, **(D)** 2.22E12 vg/kg, or **(E)** 7.4E11 vg/kg.

Each panel represents one vector/dose group, and each curve represents a single animal. Terminal VCN was quantified by qPCR on liver DNA.

As with the ET3-expressing vectors, all animals administered the highest 6E13 vg/kg dose of AAV-E06.TTR-HSQ lost fVIII activity after reaching supraphysiological levels (Fig. 3.9A). In fact, these animals showed some of the highest peak fVIII levels at 5.15 – 8.11 IU/mL that occurred slightly later between 15 – 35 days. This could indicate that either higher peak levels associated with the more potent AAV-ET3 vectors were missed, either occurring between plasma collections or even before the first day 5 collection, or that slightly less transduced cell death occurred with the less potent vector. Indeed, one animal (1507R) had a terminal liver VCN of 25.04 copies/cell, well above the upper range of VCN values for animals administered any dose of the AAV-ET3 vectors. However, 1507R was sacrificed at 64 weeks post vector delivery, and the other two animals in this dose group that survived to the end of the study were sacrificed at 69 weeks and had VCN values far lower at 1.7 (1475B) and 3.3 (1481B) copies/cell. Animal 1505B died after the day 20 plasma collection. Animals administered 2E13 vg/kg AAV-E06.TTR-HSQ peaked at 4 – 4.5 IU/mL and maintained steady state fVIII levels between approximately 1.5 – 4.5 IU/mL without any appreciable decline in fVIII activity until time of sacrifice at between 64 – 69 weeks post vector delivery (Fig. 3.9B). Animal 1505N died after the day 15 plasma collection. The fVIII activity profiles in this group proceed similarly to those of AAV-E06.TTR-ET3 dosed at 2.22E12 vg/kg or AAV-HCB-ET3 dosed at 6.67E12 vg/kg. Unfortunately, limited data are available for the 6.67E12 vg/kg AAV-E06.TTR-HSQ group due to availability of the AAV-E06.TTR-HSQ vector, which had the lowest titer of the four vectors. And, after randomized pre-study enrollment of all animals, the only animal (1483R) to receive 6.67E12 vg/kg of AAV-E06.TTR-HSQ died after the day 25 plasma collection (Fig. 3.9C). The animals administered 2.22E12 vg/kg AAV-E06.TTR-HSQ peaked at between 0.88 – 1.67 IU/mL between days 25 – 30 (Fig. 3.9D) and maintained these

therapeutic levels until they decreased drastically by the study endpoint. FVIII activity decreased to undetectable levels at the study endpoint in animals administered 7.4E11 vg/kg AAV-E06.TTR-HSQ; however, their initial peak fVIII levels were much lower at 0.11 – 0.26 IU/mL by day 30 (Fig. 3.9E). The terminal liver VCN values were also much lower in animals administered 7.4E11 or 2.22E12 vg/kg of AAV-E06.TTR-HSQ and similar to those VCN values observed at the same doses in AAV-E06.TTR-ET3 treated animals.

Keeping with the trend of decreasing vector potency, animals administered the least potent AAV-HCB-HSQ vector are the only ones to maintain initial increases in fVIII activity levels after receiving 6E13 vg/kg of the vector (Fig. 3.10A). These animals also display the highest observed liver VCN values of any animals enrolled in the study, with animal 1481R being close to 20 copies/cell and animals 1497N and 1505R having values around 40 copies/cell. The fVIII activity levels in this group peaked between 3.6 – 4.2 IU/mL at between 30 – 56 days, which is a slower increase than that observed in the over vectors dosed at 6E13 vg/kg. These fVIII levels were maintained with some fluctuation until sometime between the week 44 plasma collection and time of sacrifice at weeks 64 – 69, where fVIII activity levels decreased from ~2 – 3.5 IU/mL down to ~0.4 – 0.75 IU/mL.

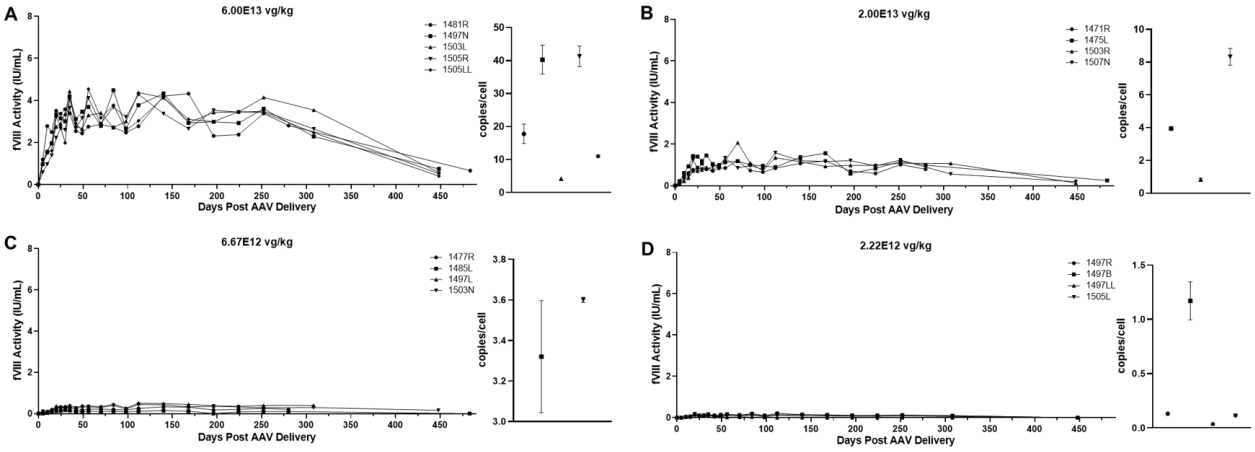


Figure 3.10. FVIII activity profiles and terminal VCN for individual animals administered AAV-HCB-HSQ.
These panels display FVIII activity over time and terminal VCN after administration of AAV-HCB-HSQ at **(A)** 6E13 vg/kg, **(B**) 2E13 vg/kg, **(C)** 6.67E12 vg/kg, or **(D)** 2.22E12 vg/kg. Each panel represents one vector/dose group, and each curve represents a single animal. Terminal VCN was quantified by qPCR on liver DNA.

In fact, all animals administered AAV-HCB-HSQ at the doses tested in this study maintained their initial fVIII activity levels until a decrease occurred by the time of sacrifice between 64 – 69 weeks post vector delivery (~72 – 81 weeks of age). The animals administered 2E13 vg/kg of AAV-HCB-HSQ initially peaked at about 1 – 2 IU/mL at between 20 – 56 days after vector delivery (Fig. 3.10B). As expected at the 3-fold lower vector dose, these animals had lower terminal liver VCN values ranging between 0.84 – 8.33 copies/cell. Animal 1471R died after the week 40 plasma collection. The animals administered 6.67E12 vg/kg AAV-HCB-HSQ had initial peak fVIII activity levels of 0.18 – 0.41 IU/mL between day 30 – 35 post vector delivery and were maintained with very little fluctuation until the study endpoint (Fig. 3.10C). Two animals (1477R and 1497L) died before sacrifice after 40 and 44 weeks, respectively. The two remaining animals had terminal fVIII levels that were either undetectable (1485L) or at 0.16 IU/mL (1503N); however, both animals had terminal liver VCN values of about 3.5 copies/cell, within range of those belonging to the animals dosed 3-fold higher at 2E13 vg/kg. The animals administered 2.22E12 vg/kg AAV-HCB-HSQ reached and maintained low levels of fVIII activity between 0.14 – 0.17 IU/mL (Fig. 3.10D). One animal, 1497LL, lost fVIII activity by week 16, while the others maintained fVIII activity until time of sacrifice at 64 weeks, at which point all had undetectable fVIII levels. The animals in this group had terminal liver VCN values between 0.04 – 1.17 copies/cell, with the lowest VCN belonging to the animal that lost fVIII activity by week 16.

While the long-term decline in fVIII activity observed may be due to a decrease in vector copies in originally transduced hepatocytes and or transduced cell death, The rapid decline in fVIII activity observed in animals initially expressing higher levels of fVIII suggested that fVIII neutralizing humoral immune responses occurred. To confirm this, both total anti-fVIII IgG and functional clot inhibition were measured by ELISA and modified Bethesda assay, respectively (Fig. 3.11). Animals produced anti-fVIII IgG after treatment with the AAV-E06.TTR-ET3, AAV-HCB-ET3, and AAV-E06.TTR-HSQ vectors but not the least potent AAV-HCB-HSQ vector. The 6E13 vg/kg dose of AAV-E06.TTR-ET3 and AAV-HCB-ET3 resulted in a 100% inhibitor incidence rate, wherein supraphysiological fVIII levels were reached within the first week after AAV administration and then rapidly declined to undetectable levels. Subsequent to loss of fVIII activity, detectable IgG titers appeared at 15 days after AAV-E06.TTR-ET3 and 20-30 days after AAV-HCB-ET3 delivery. In agreement with the vector potency and dose response trends, AAV-E06.TTR-ET3 dosed at 2E13 vg/kg produced an IgG response profile similar to that of AAV-HCB-ET3 dosed 3-fold higher at 6E13 vg/kg, while 2E13 vg/kg AAV-HCB-ET3 produced an IgG response profile more similar to AAV-E06.TTR-ET3 dosed 3-fold lower at 6.67E12 vg/kg (6E13vg/kg AAV-E06.TTR-ET3 > 6E13vg/kg AAV-HCB-ET3 ≈ 2E13vg/kg AAV-E06.TTR-ET3 > 2E13vg/kg AAV-HCB-ET3 ≈ 6.67E12vg/kg AAV-E06.TTR-ET3, comparing IgG titer incidence). Animal 1457B did not show detectable IgG or Bethesda titers throughout the study, which further supports the conclusion that this animal lost fVIII activity due to loss of genetically modified cells. AAV-E06.TTR-HSQ dosed at 6E13 vg/kg also displayed a 100% inhibitor incidence rate. While fVIII activity reached supraphysiological levels, the expression kinetics were slower and produced a larger area under the curve for fVIII exposure before the emergence of an anti-fVIII IgG titer compared to the AAV-E06.TTR-ET3 and AAV-HCB-ET3 vectors at 6E13

vg/kg. No animals administered ≤2E13 vg/kg AAV-E06.TTR-HSQ developed inhibitors within the study timeframe. For all AAV-fVIII treated animals that developed inhibitors, the fVIII activity began to decrease before the IgG titers were quantifiable, with little to no overlap in co-detectable fVIII activity and IgG titer at the time points assayed. Additionally, IgG titers uniformly appeared before measurable Bethesda titers and, in cases where the inhibitor response resolved spontaneously, the Bethesda titer abated before the IgG titer.

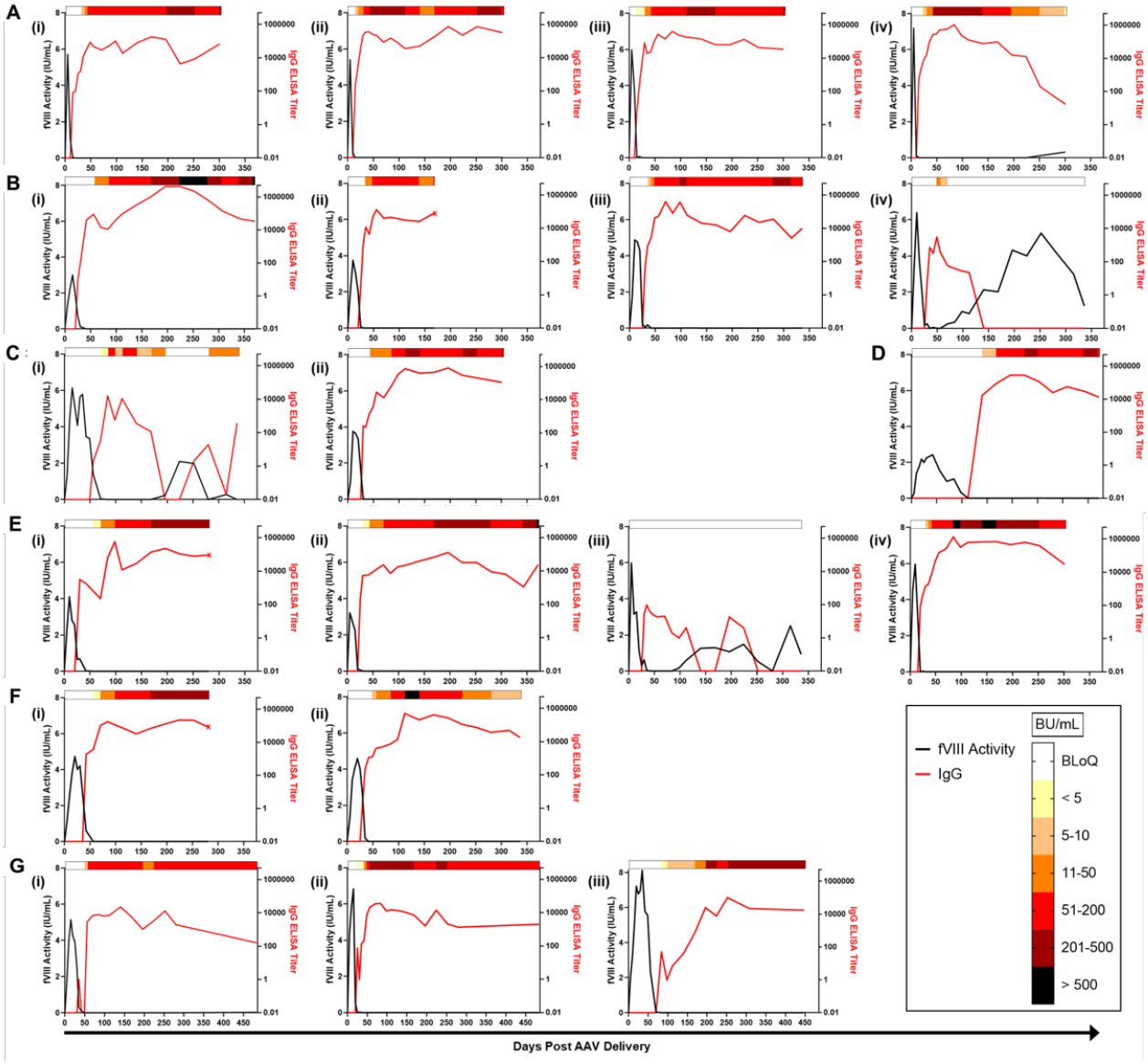


Figure 3.11. The anti-fVIII antibody response after AAV-fVIII delivery can be complex and multiphasic.
The anti-fVIII IgG titers were measured by ELISA. IgG titers are indicated by the red curve and quantified on the right y-axis. The corresponding fVIII activity levels are indicated by the black curve and quantified on the left y-axis. Bethesda (inhibitor) titers (BU/mL) were measured for every sample with a detectable IgG titer and/or loss of detectable fVIII activity. BU/mL values are indicated by a heat map at the top of each graph. Samples below the limit of quantification for any antibody assay were given a working value of zero in data analysis and graphical representation; however, the actual value could be somewhere between zero and the limit of quantification. An "X" terminating the IgG titer curve denotes death of the animal at the designated time point. **(A)** 6E13vg/kg AAV-E06.TTR-ET3. Animal IDs: (i) 1471L, (ii) 1475N, (iii) 1477B, (iv) 1483N. **(B)** 2E13vg/kg AAV-E06.TTR-ET3. Animal IDs: (i) 1451L, (ii) 1453R, (iii) 1455R, (iv) 1457L. **(C)** 6.67E12vg/kg AAV-E06.TTR-ET3. Animal IDs: (i) 1453N, (ii) 1479N. **(D)** 2.22E12vg/kg AAV-E06.TTR-ET3, animal 1437N. **(E)** 6E13vg/kg AAV-HCB-ET3. Animal IDs: (i) 1437R, (ii) 1451R, (iii) 1453LL, (iv) 1479L. **(F)** 2E13vg/kg AAV-HCB-ET3. Animal IDs: (i) 1437L, (ii) 1455N. **(G)** 6E13vg/kg AAV-E06.TTR-HSQ. Animal IDs: (i) 1475B, (ii) 1481B, (iii) 1507R.

Among the animals that developed inhibitors (n=20), there were four cases in which fVIII activity spontaneously reemerged (Fig. 3.11 A(iv), B(iv), C(i), E(iii)). Generally, these animals had the highest peak fVIII activity in their vector/dose group but generated lower IgG and Bethesda titers. Recovery of fVIII activity and apparent immune tolerance induction (ITI) appear to have occurred through a complex, multiphasic process wherein fVIII activity and IgG titer are transiently co-detectable and do not always proceed inversely. Interestingly, the two animals with peak IgG titers <5,000 and peak Bethesda titers <50 BU/mL recovered fVIII activity earlier (Fig. 3.11 B(iv), E(iii), recovery within 6 weeks after AAV-fVIII delivery) than the two animals with IgG titers >5000 and more sustained Bethesda titers >50 BU/mL (Fig. 3.11 A(iv), C(i), recovery after ≥16 weeks). Also, in all inhibitor response cases, the IgG titer fluctuated by as much as 1-2 logs between plasma collections. These data suggest that while the likelihood of developing an inhibitor response can be judged from initial fVIII exposure kinetics, the nature of the sustained anti-fVIII immune response is complex and possibly dependent on multiple immunological mechanisms, such as the interaction and clearance of immune complexes. Therefore, pharmacokinetic principles were employed to create a mathematical model of the observed

positive correlation between initial fVIII kinetics and anti-fVIII IgG incidence (Table 3.1 and Figs. 3.12 – 3.14).

AAV vectors used in the current study share the following attributes: capsid serotype 8, liver-directed promoter, liver codon-optimized transgene, titers around 4E13 vg/mL, 0.5 full/empty particle ratio, and dosed on a vg/kg body weight basis. Therefore, AAV capsid exposure and cell entry are assumed to be similar between the vectors from both a pharmacokinetic and immunological perspective. Given these assumptions and the dose response trends that persist throughout this study, the apparent plasma fVIII exposure was used for PK/PD modeling and immunogenicity risk assessment. Analysis of plasma fVIII levels facilitated the application of a single-compartment (circulation), first-order elimination (constant fraction of fVIII eliminated per unit time) pharmacokinetic model. All of the values used in the pharmacokinetic analyses are outlined in Table 3.1.

Parameter	Variable	Units	Value
Half-life of HSQ and ET3	$T_{1/2}$	hours	7
		days	0.292
Volume of distribution	V_d	mL/kg	65
		mL (in 0.025 kg mouse)	1.625
Clearance	CL	mL/day	3.857
Elimination rate constant	k_e	day^{-1}	2.373
Plasma fVIII activity	C	IU/mL	Varies
fVIII production or exposure rate	k_{fVIII}	IU/day	Varies
Total fVIII exposure	AUC_{fVIII}	IU	Varies

Table 3.1. The pharmacokinetic parameters employed in determination of the immunogenicity threshold based in initial kinetics of fVIII protein exposure following AAV-fVIII delivery.

The half-life ($T_{1/2}$) and volume of distribution (V_d) of recombinant BDD-fVIII are values obtained from the literature.[245-248] These values were used to calculate the clearance (CL, Equation

1) and elimination rate constant (k_e, Equation 2) for a 0.025 kg mouse (average weight at time of AAV delivery).

Equation 1

$$CL = 0.693\left(\frac{V_d}{T_{1/2}}\right)$$

Equation 2

$$k_e = \frac{CL}{V_d}$$

Activity (IU/mL) was chosen as the fVIII exposure input since this is how fVIII is monitored clinically and it provides feedback on timing and degree of inhibition by anti-fVIII antibodies. FVIII activity data over 24 weeks from all animals treated with AAV-fVIII thus far were analyzed using the equation for constant-rate intravenous drug infusion (Equation 3) to mimic secretion from the liver. Transduced hepatocyte fVIII production rates are not expected to be constant over time, but apparent plasma fVIII concentration (C, in IU/mL) along with the time point assayed (t, in days) can be used to calculate the fVIII production, or exposure, rate (k_{fVIII}, in IU/day) at that time point (Equation 3). These values were then plotted to show the change in fVIII production rate over time and the area under the curve was calculated to give total cumulative fVIII exposure (AUC_{fVIII}, in IU, Equation 4).

Equation 3

$$C = \frac{k_{fVIII}}{CL}\left(1 - e^{-k_e t}\right)$$

Equation 4

$$\mathrm{AUC_{fVIII}} = \int_0^t \frac{(\mathrm{C})(\mathrm{CL})}{(1 - \mathrm{e}^{-\mathrm{k_e t}})} dt, \qquad \mathrm{t} = 5 \; or \; 10$$

FVIII activity becomes rapidly immeasurable once inhibitors develop. However, the data clearly suggest the primary importance of initial fVIII exposure kinetics for determining inhibitor incidence. Since the goal is to create a model that can guide AAV gene therapy design and dosing parameters with the greatest possibility of avoiding inhibitor induction, data through day 10 were included in the risk assessments (through day 5 for the five animals whose fVIII activity decreased at day 10, preceding an inhibitor titer) (Fig. 3.12A). Thresholds above which inhibitors formed are delineated by a k_{fVIII} of approximately 1 IU/day and an AUC_{fVIII} of 50 IU over the first 10 days. Logistic regression analysis of k_{fVIII} and AUC_{fVIII} at days 5 and 10 demonstrates the significance of all four threshold values (Fig. 3.12B).

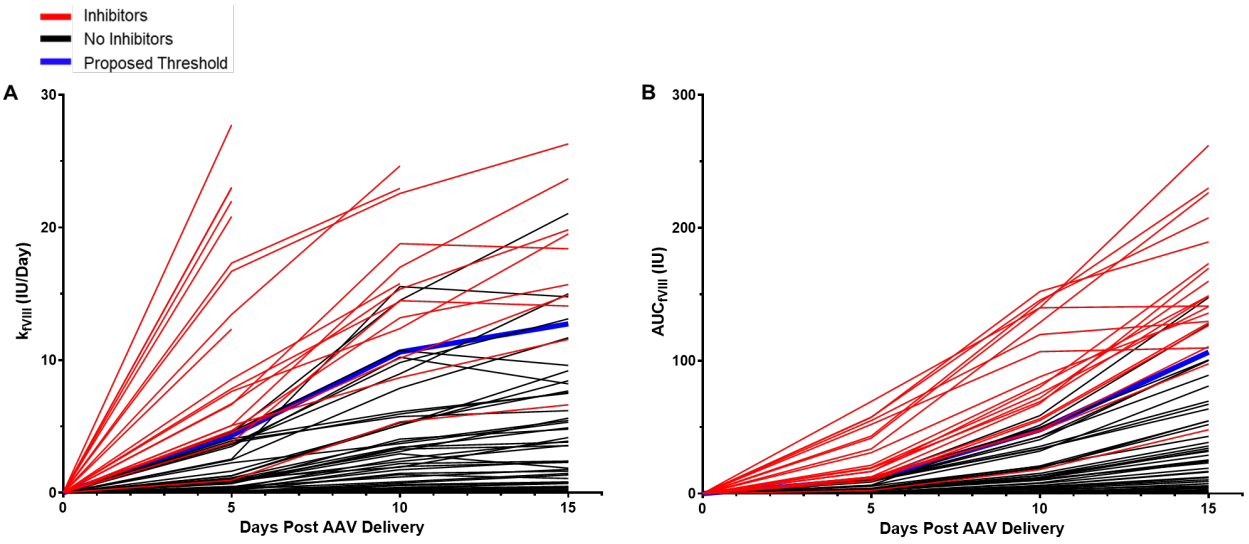


Figure 3.12. The proposed fVIII immunogenicity threshold following AAV-fVIII delivery based on initial fVIII activity levels.
These graphs display the **(A)** fVIII production (or exposure) rate, k_{fVIII}, and **(B)** the corresponding cumulative fVIII exposure, AUC_{fVIII}, for each AAV-fVIII treated animal through day 15 (n = 66). Red curves represent animals that developed an inhibitor response and black curves represent the animals that did not develop inhibitors. The proposed immunogenicity threshold is outlined in blue and was determined by linear regression with shared parameters.

However, simple logistic regression analysis of the pharmacokinetic and inhibitor incidence data revealed that day 5 k_{fVIII} is the strongest independent indicator of inhibitor incidence (Fig. 3.13A: $\beta 1$=3.2, 95%CI [1.860, 8.818], LRT $p<0.0001$). The fVIII exposure immunogenicity threshold values outlined in figure 3.12 are also validated by the 50% inhibitor development threshold (ID_{50}) values determined by the logistic regression results (Fig. 3.13 A(i) – (iv): day 5 k_{fVIII} ID_{50}= 4.49, day 10 k_{fVIII} ID_{50}= 11.12 IU/day; day 5 AUC_{fVIII}= 11.22, day 10 AUC_{fVIII}= 50.22 IU), with Table 3.2 outlining which animals developed inhibitors and whether they met the immunogenicity threshold by day 5 and/or 10.

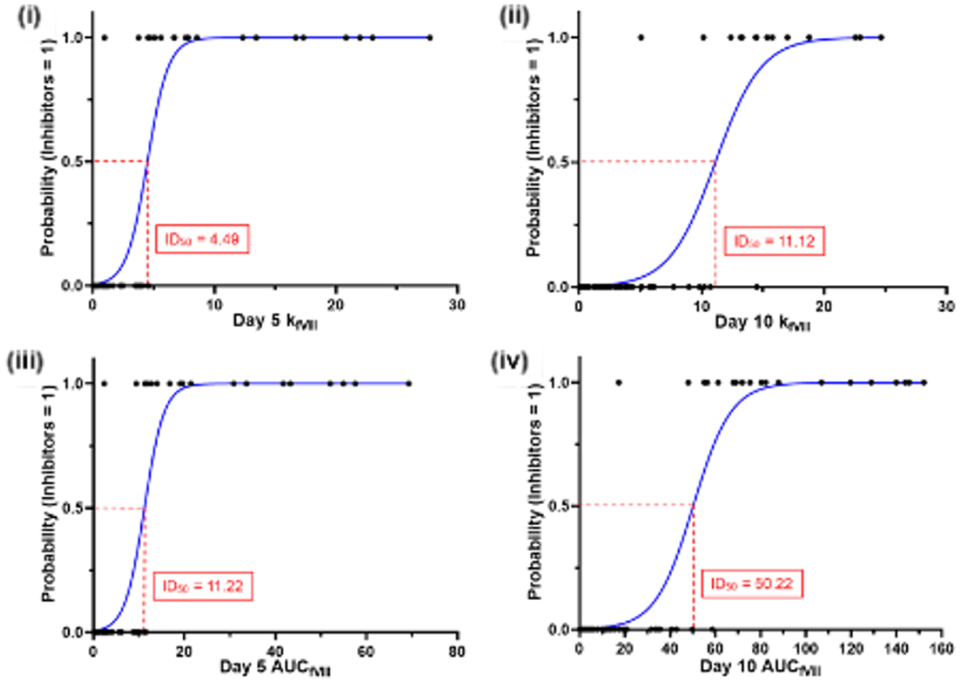
	N with inhibitors (N meet immunogenicity threshold)			
Vector	**E06.TTR-ET3**	**HCB-ET3**	**E06.TTR-HSQ**	**HCB-HSQ**
Relative potency	++++	+++	++	+
6E13 vg/kg	4 (4)	4 (4)	3 (3)	0 (1)
2E13 vg/kg	4 (4)	2 (3)	0 (0)	0 (0)
6.67E12 vg/kg	2 (2)	0 (0)		0 (0)
2.22E12 vg/kg	1 (0)	0 (0)	0 (0)	0 (0)
7.4E11 vg/kg	0 (0)		0 (0)	
2.46E11 vg/kg	0 (0)			

Table 3.2. Inhibitor incidence and immunogenicity threshold fulfillment by vector/dose group.
These counts represent animals that developed inhibitors after AAV-fVIII delivery (prior to any protein challenge). The only animal that developed inhibitors and did not meet the immunogenicity threshold was one administered 2.22E12 vg/kg of AAV-E06.TTR-ET3. All other animals counted as developing inhibitors also met the immunogenicity threshold. Shaded boxes indicate that the vector either was not tested at that dose or that the animals in that vector/dose group were not included in the PK model due to spontaneous death at an early time point.

The logistic regression ID_{50} values are those at which the risk of inhibitor development is predicted to be 50% or, alternatively, the values predicted to induce inhibitor development in 50% of AAV-fVIII treated subjects. Risk assessment by Fisher's exact test reports an attributable risk increase of 0.9065 (95%CI [0.6579, 0.9660], $p<0.0001$) and a risk ratio of 21.85 (95%CI [6.50, 79.35], $p<0.0001$) for inhibitor incidence when the k_{fVIII} threshold is met by day 10 (Fig. 3.13B).

A

Day 5 and Day 10 Odds for Inhibitor Incidence					
		ID_{50}	β0 Odds	β1 Odds	LRT
k_{fVIII}	Day 5	4.49	0.0054 [7.22E-5, 0.0504]	3.2 [1.860, 8.818]	p<0.0001
	Day 10	11.12	0.0027 [2.83E-5, 0.0323]	1.704 [1.350, 2.548]	p<0.0001
AUC_{fVIII}	Day 5	11.22	0.0054 [7.21E-5, 0.050]	1.593 [1.282, 2.389]	p<0.0001
	Day 10	50.22	0.0024 [1.97E-5, 0.032]	1.127 [1.069, 1.241]	p<0.0001



B

fVIII Exposure Rate (k_{fVIII}) and Inhibitor Incidence			
	Meets Threshold	Below Threshold	Total
Inhibitors	19	1	20
No Inhibitors	2	44	46
Total	21	45	66
ARI	0.9065	p < 0.0001 [0.6579, 0.9660]	
RR	21.85	p < 0.0001 [6.50, 79.35]	

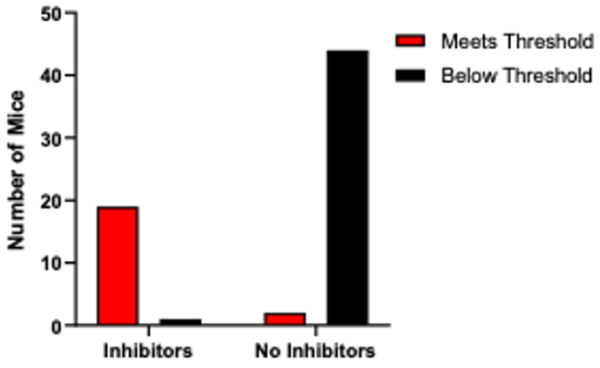


Figure 3.13. Statistical analyses support the predictive ability of the proposed fVIII exposure threshold for fVIII inhibitor incidence after AAV-fVIII gene therapy.
(A) ID_{50} values were calculated by simple logistic regression analysis (ID_{50} = the value predicted to induce inhibitor development in 50% of AAV-fVIII treated subjects): (i) Day 5 k_{fVIII}, (ii) Day 10 k_{fVIII}, (iii) Day 5 AUC_{fVIII}, (iv) Day 10 AUC_{fVIII}. (Likelihood Ratio Test, LRT, of the β1 variable was set at $p<0.05$ for significance and brackets indicate the 95% confidence interval, CI.) **(B)** Contingency analysis was performed based on the inhibitor incidence (count) of animals that met the k_{fVIII} threshold by day 10. (Two-sided Fisher's exact test with significance set at $p<0.05$. Brackets indicate 95%CI. ARI = Attributable Risk Increase. RR = Risk Ratio.)

Further supporting the significance of the day 5 k_{fVIII} threshold, contingency analysis of survival proportions (survival = no inhibitors) shows a significant survival benefit for animals with day 5 $k_{fVIII} < ID_{50}$ (Fig. 3.14A, $p < 0.0001$. Median time to inhibitors with $k_{fVIII} \geq 4.49$ = 30 days). There is also a significant inverse correlation between the day 5 k_{fVIII} and the time to anti-fVIII IgG incidence (Fig. 3.14B, $r_s = -0.7333$, 95%CI [-0.8907, -0.4189], $p < 0.0002$). In agreement with the predicted vector potency ranking, both the vector design and dose have a significant effect on the day 5 k_{fVIII} values (Fig. 3.14C, main effects two-way ANOVA, dose factor = 65.56, vector factor = 18.76, $p<0.0001$ for both factors).

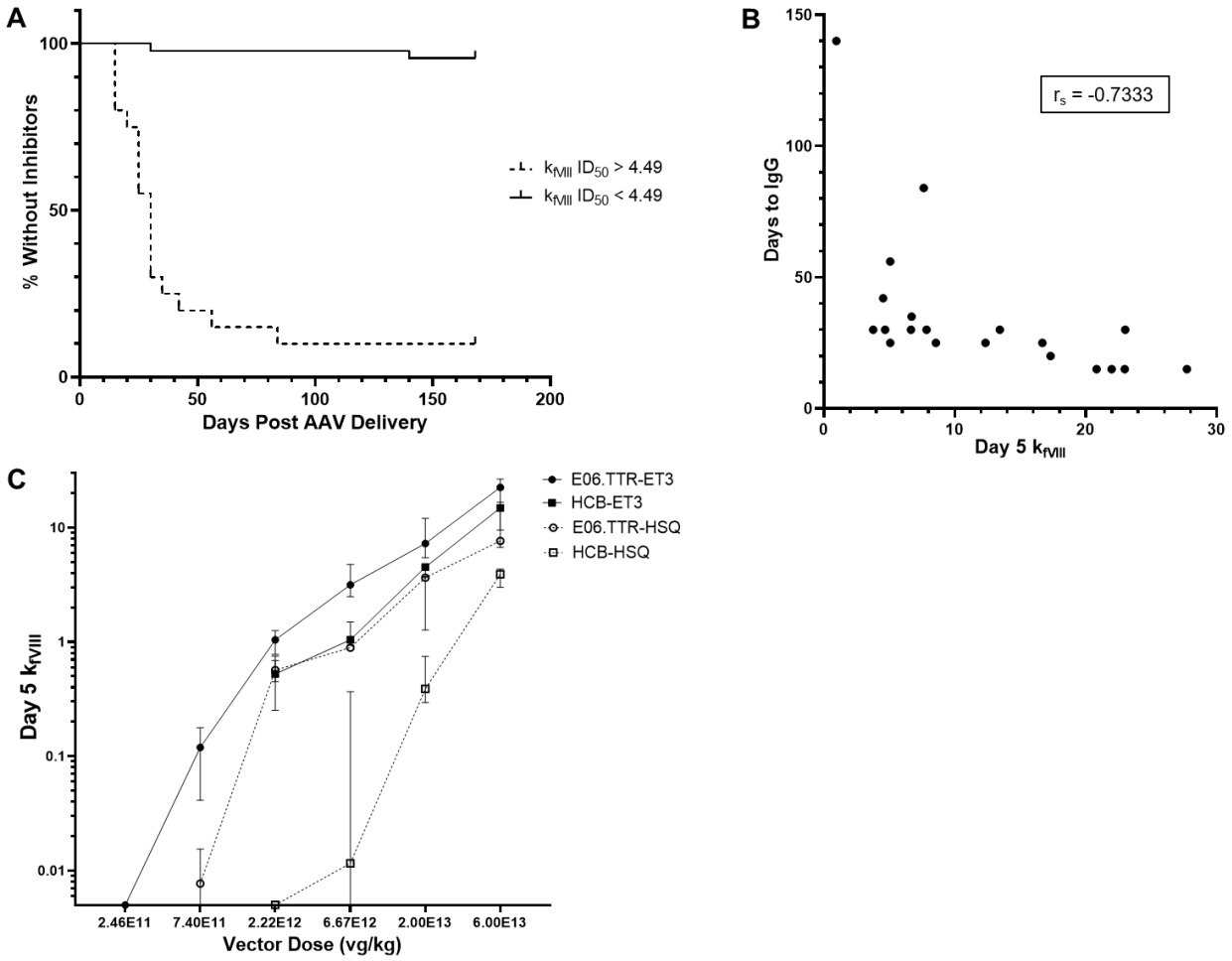
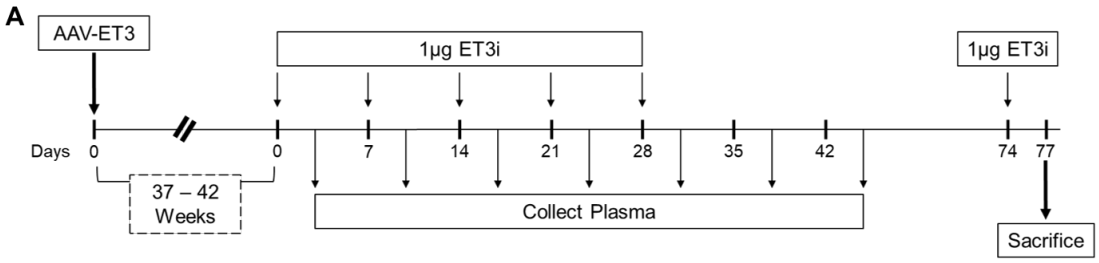


Figure 3.14. Further validation of the day 5 kfVIII value as the primary independent predictor of fVIII inhibitor incidence following AAV-fVIII delivery.

(A) The survival curve indicates occurrence of inhibitors for animals with day 5 k_{fVIII} values ≥ID_{50} compared to those below the ID_{50}. The median time to inhibitors with k_{fVIII} ≥4.49 = 30 days. (Chi-square = 66.95, df=1, $p < 0.0001$). **(B)** This graph displays the correlation of day 5 k_{fVIII} values with the time to anti-fVIII IgG incidence. (Spearman r = -0.7333, 95%CI [-0.8907, -0.4189], p = 0.0002). **(C)** This graph displays the median day 5 k_{fVIII} values by vector dose for each AAV-fVIII vector. Error bars indicate IQR. Values below the red dashed line indicate a day 5 k_{fVIII} = 0, meaning that there was no detectable fVIII activity by chromogenic assay 5 days after vector delivery. Both the vector design and dose factors have a significant impact on the day 5 k_{fVIII}. (Main effects two-way ANOVA, $p<0.0001$ for vector and dose effects.)

To test whether the AAV-ET3 treated animals that did not develop inhibitors within 37-42 weeks may have developed immune tolerance to ET3 or if they would still respond to additional exogenous ET3i (i.e., purified recombinant ET3) exposure, these animals were challenged with 5 weekly injections of 1 µg ET3i and assayed for fVIII activity and inhibitors after each challenge

(Fig. 3.15A and B). Naïve E16 mice were challenged in parallel for comparison of IgG titer incidence and magnitude in fVIII naïve animals to that in AAV-ET3 pre-treated challenge animals. In the context of an immunogenic fVIII exposure threshold model, one hypothesis was that the animals making higher endogenous levels of fVIII after gene therapy would more readily cross the immunogenicity threshold after infusion of exogenous fVIII. As this model is based on early fVIII exposure kinetics, the alternative hypothesis was that animals that do not meet this initial immunogenicity threshold must inevitably adopt some form of immune tolerance to fVIII to allow for sustained fVIII activity and, therefore, animals with higher steady state endogenous fVIII levels would display greater immunological resistance to exogenous fVIII challenge. FVIII activity and IgG titer data validate the latter hypothesis (Fig. 3.15C and Supplementary Tables S3.1 and S3.2).



B

Challenge	No Challenge	AUC Range [IU ET3]	Activity Range [IU/mL ET3]
3	1	2092 – 4138	≥ 2
4	1	1857 – 2272	1.2 – 2
3	1	525 – 1393	0.5 – 1.2
4	1	82 – 428	0.01 – 0.5
3	1	4 – 283	≤ 0.01
4	2	Naïve HA mice	

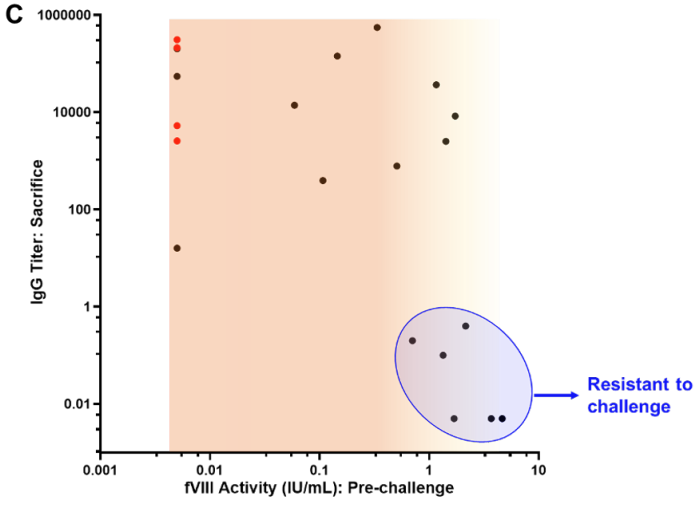


Figure 3.15. ET3i protein challenge in AAV-ET3 treated animals suggests an ideal steady state fVIII activity level for maintaining fVIII immune tolerance.

(A) Animals treated with either AAV-E06.TTR-ET3 or AAV-HCB-ET3 that did not develop inhibitors by 37-42 weeks were challenged with 5 weekly infusions of 1ug purified ET3i protein. Plasma was collected 3 days after every injection and at weeks 6 and 7 post-challenge initiation. An additional 1ug ET3i was administered 3 days before sacrifice. **(B)** Animals were subdivided based on pre-challenge FVIII activity, and one animal per group served as a no challenge control. Naïve hemophilia A animals were immunized alongside the gene therapy treated animals. **(C)** This graph displays the steady state fVIII activity prior to ET3i challenge vs. the IgG titer at time of sacrifice in ET3i challenged animals. The following animals were resistant to challenge: 1433L, 1437LL, 1443N, 1443L, 1455LL, 1463N.

In AAV-ET3 treated animals with low steady state fVIII activity levels (<0.7 IU/mL), inhibitor responses were more similar to those of the fVIII naïve challenge animals, with inhibitors developing somewhere between the 3rd and 5th challenge. Animals making at least 2 IU/mL were resistant to challenge, while inhibitor incidence results were mixed in animals making between approximately 0.7 – 2 IU/mL fVIII prior to challenge. While initial fVIII exposure kinetics are the primary predictor of inhibitor incidence after AAV-fVIII treatment (i.e., gene therapy derived fVIII immunogenicity), achieving steady state levels of around 1.5 – 4.5 IU/mL increases the probability of developing an immunological state of unresponsiveness (i.e., tolerance) to the exogenous fVIII (Fig 3.15C).

The inability to accurately quantify fVIII antigen in the presence of anti-fVIII neutralizing antibodies is an obstacle to *in vivo* investigation of immunological mechanisms involved in the fVIII inhibitor response after gene therapy. The earliest time point when fVIII activity began to decrease in AAV-fVIII treated mice that developed inhibitors was day 10; therefore, ET3 antigen could be reliably quantified by antigen ELISA in day 5 plasma. The day 5 antigen levels (ng/mL) were significantly higher in animals that proceeded to develop inhibitors after AAV-ET3 delivery (Fig. 3.16A, $p = 0.0008$). However, when the day 5 antigen levels were plotted against the day 5 fVIII activity data, there was no significant difference in the *in vivo* ET3 specific activity (IU/mg)

between those two groups, further indicating that the detectable ET3 antigen was fully active and not yet subject to significant inhibition (Fig. 3.16B).

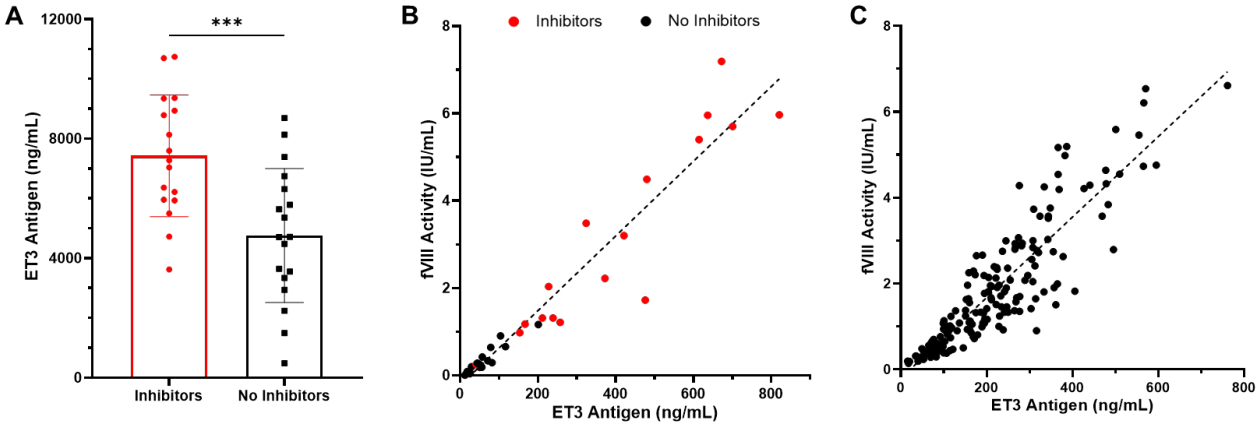


Figure 3.16. There is a significant difference in ET3 antigen levels but not *in vivo* specific activity 5 days after AAV-ET3 delivery.
(A) The mean ET3 antigen levels 5 days after AAV-ET3 delivery are significantly higher in animals that subsequently formed inhibitors compared to animals that did not. Error bars indicate standard deviation. (Two-tailed t test, p = 0.0008). **(B)** This graph plots ET3 antigen vs. FVIII activity 5 days after AAV-ET3 delivery, with a combined linear regression slope of 8559 IU/mg. Animals that subsequently formed inhibitors are indicated by red dots, and there was no significant difference in day 5 linear regression slopes between animals that did and did not form inhibitors. **(C)** This graph plots ET3 antigen vs. FVIII activity through week 28 for all animals that did not form inhibitors after AAV-ET3 delivery. The linear regression slope is 9376 IU/mg.

Plotting the ET3 antigen vs. fVIII activity values through week 28 from all AAV-ET3 treated animals that did not develop inhibitors (prior to ET3i challenge) calculated an *in vivo* ET3 specific activity of 9400 IU/mg (Fig. 3.16C, r^2 = 0.804). While *in vivo* ET3 specific activity fluctuates over time in AAV-ET3 treated animals, values above 13,000 IU/mg were calculated (Supplementary Fig. S3.4), which is consistent with previously determined *in vitro* and *in vivo* ET3i specific activity data.[161,175] ET3 antigen was detectable in AAV-ET3 treated animals that developed the multiphasic inhibitor response (n=4) and even in some animals with sustained anti-fVIII IgG titers at certain time points (Fig. 3.17A and Supplementary Figs. S3.3 and S3.4). ET3

antigen was also still measurable throughout the ET3i challenge in those animals that demonstrated immunological resistance despite observed decreases in fVIII activity and occasional IgG titers of <5. In fact, there was no significant difference in ET3 specific activity between challenge resistant animals and the no challenge AAV-ET3 controls (Fig. 3.17B and Supplementary Figs. S3.4 and S3.5). Importantly, by the time of sacrifice, there was also no significant difference in ET3 specific activity between the AAV-ET3 multiphasic response animals and the AAV-ET3 treated + no ET3i challenge controls (no inhibitors) (Fig. 3.17C).

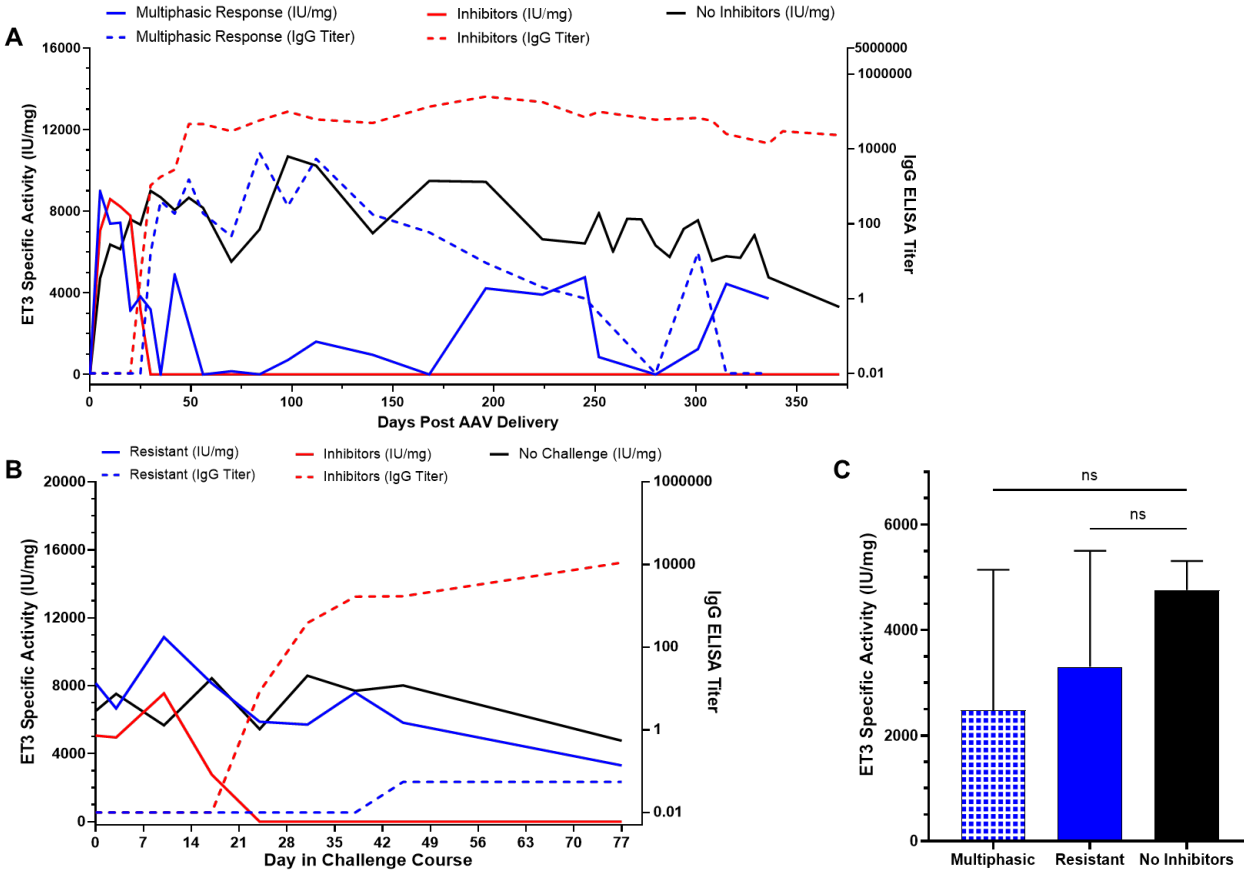


Figure 3.17. The *in vivo* ET3 specific activity in different anti-ET3 immune response scenarios after AAV-ET3 treatment.

These graphs profile the median *in vivo* ET3 specific activity and IgG titer over time **(A)** prior to ET3i challenge or **(B)** during the ET3i challenge course. These data are stratified by the type of anti-ET3 immune response observed. The IQR error bars were removed for visual clarity but are shown in Supplementary Figures S3.4 and S3.5. **(C)** There is no significant difference in *in vivo* ET3 specific activity at time of sacrifice for animals that demonstrated a multiphasic inhibitor response after AAV-ET3 delivery (blue and

white) and AAV-ET3 treated animals that were resistant to ET3i challenge (solid blue) compared to the AAV-ET3 treated + no ET3i challenge control animals (black) (Kruskal-Wallis H test, $p = 0.5874$).

Taken together, these data indicate that ET3 antigen was still being produced even in the context of an anti-ET3 neutralizing antibody response. One of the hopes associated with gene therapy development and fVIII protein engineering is that alternative fVIII proteins delivered via a constant source of therapeutic levels of endogenously produced protein (i.e., via gene therapy) would allow for effective and easier treatment of hemophilia A patients with inhibitors. For the purposes of drug development, translational research, and clinical research and/or application in the inhibitor setting, it is important to know the pharmacological properties of the engineered fVIII protein product and whether it can be detected in the plasma of patients with inhibitors against their usual prophylactic replacement fVIII (usually BDD-hfVIII). Understanding whether and to what degree the new fVIII protein can be detected via a binding assay like an antigen ELISA in the inhibitor patient plasma will provide valuable information as to whether this alternative fVIII protein could be useful in an ITI application as well as help inform what dosing of the gene therapy would be required to yield fVIII exposure kinetics and steady state levels that would have the best chance of inducing immune tolerance. Indeed, it would be extremely unfortunate for the patient's quality of life if they were to pay for AAV-fVIII gene therapy treatment without every effort being put in to determining the probability of its success and the best treatment protocol for increasing that probability as much as possible.

ET3i is a new drug candidate for fVIII replacement prophylaxis and for functional hemophilia A cure via delivery of LV-fVIII transduced HSPCs or intravenous delivery of AAV-fVIII gene therapy vector. Multiple assays were performed with ET3i in order to evaluate its relative immunogenicity and its cross-reactivity with anti-hfVIII inhibitory antibodies. As assessed

previously and discussed in this dissertation (Chapter 2, Figs. 2.1 and 2.2), like fVIII proteins in general, ET3i is similarly immunogenic compared to human fVIII (HSQ or Advate) and ancestral fVIII 53 (An53). Work performed by Jasmine Ito and Courtney Cox in the laboratory of Shannon Meeks evaluated the functional anti-hfVIII inhibitor titer of several inhibitor plasma samples from hemophilia A patients via Bethesda assay. These samples were then evaluated for the degree of cross-reactivity the anti-fVIII inhibitors have against ET3i using the Bethesda assay. The anti-fVIII and anti-ET3i Bethesda titers for these samples are listed in Table 3.2. Many of the human inhibitor plasma samples demonstrated a 20 – 75% decrease in inhibitor titer when ET3i was used in the assay, some of the titers remained relatively unchanged, but two actually displayed a 46 – 48% increase in inhibitor titer against ET3i. Domain mapping for the dominant antibody epitope was also performed on these samples. BDD porcine fVIII has 83% identity with BDD human fVIII, and with only the A1 and A3 domains of ET3i being porcine, BDD ET3i has 90% identity with BDD human fVIII. While A2 and C2 are found to be the most immunodominant epitopes in human fVIII, inhibitors do target epitopes all along the fVIII protein. Interestingly, while ET3i is porcine in the activation peptide, A, and A3 domains, two of the patients that had increases in anti-ET3i inhibitor titer had mostly A2 or C2 dominant domains. Some patients showed no domain dominance, porcine cross-reactivity, or some dominance in the A1 or A3 domains. This tells us that, indeed, while some domains are considered dominant, there are contributions to inhibitor development all along the fVIII protein and the antibody response to fVIII is polyclonal. However, some decreases in functional inhibition were observed with ET3i, suggesting that ET3i could still be effective in patients with anti-hfVIII inhibitors and perhaps even more so when provided at constant levels at or above physiological circulating concentrations of fVIII (≥ 1 IU/mL). Also,

not all anti-fVIII antibodies inhibit fVIII activity, but they could affect fVIII binding to vWF and/or fVIII clearance and half-life in circulation.

Therefore, to further assess ET3i pharmacology in the inhibitor setting, original work presented herein tests the ability to detect ET3i protein with an anti-ET3i specific antigen ELISA after addition of known concentrations of ET3i into hfVIII inhibitor patient plasmas (Table 3.2 and Figs. 3.18 – 3.19). Fourteen of the human inhibitor plasma samples that were assessed for anti-ET3i Bethesda titers and mapped for dominant epitopes were also evaluated with this antigen ELSA. Ten other inhibitor patient plasma samples with varying anti-hfVIII Bethesda titers (but no ET3i cross-reactivity data) were also evaluated. FACT was used as a control for detection of ET3i in normal human plasma (no fVIII inhibitors). ET3i was added in amounts of 300 ng/mL, 100 ng/mL (middle of the range of normal circulating fVIII protein levels, 50 – 150 ng/mL), and 10 ng/mL. The values of ET3i detected and reported by the antigen ELISA are listed in Table 3.2. The patient IDs highlighted in green are those that displayed detection accuracy above 80% for two out of the three ET3i concentrations assayed.

Sample ID	300 ng/mL ET3i	100 ng/mL ET3i	10 ng/mL ET3i	No ET3i	fVIII BU/mL	ET3i BU/mL	Dominant Epitope
IB001C	195.4	66	7.9	3.8	7.6	N/A	
IB002D	272.2	54.3	7	0	76.3	28.6	A2
IB003A	326.9	84.3	0	0	42	N/A	
IB006E	481.6	167.8	18.4	3.5	79	36.2	C2
IB008G	236.1	99.3	8.1	0.42	27.8	13.1	C2/A2
IB009C	238	0	0	0	18.1	N/A	
IB010E	450	179.6	0	9.9	115.5	71.4	C2
IB012A	282.5	64.3	2.2	2.3	105.5	68.5	C2
IB015B	358.6	114.1	0	21	29	19.8	No Dominant Epitope
IB018D	781.5	173.5	11.9	0	105	N/A	
IB020B	100.5	7.3	1.7	4.7	84	63.7	Porcine Cross-reactive
IB027A	97.5	24.7	4.7	0.57	12.1	9.5	C2/A2
IB029G	446.1	151.9	22.5	1.8	98	N/A	
IB030C	709.9	188	0	0	19.6	16.8	C2
IB037B	179.4	0	0	0	109.4	110	Slight A2
IB043A	508.3	169	31.2	44.1	48.1	54.6	C2/A1
IB047B	208.7	62.6	6.4	0.75	22	25.4	A2
IB050A	207.6	42.1	3.7	0.28	88.4	129	A2/Small C2 and A3
IB050B	176.4	82.9	10.6	2	259.4	N/A	
1B053A	398.4	49.3	1.7	0	33.3	49.3	C2/Small A2
IB057B	150.9	30.2	3.4	0	727	N/A	
IB057C	127.1	41	3	3.1	773.1	N/A	
IB062A	623.6	126.7	11.4	1.6	814	N/A	
IB094A	568.3	206.8	22	1.8	260	N/A	
FACT 1	392.8	98.1	10.2	0	0	0	
FACT 2	413.3	97.5	9.4	1.5	0	0	
FACT 3	613.3	117.9	0	32.1	0	0	

Table 3.3. ET3i antigen levels detected in human fVIII inhibitor patient plasma samples.

This table lists the patient sample IDs used in the ET3i antigen ELISA. The ET3i concentrations measured by the ELISA are listed below the actual ET3i concentrations added to the plasma samples. The values of ET3i reported by the assay when no ET3i protein was added to the samples are also listed. The anti-fVIII and anti-ET3i Bethesda titers and ET3i epitope domain mapping results where available are listed in the final three columns. *The anti-fVIII and anti-ET3i Bethesda titers along with the epitope domain mapping results were performed by Jasmine C Ito and Courtney L Cox, and these results were presented in a poster by Jasmine C Ito, et. al. at the 2017 annual ASH meeting.*

When the accuracy of detection of the ET3i protein added to the inhibitor patient plasmas is plotted against the anti-fVIII inhibitor (Bethesda) titers for each sample, one can see that there

is no correlation in the population between the magnitude of the fVIII inhibitor titer and the accuracy with which ET3i can be quantified by this antigen ELISA at either 10 ng/mL, 100 ng/mL or 300 ng/mL (Fig. 3.18). This is interesting and encouraging, as one might expect that the higher the inhibitor titer, the more difficult it would be to bind ET3i protein in the ELISA. This points to the fact that it is likely that the epitopes that the inhibitors bind to are more significant than the magnitude of the titer. ET3i was able to be detected with at least 80% accuracy for two out of the three concentrations tested in 5 of the patient samples (highlighted in green in Table 3.2). Additionally, all 5 of these samples detected the 100 ng/mL ET3i concentration accurately. It does appear that the assay developed here detects the 100 ng/mL (physiological) concentration of ET3i with greater accuracy than higher (300 ng/mL) or lower (10 ng/mL) concentrations. Two of the patients, IB050 and IB057, gave two samples from separate blood draws. For patient IB050, their fVIII inhibitor titer did increase from sample IB050A (88.4 BU/mL) to sample IB050B (259.4 BU/mL); however, the accuracy with which ET3i was detected in this patient's plasma actually improved in the plasma with the higher fVIII inhibitor titer. The inhibitor titer for patient IB057 remained relatively steady, albeit very high, from sample IB057B (727 BU/mL) to IB057C (773.1), and the accuracy with which ET3i was detected at every concentration also remained relatively steady, fluctuating by at most 11% within the range of approximately 30 – 50% accuracy.

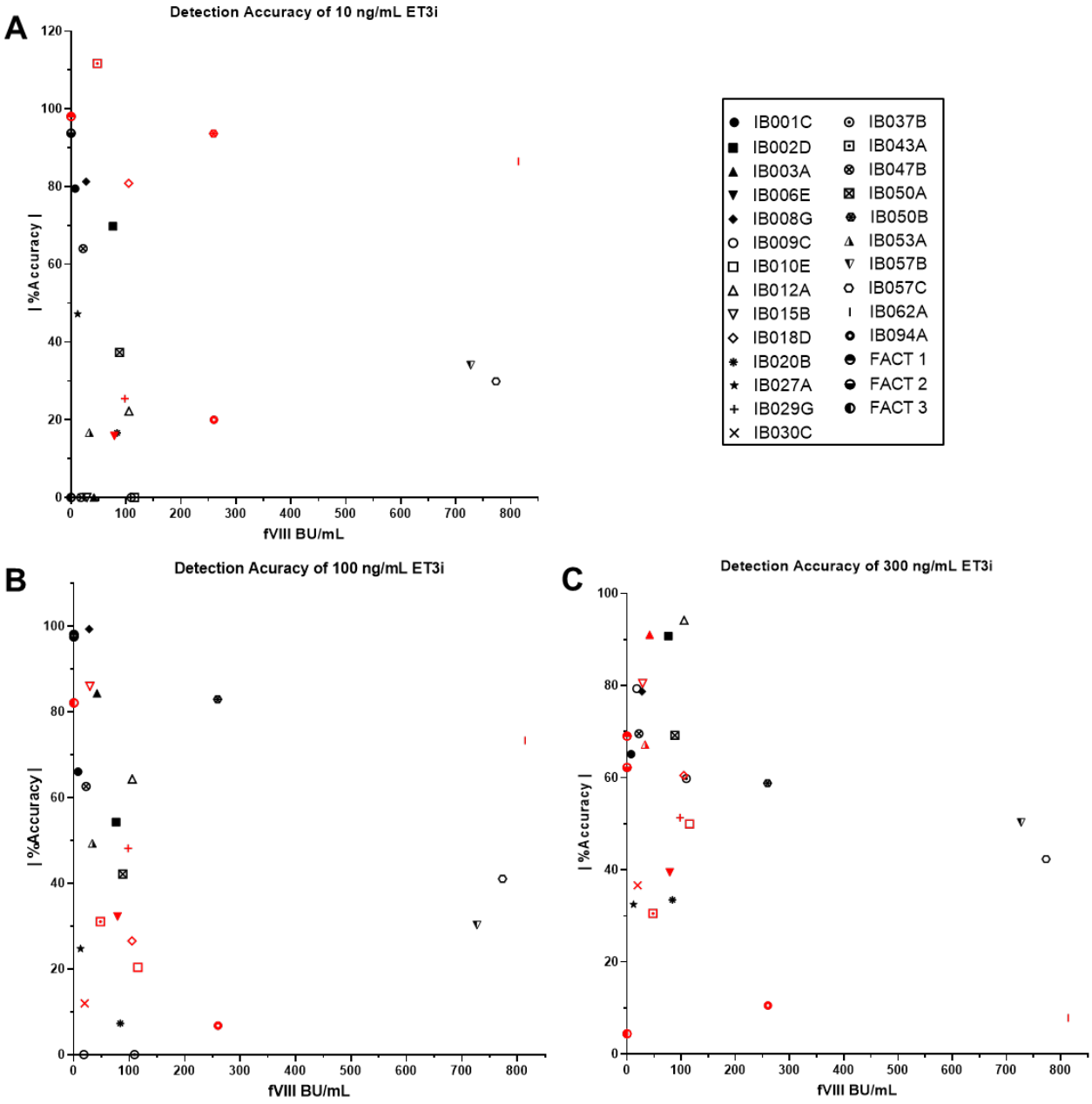


Figure 3.18. ET3i antigen detection accuracy in human fVIII inhibitor patient plasma samples compared to the fVIII inhibitor (Bethesda) titer.

These panels display the accuracy of detection of the known added concentration of ET3i to human fVIII inhibitor patient plasma samples plotted against the anti-fVIII inhibitor titers quantified by Bethesda assay for that sample. These plots are shown after addition of **(A)** 10 ng/mL, **(B)** 100 ng/mL) and **(C)** 300 ng/mL of ET3i. The identification numbers for the human plasma samples are listed in the figure legend at the top right. The |%Accuracy| was calculated by 100 – Error Rate (see the *Materials and Methods* section for details). Samples shown in red report detected ET3i concentrations above what was added to the sample.

The 14 human inhibitor plasma samples that were also evaluated for anti-ET3i inhibitor (Bethesda) titers and dominant epitope domain mapping, their ET3i detection accuracy vs. ET3i inhibitor titers also show no overall correlation between the ET3i inhibitor titer magnitude and the accuracy with which ET3i protein is detected by ELISA after being spiked into the plasma (Fig. 3.19). However, the 2 patient samples among these 14 samples that had accurate ET3i detection for two out of the three concentrations detected to have ET3i inhibitor titers on the lower end (IB008G at 13.1 BU/mL and IB015B at 19.8 BU/mL) with some of the greatest % differences between their anti-fVIII inhibitor titers and cross-reactive anti-ET3i inhibitor titers. However, there are of course patient samples that fall within those parameters and detect the ET3i protein spiked into the plasma less accurately. Additionally, there are multiple samples that displayed accurate detection for one or more ET3i concentrations but for which the ET3i Bethesda titers and dominant epitope mapping have not been assessed. While it seems that the immunodominant epitope of the fVIII inhibitors would be important for predicting the accuracy with which ET3i added to the plasma could be detected, most of the epitope mapping is A2 or C2 dominant, which is common, and does not seem to have strong predictive value for the ET3i antigen detection results. There is, however, a slight general trend to the 300 ng/mL ET3i concentration being more accurately detected on a population level when the data are plotted against the anti-ET3i inhibitor titers than against the anti-fVIII inhibitor titers, but this does not correlate specifically with the magnitude of the anti-ET3i inhibitor titer.

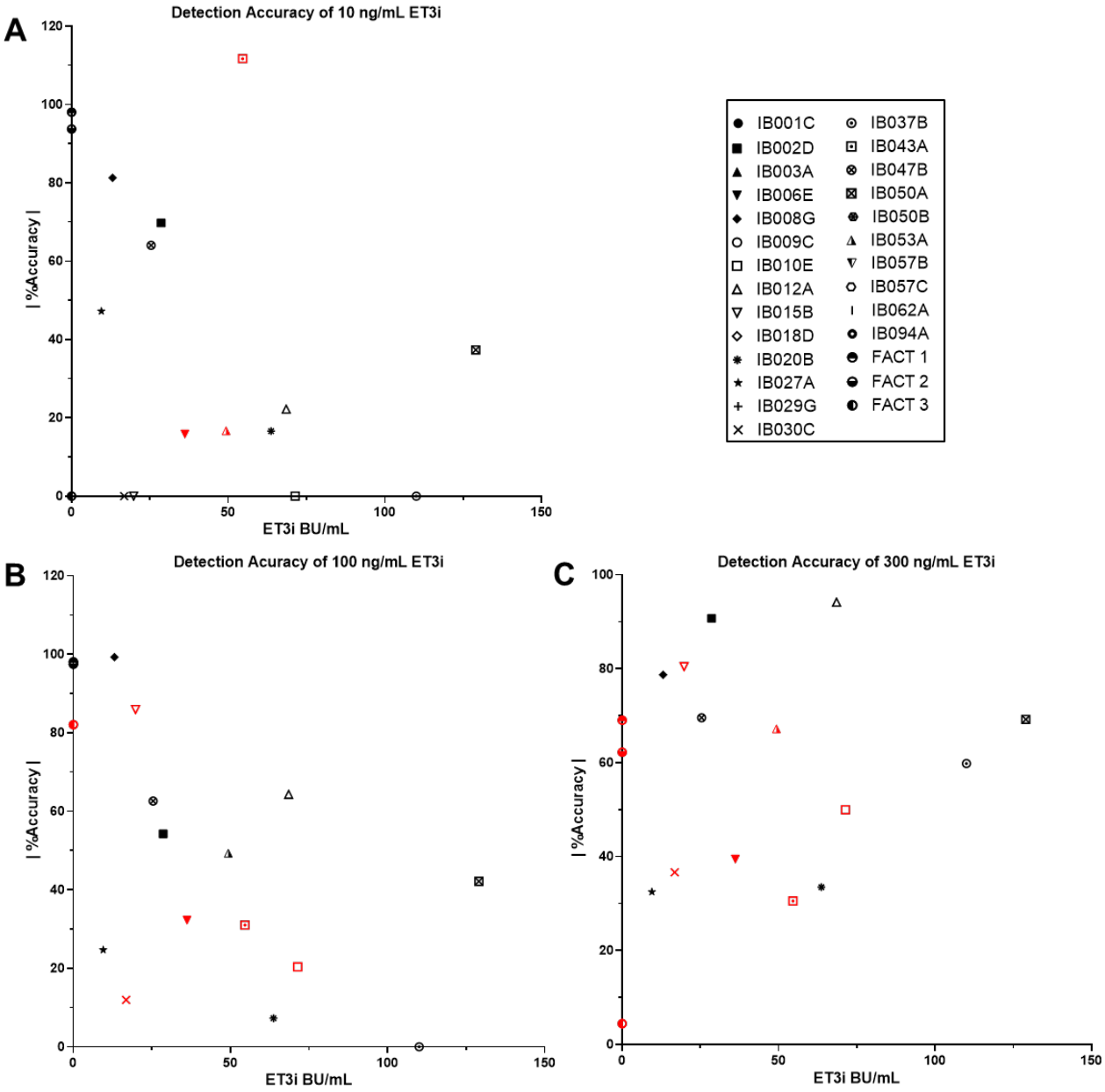


Figure 3.19. ET3i antigen detection accuracy in human fVIII inhibitor patient plasma samples compared to the ET3i inhibitor (Bethesda) titer.

These panels display the accuracy of detection of the known added concentration of ET3i to human fVIII inhibitor patient plasma samples plotted against the anti-ET3i inhibitor titers quantified by Bethesda assay for that sample. These plots are shown after addition of **(A)** 10 ng/mL, **(B)** 100 ng/mL) and **(C)** 300 ng/mL of ET3i. The identification numbers for the human plasma samples are listed in the figure legend at the top right. The |%Accuracy| was calculated by 100 – Error Rate (see the *Materials and Methods* section for details). Samples shown in red report detected ET3i concentrations above what was added to the sample.

While it is encouraging that ET3i can be detected by ELISA in inhibitor patient plasma samples, the accuracy with which it is quantified varies widely and for reasons that remain elusive and seemingly not common to the fVIII inhibitor patient population as a whole (i.e., the reasons may vary between individuals much like the fVIII inhibitor response itself). It also remains unclear as to why more ET3i than was added would be detected in a sample, as the ET3i binding antibody is specific to porcine regions of ET3i and both the binding and detection antibodies are murine.

Beyond the primary clinical challenge of fVIII inhibitor formation, for liver-directed AAV-fVIII gene therapy specifically, another clinical concern is short- and long-term liver health. In preliminary AAV-fVIII studies discussed in Chapter 2 of this dissertation, an abnormal liver growth was observed upon sacrifice of an E16 mouse treated with 1.6E13 vg/kg of AAV-HCB-HSQ (Fig. 2.7 and Supplemental Fig. S2.4). This animal (1389R) maintained fVIII activity levels between approximately 1 – 2 IU/mL and was sacrificed at 48 weeks post AAV-HCB-HSQ delivery. The official pathology report from the University of Georgia diagnosed the growth as an "hepatocellular hyperplasia with hepatocellular vacuolar degeneration and cholangiolar hyperplasia." This indicates a noncancerous hyperplasia in the nodule that was replacing and compressing sinusoids and adjacent liver tissue, which would usually result from hepatic injury. While liver tissue from the animals in the AAV-fVIII dose response study was not submitted for professional pathology analysis, photographs were taken of any abnormal liver physiology observed during necropsy of the animals in this study. Photographs were also taken at necropsy of AAV-ET3 treated mice as well as naïve ET3i immunized mice and no treatment controls from the ET3i challenge study with visibly normal liver physiology, with "visibly normal" indicating no observable pathology on the liver tissue.

Upon sacrifice, three of the AAV-ET3 treated animals had large growths on the liver (Fig. 3.20). While the sample size is too small to draw firm general conclusions, there does not seem to be a unifying reason why these animal in particular display liver pathology after AAV-ET3 gene therapy. Animals 1437B (Fig. 3.20A) and 1435N (Fig. 3.20C) were both treated with the AAV-E06.TTR vector at doses 2.46E11 and 6.67E12 vg/kg, respectively. While animal 1437LL was administered 2.22E12 vg/kg of AAV-HCB-ET3 (Fig.3.20B). Animal 1437B did not form inhibitors after AAV-ET3 delivery but did form inhibitors after ET3i challenge. Animal 1437LL also did not form inhibitors after AAV-ET3 delivery but was also resistant to ET3i challenge. And animal 1453N had a multiphasic inhibitor response following AAV-ET3 delivery, and hence was not challenged with ET3i protein. Neither vector dose, promoter, nor type of immune response unify these 3 animals. There are also many animals in these treatment and/or immune response groups that did not display liver pathology at time of sacrifice. However, it is worth noting that 2 animals in the 1437 litter died before the study ended and two of the animals displaying liver pathology here are also from the 1437 litter. It is therefore possible that even though the E16-/- hemophilia A mice are bred to be genetically homogenous, there may have been some genetic predisposition in the 1437 litter that made them more susceptible to liver pathology. None of the animals that had sustained inhibitor titers after AAV-ET3 delivery and that survived until the study endpoint also had abnormal liver growths at the time of sacrifice.

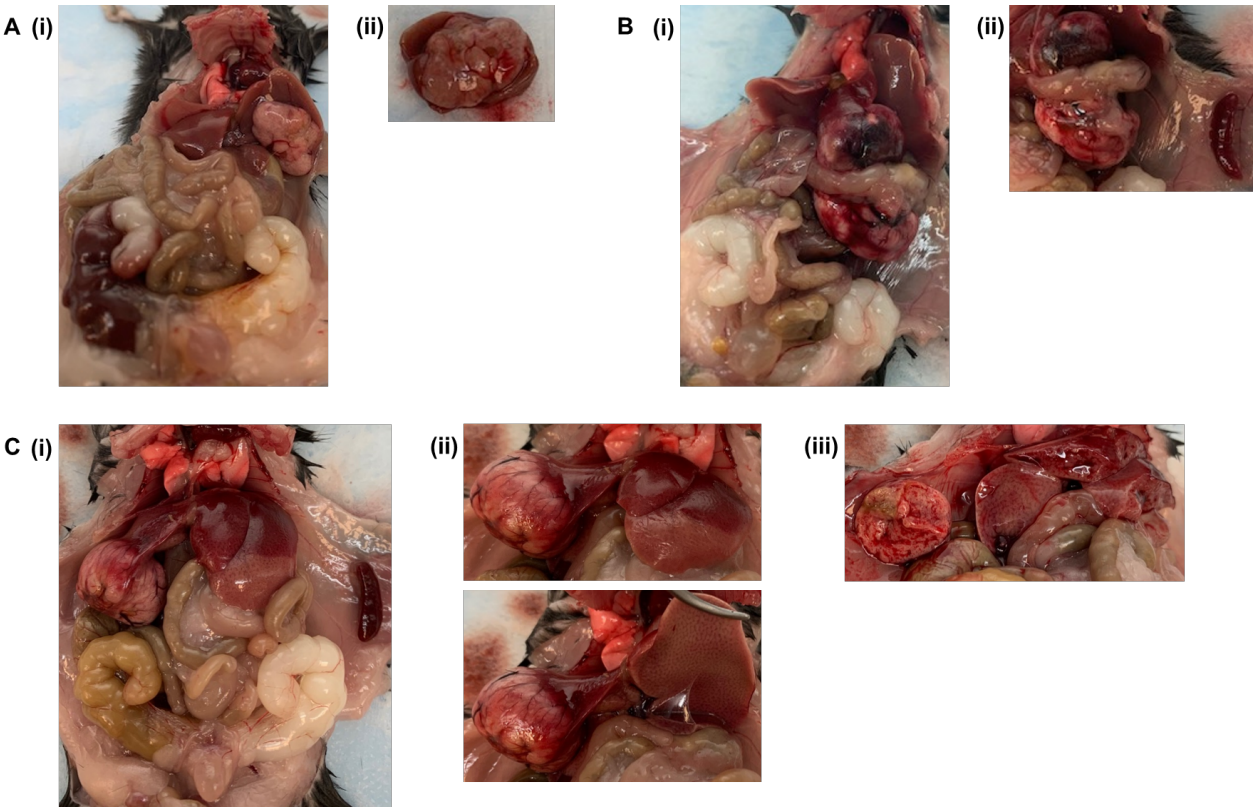


Figure 3.20. Necropsy results from mice administered AAV-ET3 vectors with visible liver pathology. Three animals administered AAV-ET3 vectors demonstrated abnormal growths on the liver at time of sacrifice. **(A)** Animal 1437B was administered 2.46E11 vg/kg AAV-E06.TTR-ET3, where (i) displays the full anatomy and (ii) shows the excised abnormal growth. **(B)** Animal 1437LL was administered 2.22E12 vg/kg AAV-HCB-ET3, where (i) displays the full anatomy and (ii) highlights the abnormal growth. **(C)** Animal 1453N was administered 6.67E12 vg/kg AAV-E06.TTR-ET3, where (i) displays the full anatomy with an excised spleen, (ii) highlights the liver tissue and growth, and (iii) shows a cross-section of the abnormal growth and adjacent normal liver tissue.

Animal 1457B displayed high fVIII activity levels after AAV-ET3 delivery but eventually lost both detectable fVIII activity and ET3 antigen without a concomitant anti-fVIII antibody response. qPCR on liver DNA after sacrifice also demonstrated that this animal no longer had detectable AAV-ET3 VCN. There was no visible pathology on the liver during necropsy despite having lost vector expression (Fig. 3.21). This could suggest the possibility that a cytotoxic T cell response against the AAV-ET3 transduced hepatocytes or cellular stress responses activated apoptosis pathways in those cells.

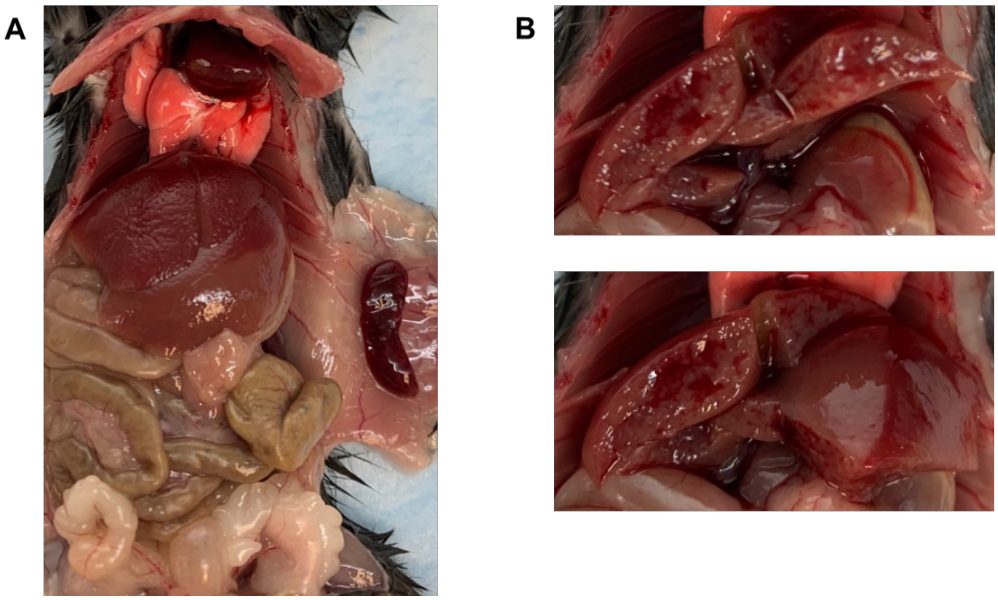


Figure 3.21. Necropsy of the only AAV-ET3 treated animal to lose fVIII VCN (1457B) reveals no discernable liver pathology.
Animal 1457B was administered 6.67E12 vg/kg AAV-E06.TTR-ET3, had initial peak fVIII activity levels of 6.35 IU/mL by day 30 post vector delivery, and subsequently lost fVIII activity after week 32. There was no detectable anti-fVIII antibody response; however, upon sacrifice this was the only animal in that treatment group to have completely lost liver VCN. Despite losing fVIII protein expression and AAV vector transduction, no abnormal liver physiology was observed **(A)**, even in the liver cross-section **(B)**.

The AAV-ET3 dose response and subsequent ET3i challenge studies resulted in multiple experimental groups:

1. AAV-ET3 treated animals that developed sustained inhibitors after vector delivery.
2. AAV-ET3 treated animals that developed complex, multiphasic inhibitor responses.
3. AAV-ET3 treated animals that did not develop inhibitors after vector delivery but did develop inhibitors after subsequent ET3i protein challenge.
4. AAV-ET3 treated animals that did not develop inhibitors after vector delivery and showed immunological resistance to ET3i challenge.
5. Naïve hemophilia A mice that were subjected to ET3i protein challenge alongside the gene therapy treated mice.

6. Naïve hemophilia A no treatment control animals.

There were animals from each of these treatment groups that did not display any abnormal liver growths at time of sacrifice (Fig. 3.22). The anatomy of aged mice treated with the liver-directed AAV gene therapies does appear different to that of younger mice without AAV gene therapy exposure (e.g., Fig. 3.22B compared to 3.22D and E).

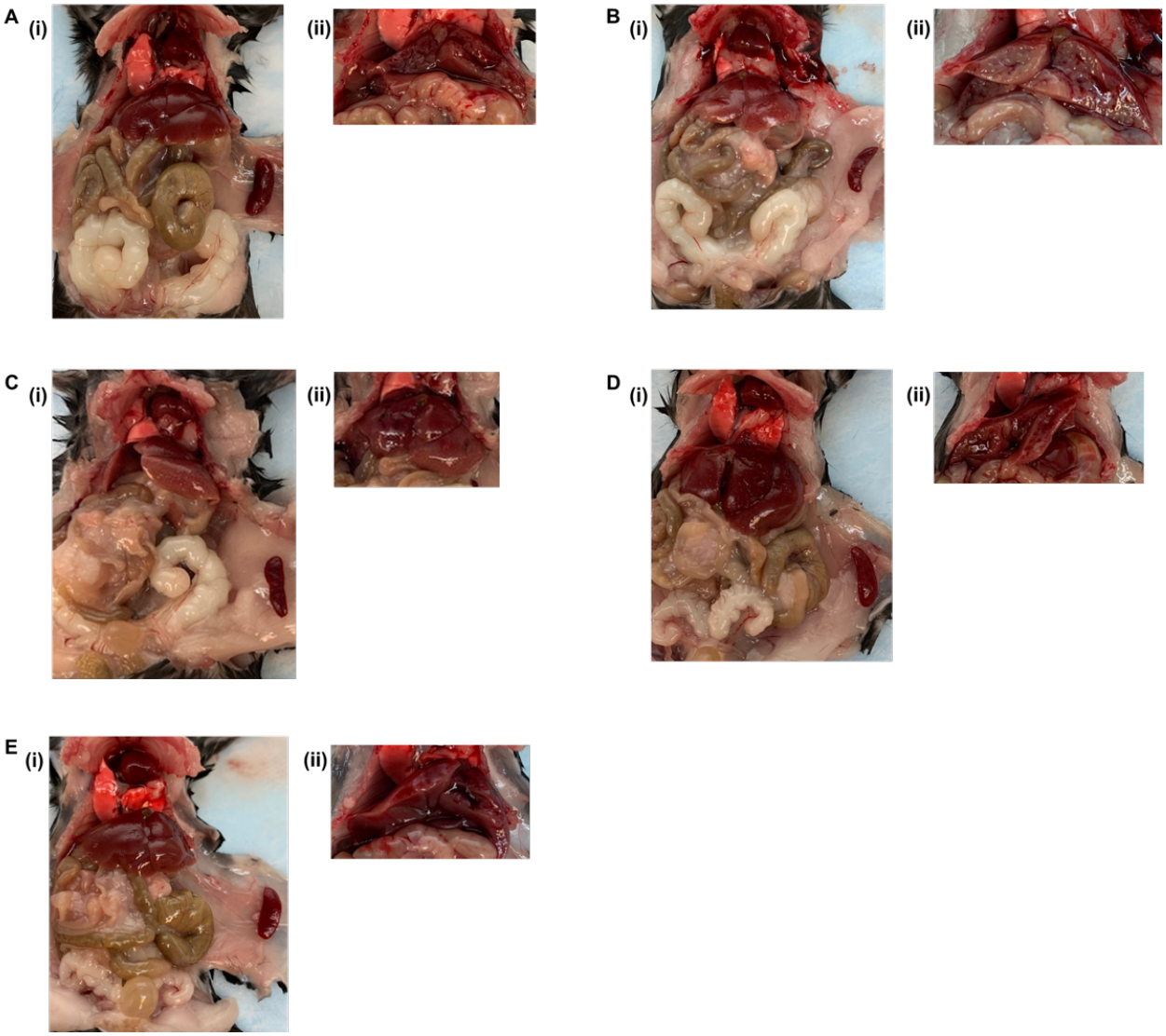


Figure 3.22. Representative images from necropsy of ET3i challenge and AAV-ET3 treated mice with no discernable liver pathology.
These panels are representative necropsy images from animals in various treatment groups for AAV-ET3 vector dose response and ET3i challenge studies. In all panels, (i) displays the full anatomy and (ii)

highlights a cross-section of the liver tissue. **(A)** Animal 1427N is a male E16 mouse that is age-matched to the AAV-ET3 dose response study mice but received no AAV-fVIII nor any other treatment. **(B)** Animal 1439R was administered 2.46E11 vg/kg E06.TTR-ET3 and was not challenged with ET3i. **(C)** Animal 1451L was administered 2E13 vg/kg E06.TTR-ET3 and had an anti-fVIII inhibitor response post vector delivery. **(D)** Animal 1603B is a male, naïve E16 mouse that received neither AAV-fVIII nor ET3i protein but is age-matched to the ET3i immunized only animals. **(E)** Animal 1597N is a male E16 mouse that was immunized with ET3i protein (no AAV-fVIII).

The AAV-HSQ treated animals were sacrificed at slightly later time points (64 – 69 weeks) compared to their AAV-ET3 counterparts (48 – 53 weeks). Table 3.4 shows the number of animals in each vector/dose group that had visible liver pathology at necropsy.

Liver Pathology at Necropsy				
Vector	**E06.TTR-ET3**	**HCB-ET3**	**E06.TTR-HSQ**	**HCB-HSQ**
Relative potency	++++	+++	++	+
6E13 vg/kg			**2**	**2**
2E13 vg/kg				**1**
6.67E12 vg/kg	**1**			**1**
2.22E12 vg/kg		**1**	**1**	**1**
7.4E11 vg/kg			**1**	
2.46E11 vg/kg	**1**			

Table 3.4. Incidence of liver pathology at necropsy by vector/dose group.
These counts represent the number of animals in each treatment group that displayed visible liver pathology at necropsy. Shaded boxes indicate that the vector either was not tested at that dose or that the animals in that vector/dose group were not included in the PK model due to spontaneous death at an early time point.

Almost none of the AAV-HSQ treated animals developed inhibitors after vector delivery and inhibitors only appeared in the animals administered 6E13 vg/kg of AAV-E06.TTR-HSQ. However, at time of sacrifice, more mice treated with AAV-HSQ vectors displayed abnormal liver growths. These growths appeared in 5 of the animals administered AAV-HCB-HSQ (Fig. 3.23). These animals were administered anywhere from 2.22E12 to 6E13 vg/kg and only two of them were from the same litter. However, the growths observed in panels 3.23A and 3.23B appear different in morphology than the growth observed in preliminary studies (Fig. S2.4) and in the AAV-ET3 treated animals.

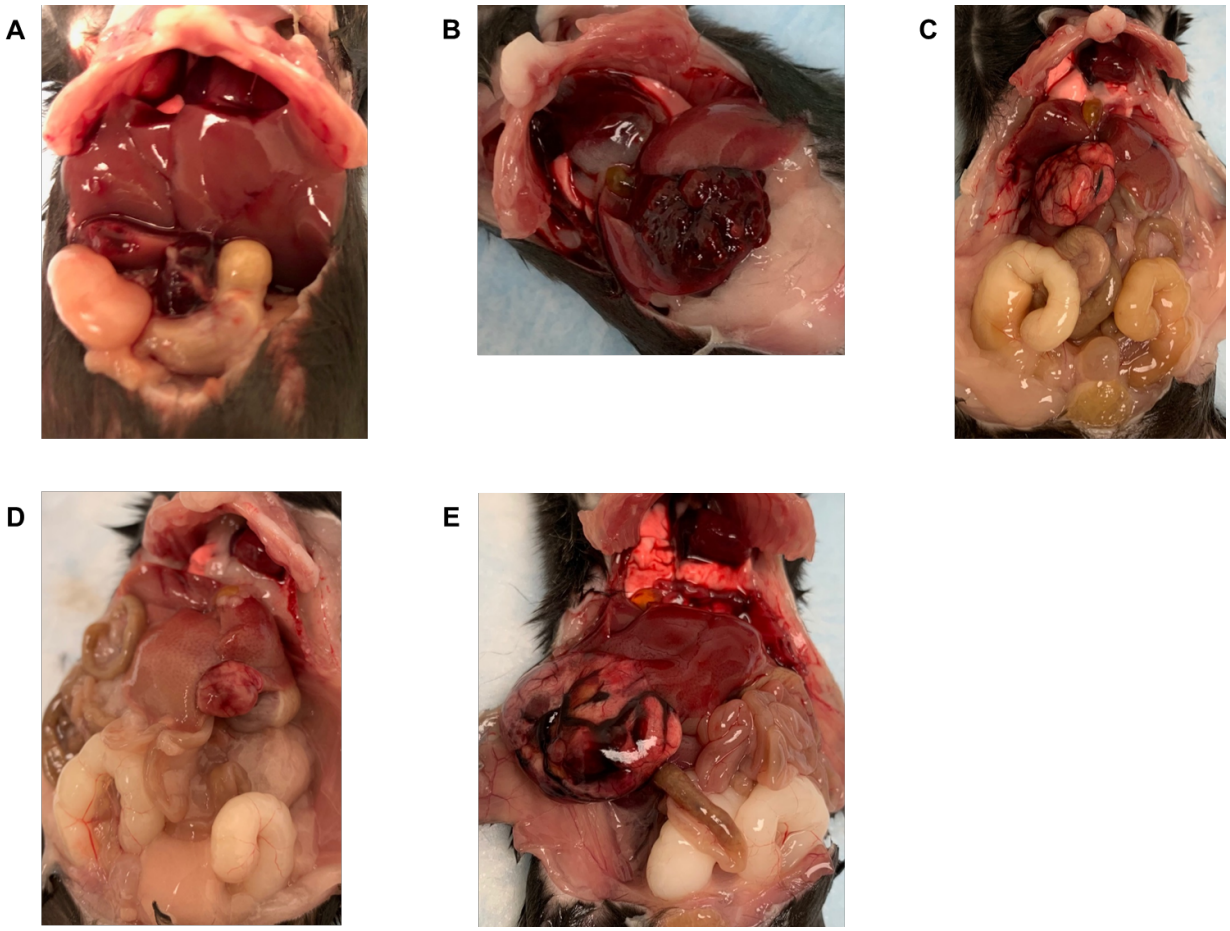


Figure 3.23. Necropsy results from mice administered AAV-HCB-HSQ with visible liver pathology. Five animals treated with the AAV-HCB-HSQ vector displayed liver pathology at time of sacrifice. **(A)** Animal 1475L was administered 2E13 vg/kg. **(B)** Animal 1485L was administered 6.67E12 vg/kg. **(C)** Animal 1497N was administered 6E13 vg/kg. **(D)** Animal 1497LL was administered 2.22E12 vg/kg. **(E)** Animal 1503L was administered 6E13 vg/kg.

Four animals administered the AAV-E06.TTR-HSQ vector also displayed abnormal liver growths at time of sacrifice (3.24). Here the animals were administered vector at doses between 7.4E11 and 6E13 vg/kg. Again, only two of the animals were from the same litter and they received vector doses at the two extremes of the dose range (Fig. 3.24B and 3.24D). Interestingly, the animal that received the lower 7.4E11 vg/kg dose also displayed extreme splenomegaly (Fig. 3.24B).

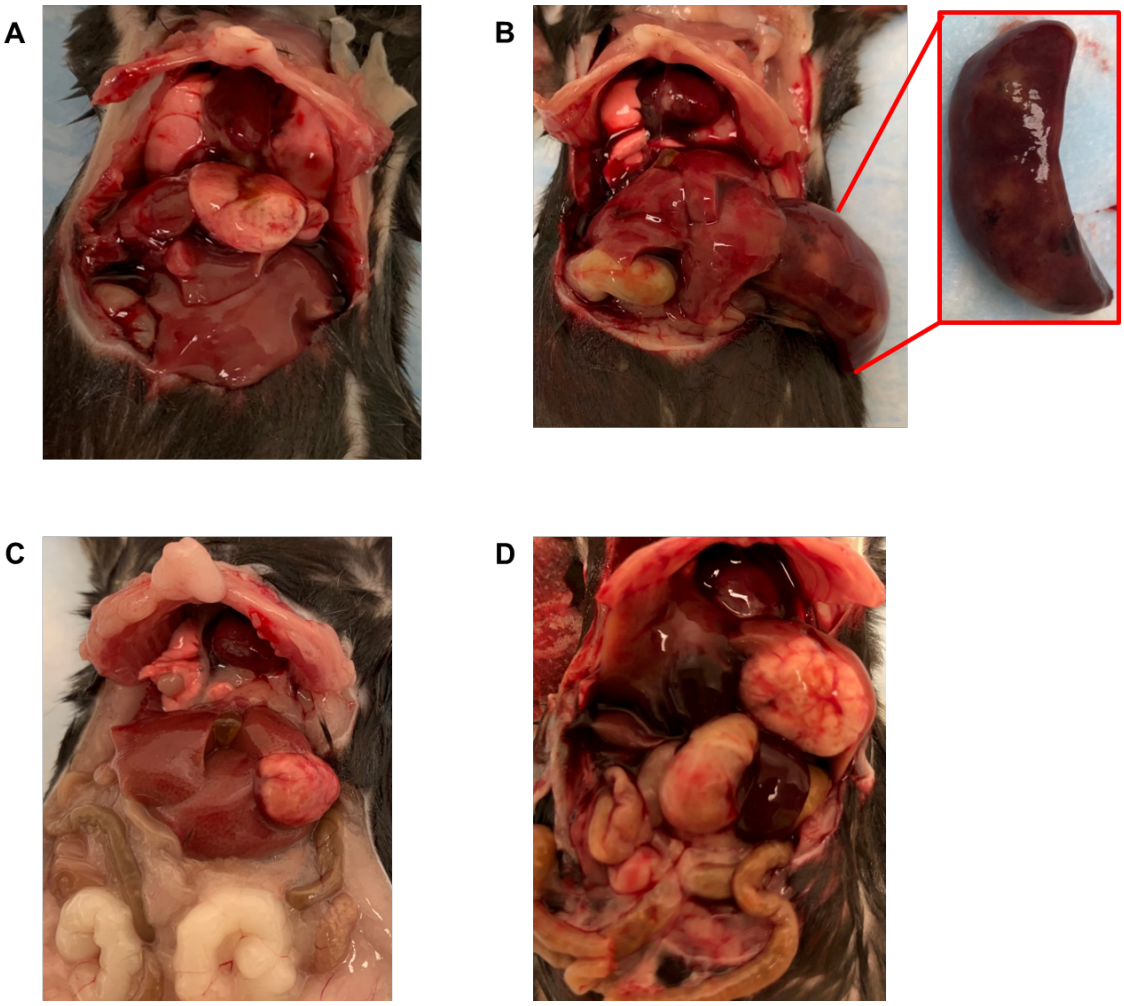


Figure 3.24. Necropsy results from mice administered AAV-E06.TTR-HSQ with visible liver pathology.
Four animals treated with the AAV-E06.TTR-HSQ vector displayed liver pathology at time of sacrifice. **(A)** Animal 1475B was administered 6E13 vg/kg. **(B)** Animal 1481N was administered 7.4E11 vg/kg, where the subpanel highlights the excised spleen. **(C)** Animal 1477N was administered 2.22E12 vg/kg. **(D)** Animal 1481B was administered 6E13 vg/kg.

3.5 Discussion

Establishment of a logical and effective framework for the assessment of genetic medicine pharmacology has become a high priority in drug development. The classical absorption, distribution, metabolism, and excretion (ADME) parameters apply in some contexts but are less clear in others. Gene therapy performance is dependent on both the pharmacology of the gene

therapy vector (e.g., AAV) and the expression of the transgene product (e.g., fVIII), while only the former can be treated as an independent variable. Furthermore, gene therapy product development has progressed to the state where customization of individual candidates has become standard practice. Common design elements include vector packaging (e.g., lipid nanoparticle composition, AAV serotype, lentiviral vector pseudotype), enhancer/promoter sequence(s), transgene sequence (e.g., codon optimization, bioengineered protein products), introns, polyadenylation sequences, and terminal elements (inverted terminal repeats or long terminal repeats). Although characterization of the effects of design elements on efficacy parameters is standard practice, currently, there exists little understanding of the impact these factors have on the immune response to gene therapy products, i.e., their immunogenicity. This represents a major gap in knowledge and safety risk as genetic medicines, including AAV-fVIII gene therapy, quickly progress through clinical trials in human subjects. Indeed, immunogenicity represents a significant barrier to many biopharmaceuticals due to effects on therapeutic efficacy (inhibitors and/or increased clearance) as well as potentially deadly allergic reactions. While immunogenicity assessment is a standard component of protein drug development, its role in preclinical gene therapy studies is not well established. Many preclinical studies demonstrate significant immunogenicity of both the vector and the transgene product, but the specific properties of a given gene therapy candidate that are responsible for this immunogenicity are unclear and often not rigorously interrogated.

The current study represents a first attempt to address rigorously and directly both the determinants of gene therapy transgene product immunogenicity and the development of an appropriate pharmacological framework for preclinical evaluation of gene therapy candidates in the setting of one of the most active areas of clinical gene therapy research, hemophilia A.

Although 30-40% of severe hemophilia A patients develop inhibitory immune responses to fVIII protein drug products within the first 20 exposures, gene therapy clinical trials have focused exclusively on adult subjects with extensive prior fVIII exposure and no history of fVIII inhibitors. Although no inhibitors have been observed in AAV-fVIII clinical trials, the immunogenicity risk in previously untreated patients as well as an understanding of the factors governing immunogenicity risk in both the untreated and pretreated populations remain elusive. Murine models of hemophilia A are a component of all preclinical hemophilia A product development programs and have been shown to replicate many aspects of the immune response to fVIII.[48] While some previous studies have directly assessed the relationship between gene therapy dose, transgene expression kinetics and protein product immunogenicity,[249-251] most speculation on factors affecting protein drug immunogenicity have focused on parameters other than dose, including the patient's disease status, human leukocyte antigen genetics, and microbiome, or the drug-specific factors, such as function, target, T cell epitope characterization, and degree of "self-ness".[252]

This study used a murine hemophilia A model to evaluate four vectors similar to those in clinical testing at a range of clinically relevant doses. The result was that these four vectors have different apparent potencies that resulted in initial fVIII protein product exposure kinetics and steady state profiles that followed a dose-response pattern based on a combination of vector potency and dose. These fVIII exposure profiles as quantified by plasma fVIII activity also resulted in a dose-response effect in inhibitor incidence. Combining these data, a pharmacokinetic model of the fVIII activity levels and immunogenicity outcomes was developed that provided a robust assessment of the relative immunogenicity risk determined strictly by initial transgene product exposure kinetics. No other variables appeared to correlate directly and dominantly with immunogenicity risk other than their relationship with and influence on transgene product protein

exposure kinetics. Indeed, the day 5 k_{fVIII} presented itself as the strongest independent predictor of inhibitor incidence. While rapid increase to high transgene product exposure levels strongly correlated with immunogenicity, slower kinetics appears favorable for reducing immunogenicity risk and supporting a pseudo-steady state at clinically relevant levels of fVIII over the study lifetimes of the experimental animals. Furthermore, animals with sustained and predictably curative levels of fVIII demonstrated immunological resistance to exogenous fVIII challenge, supporting the concept of a therapeutic window that supports both efficacy and safety (Fig. 3.25). The initial fVIII kinetics and steady state fVIII levels that remain below the immunogenicity threshold but are high and sustained enough to establish a level of fVIII immune tolerance outline this therapeutic window in which the immunogenicity risk is lower and the probability of maintaining durable fVIII efficacy and immune tolerance is higher.

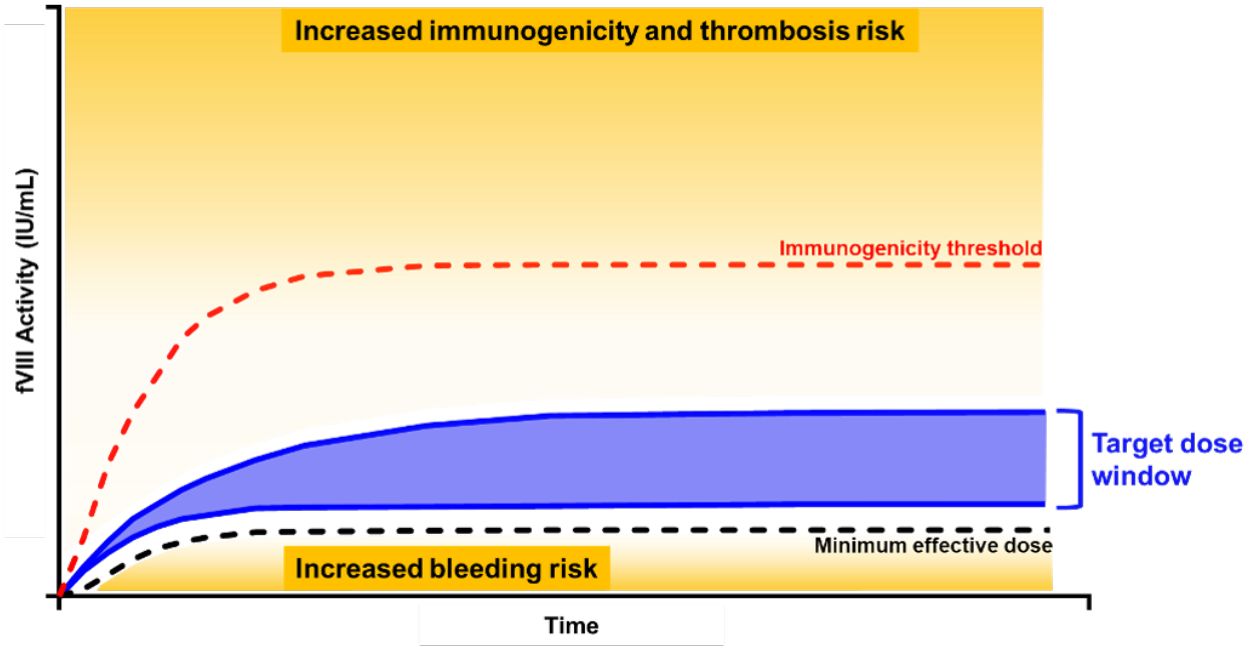


Figure 3.25. Combined results from the AAV-fVIII dose response and ET3i challenge studies suggest a therapeutic window for AAV-fVIII treatment.
This graphic suggests a target AAV-fVIII dose window based on initial fVIII exposure kinetics and steady state levels relative to thresholds for therapeutic efficacy and immunogenicity in order to mitigate the risks associated with fVIII levels significantly outside of the normal range. The "minimum effective dose"

indicates the minimum dose at which durable and therapeutic steady state fVIII levels are expected. The "immunogenicity threshold" is determined from the pharmacokinetic model described herein.

However, while it appears that the animals that achieved therapeutic steady state levels of fVIII activity maintained them for the lifetime of the study, especially in the case of AAV-ET3 treated animals that were not ET3i challenged and sacrificed at 53 weeks after vector delivery, the AAV-HSQ animals that were sacrificed months later at 64 – 69 weeks demonstrated a significant drop in fVIII activity. This mirrors the data seen in AAV-fVIII clinical trials. For example, in the multi-year follow-up of valoctocogene roxaparvovec (AAV5-hfVIII-SQ), a relatively rapid increase to therapeutic levels of fVIII activity is seen over the first 20 – 40 weeks, but then this gradually declines up to 3 years after vector delivery, placing some patients back into the moderate hemophilia A range.[253] There is major concern about the durability of fVIII expression after AAV-fVIII delivery, and this decrease over time is also observed in the AAV-HSQ mice treated in this study. Only the 2E13 vg/kg of AAV-E06.TTR-HSQ and 6E13 vg/kg of AAV-HCB-HSQ treatment groups still had what would be considered therapeutic levels of fVIII activity at up to 69 weeks post vector delivery, and even still this was far below the steady state levels seen between 40 – 45 weeks post vector delivery. Since these animals maintained fVIII activity for the majority of the study, never developed inhibitors, and VCN was still detectable in the liver after sacrifice, it is unlikely that this decline was due to a cytotoxic CD8 lymphocyte response. Some of these animals developed large, abnormal liver growths which could certainly have an effect on the ability of the transduced hepatocytes to survive and produce fVIII. However, this was not seen in all of the animals when all of the animals did show a decrease in fVIII activity at sacrifice. It is also suggested that not all regions of the liver transduce with the same efficiency and that different regions of the liver have different rates and degrees of hepatocyte turnover. It would also be

expected that as the organ ages, it may no longer be able to produce fVIII at the same efficiency from transduced cells. One limitation of the study is that the initial VCN values were after transduction with each vector at each dose, and so it cannot be determined to what degree these values decreased. Still, some factor or combination of factors is leading to a decrease in fVIII activity relatively soon after delivery of current AAV-fVIII candidates in humans and toward the end of the lifetime of the animals in this murine hemophilia A model after AAV-fVIII delivery. This concern merits preclinical studies aimed directly at answering this question and is one of the reasons why liver biopsies are being considered as an optional component of liver directed AAV gene therapy studies.

There are other apparent limitations to this study, but many of these are clearly recognized as common to all preclinical immunogenicity testing. Given the differences observed in AAV transgene product expression kinetics in different species,[179,188,209,210,251,254-256] as well as the effect of genetic background on murine preclinical immunogenicity,[73,74] it seems likely that dose-response relationships and immunogenicity threshold value deviations must exist. However, we predict that transgene product expression kinetics remain a universal and primary driver of immunogenicity in the gene therapy setting. Ideally, it would be advantageous to conduct a comprehensive retrospective analysis of all published preclinical gene therapy data. However, several confounding issues arise including common use of immunodeficient or immunosuppressed animals, limited early time point data, variations in reagents and assays used to assess fVIII activity and inhibitors, and small sample sizes particularly in large animal studies. Also, detecting and monitoring fVIII antigen after protein infusion, delivery of transduced cell therapies, or of direct infusion of gene therapy vector has been a major challenge for the field. Being able to monitor fVIII antigen, particularly in the presence of an inhibitor response, would be helpful from both a

drug development perspective and a clinical treatment perspective. It is well known that fluctuations in antigen exposure, meaning the kinetics of exposure, where in the body the antigen is seen and in what amounts, the quality of the antigen itself, and fluctuations in any of these parameters alter how the immune system perceives the exogenous protein and subsequently affects the nature of the immune response. Further development of flow cytometry protocols and assays like the fVIII antigen ELISA are warranted.

The most robust data sets outside of the current study are the later stage clinical trial data. Inspection of these data reveal the much slower kinetics of fVIII appearance in the clinical setting (~40 weeks) compared to murine studies (2-6 weeks). It can be concluded that current clinical trial AAV-fVIII dosing supports fVIII expression within or below the therapeutic window established to be safe based on immunogenicity threshold values obtained in the current study. Although clinical AAV-fVIII gene therapy studies differ in their selection of subjects previously treated with fVIII products and no history of inhibitor development, it is reassuring that the lack of immunogenicity observed clinically is consistent with the current model predictions in previously untreated animals. Furthermore, this preclinical pharmacokinetic model could be used to support the safe indication of current AAV-fVIII vector designs and dose levels to previously untreated patients. In general, pharmacokinetic/pharmacodynamic studies and models like the one presented herein are clearly applicable and critical across the rapidly advancing field of genetic medicine.

3.6 Acknowledgments

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views of the National Institutes of Health. The deidentified human samples assayed in this work were kindly provided by the laboratory of Shannon Meeks, PhD. Support was also provided by the Aflac Cancer & Blood Disorders Center at Children's Healthcare of Atlanta and the Emory University Department of Pediatrics. Original schematic figures were created with Biorender.com or GraphPad Prism and Microsoft PowerPoint.

3.7 Supplemental Information

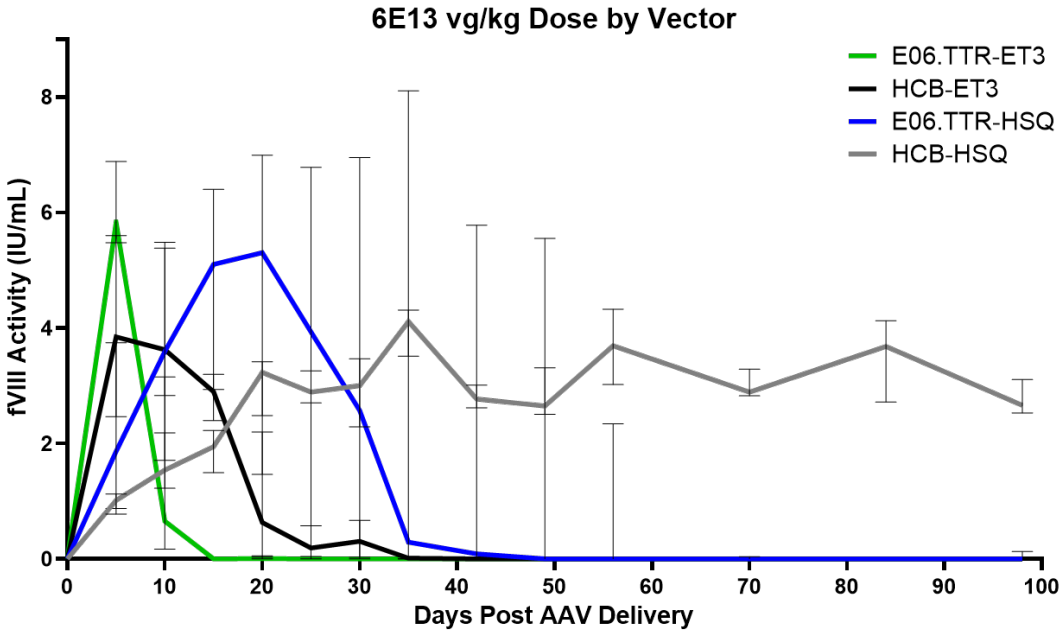

Figure S3.1. FVIII activity after administration of 6E13 vg/kg of each vector.
This graph profiles the median FVIII activity up to 98 days after administration of 6E13 vg/kg of AAV-E06.TTR-ET3 (n=4), AAV-HCB-ET3 (n=4), AAV-E06.TTR-HSQ (n=3), and AAV-HCB-HSQ (n=5). The error bars indicate interquartile range (IQR).

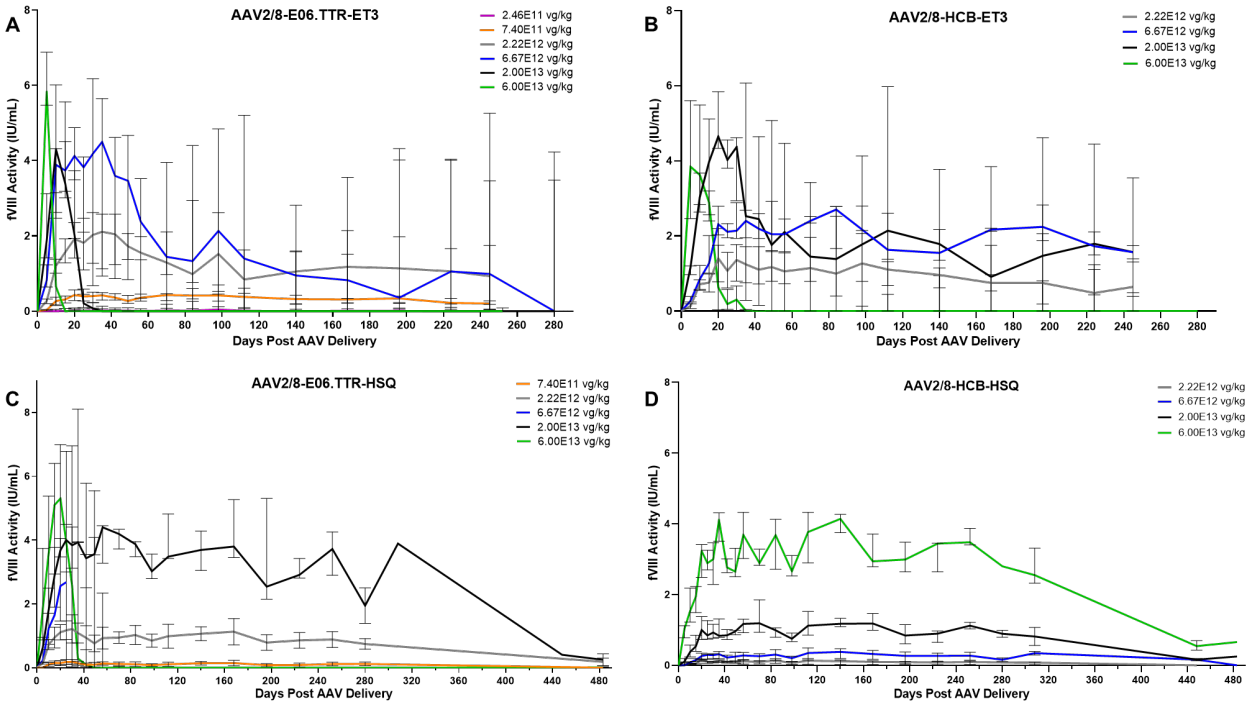

Figure S3.2. FVIII activity after dose response administration of four AAV-fVIII vectors.
These graphs profile the median fVIII activity after administration of **(A)** 2.46E11 – 6E13 vg/kg AAV-E06.TTR-ET3, **(B)** 2.22E12 – 6E13 vg/kg AAV-HCB-ET3, **(C)** 7.40E11 – 6E13 vg/kg AAV-E06.TTR-HSQ, and **(D)** 2.22E12 – 6E13 vg/kg AAV-HCB-HSQ. FVIII activity profiles after AAV-ET3 delivery **(A, B)** end prior to initiation of the ET3i challenge course. FVIII activity profiles continue until the time of sacrifice for AAV-HSQ treated animals **(C, D)**. Error bars indicate IQR.

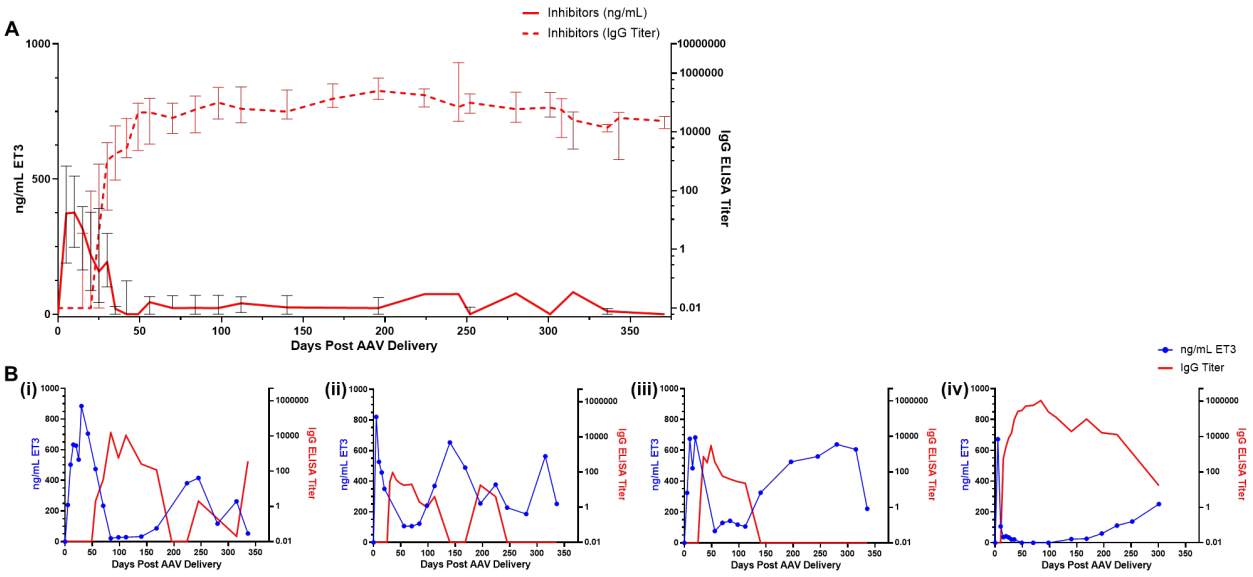

Figure S3.3. ET3 antigen levels in animals that formed a sustained or multiphasic fVIII inhibitor response after AAV-ET3 delivery.

(A) This graph displays the median ET3 antigen levels and anti-ET3 IgG titers over time in animals that developed a sustained inhibitor response after AAV-ET3 delivery. Error bars represent IQR. Antigen or specific activity error bars are solid black and IgG titer error bars are color coded with the type of inhibitor response. **(B)** These graphs display ET3 antigen levels and anti-ET3 IgG titers for the four animals that developed a multiphasic inhibitor response. Points on the antigen curves indicate time points at which plasma was assayed for antigen. Animal IDS: (i) 1453N, (ii) 1453LL, (iii) 1457L, (iv) 1483N.

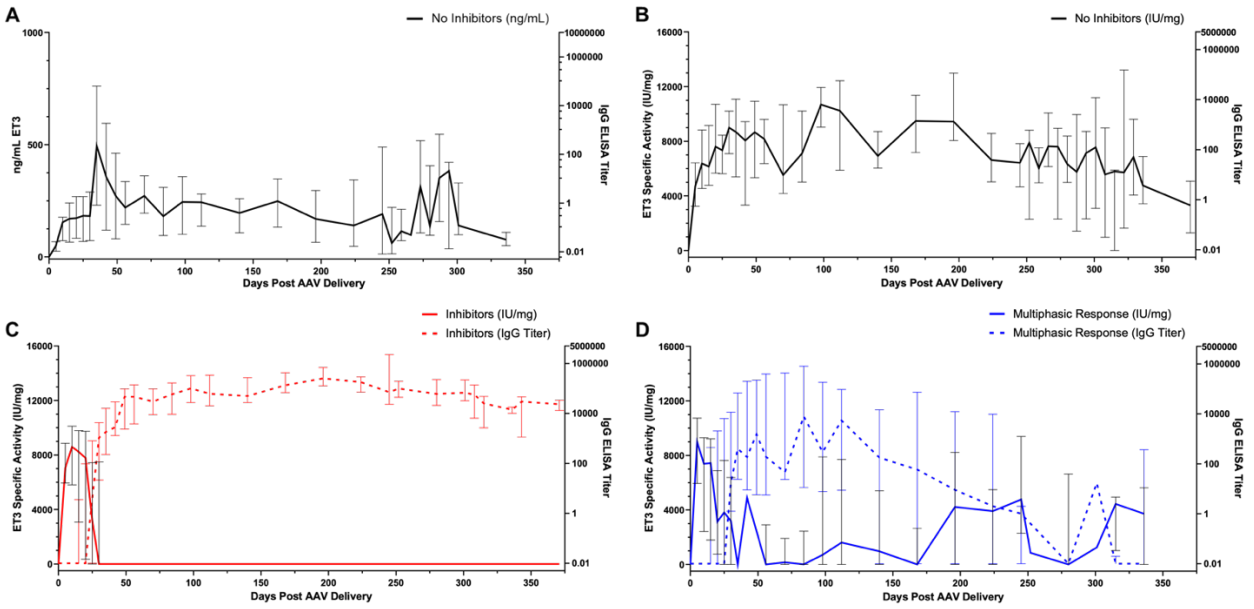

Figure S3.4. *In vivo* ET3 specific activity in inhibitor response animals vs. animals that did not form inhibitors after AAV-ET3 delivery.

Panel **(A)** displays median ET3 antigen and panel **(B)** displays median *in vivo* specific activity over time for AAV-ET3 treated animals that did not develop inhibitors prior to the ET3i challenge course. **(A, B)** The data shown after 252 days represent the no-ET3i challenge controls only. **(C, D)** These graphs display the median ET3 *in* vivo specific activity and anti-ET3 IgG titers from animals that developed either a sustained inhibitor response **(C)** or a multiphasic inhibitor response **(D)** after AAV-ET3 delivery. Error bars represent IQR. Antigen or specific activity error bars are solid black and IgG titer error bars are color coded with the type of inhibitor response.

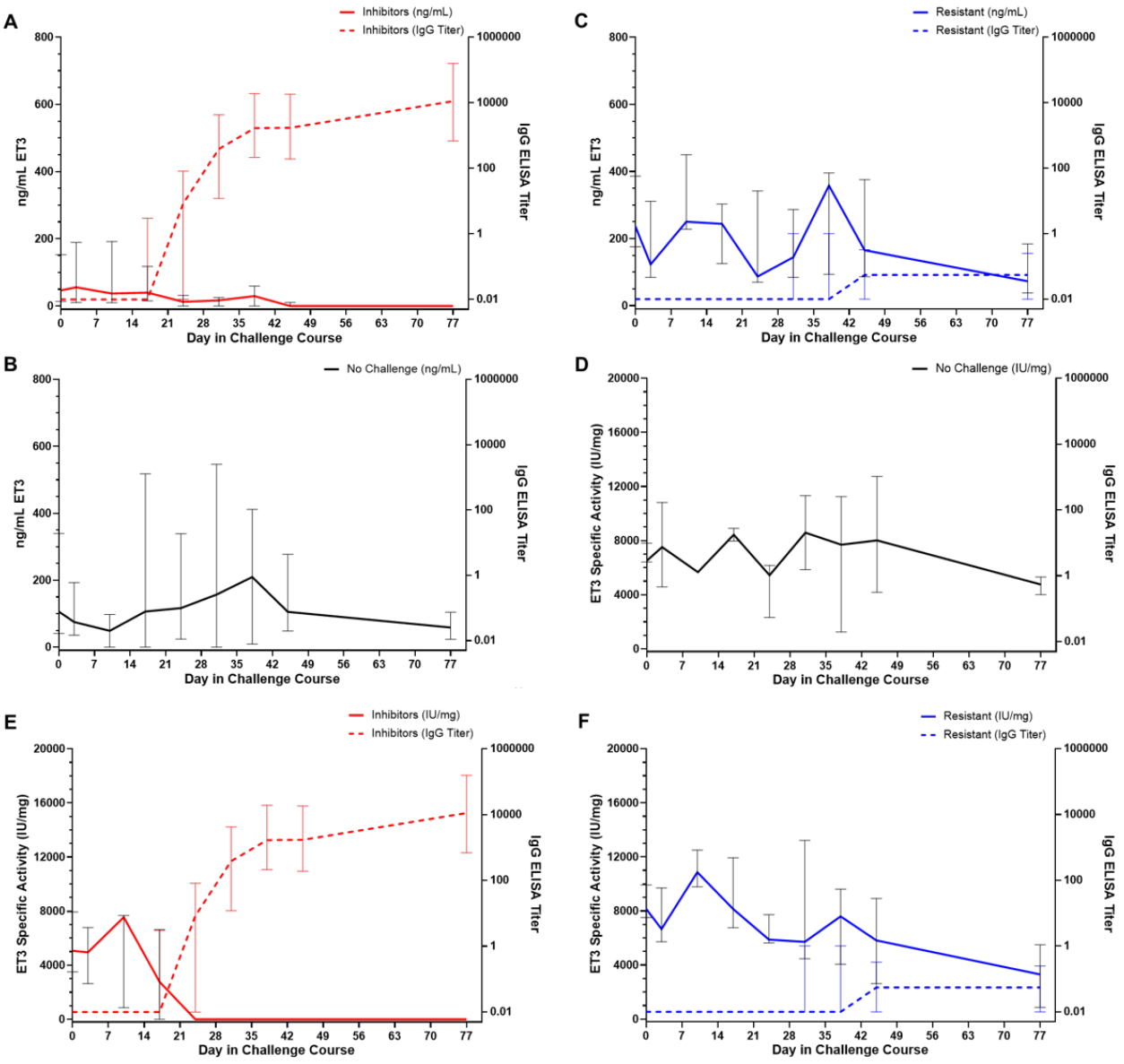

Figure S3.5. ET3 antigen, *in vivo* specific activity, and IgG response data during ET3i challenge. **(A, B)** Mean ET3 antigen levels and anti-ET3 IgG titers in animals that either **(A)** developed inhibitors in response to ET3i challenge or **(B)** demonstrated immunological resistance to ET3i challenge. **(C)** Mean ET3 antigen levels and **(D)** *in* vivo specific activity for AAV-ET3 pre-treated, no-ET3i challenge control animals. **(E, F)** Mean ET3 *in* vivo specific activity and anti-ET3 IgG titers in animals that either **(E)** developed inhibitors in response to ET3i challenge or **(F)** demonstrated immunological resistance to ET3i challenge. Error bars represent value range. Antigen or specific activity error bars are solid black and IgG titer error bars are color coded with the type of inhibitor response.

I.D.	Sac. Week Post-AAV	i.v. ET3	FVIII AUC	FVIII IU/mL	Day 3 (1st i.v. ET3)			Day 10 (2nd i.v. ET3)			Day 17 (3rd i.v. ET3)			Day 24 (4th i.v. ET3)		
					IU/mL	ng/mL	IgG Titer	IU/mL	ng/mL	IgG Titer	IU/mL	ng/mL	IgG Titer	IU/mL	ng/mL	IgG Titer
1443N	53	Y	4138	4.64	3.9	369.9	--	3.13	250.2	--	4.03	312.7	--	0.48	81.71	--
1455LL	48	Y	3790	3.68	0.47	75.95	--	4.89	450.2	--	4.06	NA	--	3.26	389.5	--
1453L	48	N	3741	3.14	0.98	153.3	--	0.553	97.54	--	4.615	517.8	--	0.32	137.6	--
1443L	53	Y	2092	2.15	0.965	135.3	--	2.23	227.8	--	1.81	274.3	--	1.92	326.1	--
1439L	53	Y	1892	1.73	1.19	166.4	--	1.285	NA	--	1.215	139.1	--	0	--	11
1433L	53	Y	2009	1.69	1.665	NA	--	1.585	NA	--	1.935	213.7	--	0.552	73.55	--
1455B	48	N	2272	1.49	2.675	231.8	--	2.48	NA	--	2.2	NA	--	2.21	406.1	--
1427L	53	Y	2252	1.42	1.71	256.8	--	1.72	167.6	--	0.175	45.23	1	0	11.16	55
1437LL	53	Y	1857	1.34	1.335	NA	--	1.615	NA	--	1.305	NA	--	0.336	58	--
1455L	48	Y	1393	1.16	1.345	266.4	--	2.04	265.5	--	1.3	143.7	--	0.042	79.07	6
1457LL	48	N	884.3	0.78	0.616	71.22	--	0.815	NA	--	0.85	106.6	--	0.59	95.65	--
1463N	48	Y	798.8	0.705	0.618	110.9	--	0.785	NA	--	0.7	96.69	--	0.473	92.28	--
1467B	48	Y	525.1	0.508	0.505	103.8	--	0.513	NA	--	0.15	54.22	--	0	--	43
1451B	53	Y	427.7	0.335	0.261	77.39	--	0.334	53.67	--	0	24.43	50	0	13.89	271
1453B	48	N	357.3	0.266	0.299	75.14	--	0.322	NA	--	0.309	NA	--	0.271	NA	--
1451N	53	Y	824	0.145	0.133	34.07	--	0.011	12.9	--	0.156	34.42	--	0	15.64	--
1439B	53	Y	187.2	0.107	0.142	14.57	--	0.179	23.71	--	0	13.12	3	0	NA	4
1467R	48	Y	82	0.059	0.095	15.77	--	0.155	20.44	--	0.011	12.07	3	0	NA	163
1437B	53	Y	3.61	0.006	0.006	13.6	--	0.026	--	--	0.006	--	--	0	NA	--
1439N	53	Y	283.4	0	0	--	--	0	NA	--	0	NA	--	0	NA	--
1439R	53	N	9.4	0	0	--	--	0	NA	--	0	NA	--	0	NA	--
1457N	48	Y	8.8	0	0.043	NA	--	0.083	NA	--	0	NA	--	0	--	33
1597N	--	Y	--	Naïve	0		--	0		--	0		113	0		12655
1597L	--	Y	--	Naïve	0		--	0		--	0		1	0		5377
1603L	--	Y	--	Naïve	0		--	0		--	0		30	0		822
1603R	--	Y	--	Naïve	0		--	0.001		--	0		28	0		353
1603B	--	N	--	Naïve	0		--	0.003		--	0		--	0		--
1603LL	--	N	--	Naïve	0		--	0.006		--	0		--	0		--

Table S3.1. Plasma assay parameters through day 24 of the ET3i challenge course in AAV-ET3 treated and naïve hemophilia A animals.

This table displays the fVIII activity, antigen levels and anti-fVIII IgG titers 3 days after each ET3i challenge through the 4th challenge. A Bethesda titer is also included at the sacrifice time point. The pre-challenge FVIII activity and AUC are listed for each AAV-ET3 pre-treated animal. Animals shaded in gray are no-challenge controls. Animals shaded in blue demonstrated resistance to ET3i challenge. "Naïve" in the FVIII IU/mL column indicates the FVIII-naïve (no gene therapy or pervious exogenous FVIII exposure) hemophilia A animals that were included in the ET3i challenge course. "--" indicates that the assay result was below the limit of detection. "N/A" indicates that the assay was not performed on that sample.

I.D.	Sac. Week Post-AAV	i.v. ET3	FVIII AUC	FVIII IU/mL	Day 31 (5th i.v. ET3) IU/mL	Day 31 ng/mL	Day 31 IgG Titer	Day 38 IU/mL	Day 38 ng/mL	Day 38 IgG Titer	Day 45 IU/mL	Day 45 ng/mL	Day 45 IgG Titer	Week of Sacrifice IU/mL	Week of Sacrifice ng/mL	Week of Sacrifice IgG Titer	Week of Sacrifice BU/mL
1443N	53	Y	4138	4.64	3.52	409.9	--	3.8	395.5	--	2.83	360.1	0.1	1.12	226.9	--	--
1455LL	48	Y	3790	3.68	3.43	NA	--	2.72	358	--	5.13	423.1	--	1.12	169.4	--	--
1453L	48	N	3741	3.14	3.2	546.3	--	4.74	421.4	--	3.61	381.2	--	0.52	114.3	--	N/A
1443L	53	Y	2092	2.15	0.206	62.92	4	0.379	93.5	4	0.507	127.9	0.2	0.1	85.96	0.4	--
1439L	53	Y	1892	1.73	0	--	13	0.005	NA	952	0	--	1460	0	--	8359	10 – 50
1433L	53	Y	2009	1.69	2.58	144.5	--	1.44	NA	--	1.56	203	--	0.26	50.67	--	4
1455B	48	N	2272	1.49	1.78	157.2	--	2.95	383.3	--	2.4	173.5	--	0.51	94.01	--	N/A
1427L	53	Y	2252	1.42	0	NA	359	0	NA	673	0	--	246	0	--	2512	50 – 200
1437LL	53	Y	1857	1.34	0.933	163.2	--	1.125	NA	--	0.085	39.1	0.7	0.1	59.78	0.1	--
1455L	48	Y	1393	1.16	0	16.85	36	0.002	58.87	274	0	10.78	536	0	--	36762	50 – 200
1457LL	48	N	884.3	0.78	0.544	NA	--	0.045	35.91	--	0.695	105.7	--	0.225	58.61	--	N/A
1463N	48	Y	798.8	0.705	0.604	106.6	--	0.59	NA	--	0.285	102.3	--	0	--	0.2	--
1467B	48	Y	525.1	0.508	0	NA	820	0	NA	2413	0	--	1970	0	--	783	10 – 50
1451B	53	Y	427.7	0.335	0	25.24	2803	0	NA	35531	0	23.56	73901	0	--	553187	> 500
1453B	48	N	357.3	0.266	0.275	NA	--	0.307	NA	--	0.326	96.36	--	0.232	46.71	--	N/A
1451N	53	Y	824	0.145	0	NA	416	0	NA	17559	0	--	19388	0.121	--	142673	50 – 200
1439B	53	Y	187.2	0.107	0	NA	1	0	NA	7	0	--	6	0	--	394	5 – 10
1467R	48	Y	82	0.059	0	NA	17738	0	NA	23365	0	NA	9593	0	--	13943	50 – 200
1437B	53	Y	3.61	0.006	0	NA	9	0	NA	30	0	--	30	0	--	16	5
1439N	53	Y	283.4	0	0	NA	8548	0.02	NA	10261	0	NA	17639	0	--	202265	200 – 500
1439R	53	N	9.4	0	0	NA	--	0	NA	--	0	NA	--	0	--	--	N/A
1457N	48	Y	8.8	0	0	NA	299	0	NA	1598	0	NA	2210	0	--	54653	200 – 500
1597N	--	Y	--	Naïve	0		50500	0		12360	0		11614	0		5306	50 – 200
1597L	--	Y	--	Naïve	0		232338	0		50541	0		101043	0		212777	200 – 500
1603L	--	Y	--	Naïve	0		49928	0		46424	0		117608	0		311135	200 – 500
1603R	--	Y	--	Naïve	0		2981	0		3459	0		8592	0		2570	50 – 200
1603B	--	N	--	Naïve	0		--	0		--	0		--	0		--	N/A
1603LL	--	N	--	Naïve	0		--	0		--	0		--	0		--	N/A

Table S3.2. Plasma assay parameters from day 31 to sacrifice of the ET3i challenge course in AAV-ET3 treated and naïve hemophilia A animals.

This table displays the fVIII activity, antigen levels and anti-fVIII IgG titers 3 days after the 5th ET3i challenge, weeks 6 and 7 post challenge initiation, and at the time of sacrifice. A Bethesda titer is also included at the sacrifice time point. The pre-challenge FVIII activity and AUC are listed for each AAV-ET3 pre-treated animal. Animals shaded in gray are no-challenge controls. Animals shaded in blue demonstrated resistance to ET3i challenge. "Naïve" in the FVIII IU/mL column indicates the FVIII-naïve (no gene therapy or pervious exogenous FVIII exposure) hemophilia A animals that were included in the ET3i challenge course. "--" indicates that the assay result was below the limit of detection. "N/A" indicates that the assay was not performed on that sample.

Chapter 4

General Discussion

4.1 Discussion of Results

As previously stated, gene therapy product development has progressed to the state where customization of individual candidates has become standard practice. Customization of any of the individual design elements has the potential to affect the performance of the gene therapy, from target cell entry to the biodistribution and level of transgene product expression. Characterization of the effects of design elements on efficacy parameters is standard practice. However, understanding the impact that these factors have on the immune response to the gene therapy vector and its transgene protein product is much more challenging and often not rigorously or directly interrogated in preclinical research. The result is a large gap in knowledge regarding the immune response to fVIII in the context of AAV gene therapy as well as in the immune response to this class of biotherapeutics in general. Much of the evaluation of the immune response to gene therapies thus far focuses on factors inherent to the patient or preclinical research model animal or the protein drug function and target, but few studies interrogate effects of vector dose and design on the downstream pharmacokinetics and immunological outcomes.

Investigations began by preliminary evaluation of multiple vector design factors for liver-targeted AAV-fVIII vectors that could influence the immune response to the fVIII protein product. One factor is the design of the fVIII protein product. However, thus far fVIII seems to be immunogenic when delivered as an exogenous, xenogeneic protein regardless of its bioengineering. Hemophilia A mice responded by forming fVIII inhibitors whether they received BDD fVIII (HSQ), full-length human fVIII (Advate), or the porcine-human chimeric BDD fVIII (ET3i). Biodistribution of vector transduction and fVIII protein product expression is another factor that could affect immunological outcomes and it was assessed here by biodistribution qPCR (DNA) and RT-qPCR (RNA), both of which demonstrated that expression is specific to the liver

as desired. A bicistronic vector expressing FLUC and GFP was designed with each of the promoters used in the dose response study (HCB and E06.TTR) was also tested in mice to look at *in vivo* expression. Again, luciferase signal was seen only in the region of the liver. A preliminary experiment with the four vectors used in this study (In order of decreasing predicted potency: AAV-E06.TTR-ET3, AAV-HCB, AAV-E06.TTR-HSQ, and AAV-HCB-HSQ) was conducted in E16-/- hemophilia A mice wherein one animal administered AAV-E06.TTR-HSQ and one administered AAV-HCB-ET3, as well as two animals administered AAV-E06.TTR-ET3 developed inhibitors. This suggested that inhibitor development could have something to do with vector potency. Additionally, total F8 knockout (TKO) mice were also treated with AAV-E06.TTR-HSQ and AAV-HCB-HSQ, after which all animals receiving AAV-E06.TTR-HSQ developed inhibitors. This supports the hypotheses that less fVIII CRM and host genetics contribute to establishing the threshold for inhibitor development.

While genetics are an extremely important factor that merit further study, both for understanding their effect on the fVIII inhibitor response and for the clinical success of fVIII gene therapies, this project moved forward to focus on the effects of vector potency and dose on the pharmacokinetics of fVIII exposure and immunogenicity outcomes. An extensive, longitudinal dose response study was undertaken using a murine model of hemophilia A and four vectors similar to those currently in clinical trials and at a range of clinically relevant vector doses. It became immediately apparent that the four vectors have different apparent potencies: AAV-E06.TTR-ET3 > AAV-HCB-ET3 > AAV-E06.TTR-HSQ > AAV-HCB-HSQ. At each dose tested, peak fVIII activity values were higher and arrived at more quickly (faster expression kinetics) in correlation with vector potency. The same dose response trend was also observed among different doses of the same vector. For vectors that rapidly reached supraphysiological fVIII activity levels,

they also subsequently lost fVIII activity in direct correlation with the combined vector potency and dose. Evaluation of anti-fVIII IgG titers and functional inhibitor titers (Bethesda titers) revealed that activity was almost exclusively being lost due to the development of an inhibitor response. One animal lost fVIII activity and liver VCN without a concomitant inhibitor response. The other 20 animals that lost fVIII activity were accompanied by the development of anti-fVIII neutralizing IgG. Over time, 4 of these animals displayed a complex, multiphasic inhibitor response in which they endeavored to resolve these inhibitors independently. The result was inverse fluctuations in fVIII activity and anti-fVIII IgG titer. The other 16 animals displayed a sustained inhibitor response for the lifetime of the study. The fVIII activity profiles and inhibitor outcomes suggested that whether or not inhibitors developed was dependent upon the level and kinetics of initial fVIII protein exposure following gene therapy delivery. Pharmacokinetic analysis of this trend revealed this to be correct. In fact, the day 5 fVIII exposure rate, k_{fVIII}, was revealed to be the strongest independent predictor of fVIII inhibitor incidence following AAV-fVIII delivery by multiple validation statistics and contingency analyses. As fVIII activity can only be reliably measured in the absence of inhibitors, the early fVIII activity levels were used in development of the model, and day 5 k_{fVIII} and AUC_{fVIII}, as well as day 10 k_{fVIII} and AUC_{fVIII}, were all found to be significant by logistic regression analysis.

The animals that did not develop inhibitors after AAV-ET3 delivery were subsequently challenged with ET3i protein infusions. The result revealed that, if the gene therapy treated animals stayed below the initial immunogenicity threshold, those expressing higher steady state levels of ET3 (as measured by fVIII activity) were less likely to respond with inhibitors during ET3i challenge and hence developed a stronger state of immune tolerance to ET3. In fact, some of the animals that developed the multiphasic response or showed resistance to challenge also had higher

liver VCN values than other animals in their vector/dose treatment groups. However, this was not uniformly the case. It would be ideal at this point to be able to directly measure fVIII antigen in both animals that did and did not develop inhibitors after AAV-ET3 delivery and/or ET3i challenge. This would provide valuable information about immunological mechanisms of inhibitor formation and help provide an explanation as to why some animals developed inhibitors, some were able to resolve them while others were not, and some did not develop inhibitors with subsequently mixed results on whether those animals were tolerized to ET3. An anti-ET3 antigen ELISA was developed and performed on plasma samples from all AAV-ET3 treated and/or ET3i challenged animals in this study at multiple time points. In agreement with the immunogenicity threshold, day 5 antigen levels were higher in animals that went on to develop inhibitors than in animals that did not. Additionally, the *in vivo* ET3 specific activity was not significantly different between AAV-ET3 treated animals that developed a multiphasic inhibitor response, AAV-ET3 treated animals that did not develop inhibitors and were not challenged with ET3i, and AAV-ET3 treated animals that demonstrated immunological resistance after challenged with ET3i at the time of their sacrifice. However, as expected, it was difficult and usually impossible to measure ET3 antigen once a robust IgG response was established. Yet, taken together, these data suggest that ET3 antigen was still being produced after gene therapy delivery whether the animals developed inhibitors or not.

Indeed, it is well understood that antigen exposure kinetics, steady state expression levels, duration of exposure, and spatial/temporal localization are all key in both induction of an immune response and tolerance.[257] These factors are integrated along with inhibitory and stimulatory signals to determine the immunological outcome. Having defined an immunogenicity threshold based on initial antigen exposure kinetics, it is therefore interesting to consider the nature of the

immune response in animals above the threshold vs. those that fell below it. Fluctuation in the structure or availability of the antigen (antigen discontinuity) can be immunogenic, especially if the change is large per unit time. These principles hold in spite of the manifold differences between periodic intravenous injection of fVIII protein and liver-directed AAV-fVIII gene therapy. Elucidating the mechanisms behind the varied and complex immune responses to the different AAV-fVIII doses and downstream fVIII expression will require further targeted studies at multiple time points; however, it may be that the initial antigen exposure kinetics and resulting steady state levels also set the stage for a certain type of response, the nature of which is further mitigated by other factors. In the case that initial fVIII exposure kinetics reach above or near the immunogenicity threshold defined here, the sudden, high levels of fVIII released from the liver induce a robust immune response and rapid increase in anti-fVIII IgG titer. Unlike the case of an acute infection or single bolus injection, fVIII antigen likely continues to be produced by the liver, evidenced by the fact that the IgG titers fluctuate over time and some animals spontaneously recover their fVIII activity and struggle to establish tolerance.

Differences in normal fVIII action and processing in the plasma due to such high rates and levels of production have yet to be investigated. The apparent delay often observed between the initial drop in fVIII activity and rise of an IgG titer could be the result of immune complex formation or BCR receptor cross-linking that quickly neutralizes fVIII activity while igniting an fVIII neutralizing IgG response. The high excess in fVIII protein could change the ratio with which fVIII binds von Willebrand Factor (vWF) in circulation. Some plasma samples were also assayed for anti-fVIII IgM at early time points and showed very low or absent IgM titers (*data not shown*). Both IgM and vWF could be contributing to early immune complex formation. Additionally, fVIII has a short half-life when not bound to vWF and is processed into many different protein fragments

in circulation ahead of clearance. The fluctuating ratios of free to bound fVIII protein would have effects on employment of different fVIII clearance pathways and fVIII pharmacokinetic parameters, which could also alter the way that the immune system perceives fVIII. Even after vWF or immune complex binding, high levels of free fVIII protein could still be in circulation, which may lead to differential processing and different motifs being available at higher frequency in lymphoid tissues during their rapid clearance. The initial, robust appearance of fVIII could strongly activate naïve T and B cells; however, contributions from the innate immune system, for example via complement activation, and the unique immunological microenvironment of the liver cannot be ruled out as important players. Subsequently, apoptosis of transduced hepatocytes due to cellular stress from the burden of high AAV transduction and fVIII protein production could lead to changes in fVIII levels over time. This possibility is supported by the relatively low VCN seen in animals up to a year after AAV delivery in spite of early high fVIII activity and persistent IgG titers. There has also been evidence of AAV vector integration and clonal expansion of AAV-fVIII transduced hepatocytes, which could lead to episodes of increased fVIII levels. During the year after AAV-fVIII delivery, along with the possible fluctuations in fVIII protein production from the liver, it follows that there would also be downstream fluctuations in fVIII immune complex formation and dissociation, protein processing and clearance rates. It is also possible that hepatocytes that are chronically strained or have high activation of the unfolded protein response could produce fVIII with differences in structure and post-translational modifications. For inhibitory IgG titers to persist over time while also fluctuating in magnitude, the immune cells could be responding to continuously present fVIII that also fluctuates (i.e., is discontinuous) enough in the antigen availability and motifs encountered that it continues to feed the antibody response in the majority of animals above the fVIII immunogenicity threshold. Sustaining the

antibody response this way may occur through phasic activation of a naïve B cell response that correlates with the peaks and troughs in antigen availability plus any significant changes in protein motifs in the microenvironment used for antigen presentation by T cells. It is also possible that memory cells formed during the initial immune response and the discontinuous antigen levels trigger a memory B cell response as fVIII availability increases. If at some point fVIII expression is lost but the IgG titer persists, then long-lived plasma cells could have formed by either the germinal center or extra-follicular pathway.

There were three animals that formed inhibitors after AAV-fVIII delivery and proceeded to display a complex attempt at establishing immune tolerance. In these cases, the animals have among the highest initial peaks in fVIII activity, but the subsequent IgG titers peaked approximately 2-4 logs lower, overlapped more with detectable fVIII activity, and coincided with lower, transient Bethesda titers (one mouse never developed a quantifiable Bethesda titer) compared to animals with sustained inhibitor responses. This may be because the initial effector response was skewed more toward antigen-induced cell death rather than memory and/or plasma cell formation, leaving behind a lower affinity effector cell population and generating an overall weaker IgG response. Perhaps restimulation of naïve immune cells in this context leads to more effective recovery of fVIII activity; however, it remains unknown whether this recovery would eventually have become permanent peripheral tolerance to fVIII. A fourth animal that was administered 6E13 vg/kg AAV-E06.TTR-ET3 had the highest peak fVIII activity and sustained a high-titer IgG response in that vector/dose group but began to recover fVIII activity just prior to sacrifice (after 250 days). It is possible that some of the fVIII-specific T cell population either became exhausted or, given the late onset and age of the animal, began to senesce. Interestingly, the one animal that never developed a Bethesda titer also had the lowest IgG titer (never >100)

and overall highest VCN at sacrifice. This could indicate that this animal had steadier continuous and chronic antigen exposure than the other AAV-fVIII treated animals that developed inhibitors, a state that would lower the threshold for establishing tolerance after the initial effector response.

Indeed, chronic steady state presence of an antigen that is continuous (i.e., without significant changes in availability and composition) is critical for establishment and maintenance of tolerance by any known mechanism. AAV-fVIII doses that resulted in high/middle fVIII activity levels (2.22E12 vg/kg – 2.00E13 vg/kg depending on vector potency) displayed frequent, higher magnitude oscillations in activity over time and approach a 50% probability of developing inhibitors. Inhibitor incidence became more unpredictable in a narrow range around the immunogenicity threshold where this fVIII activity oscillation occurs. In the middle range of AAV-fVIII doses that resulted in fVIII exposure that increased gradually in a more continuous manner, reaching steady state levels between 1.5 – 5 IU/mL, inhibitor incidence approached zero and the likelihood of maintaining fVIII tolerance after protein challenge increased with increased steady state levels. Peripheral tolerance mechanisms such as anergy, desensitization, iTreg and perhaps Breg formation, are perhaps given the time and space they require to establish themselves when fVIII antigen appears gradually without the initial vigorous inflammatory response and antigen fluctuations described above. This may create a complex population of T and B cells maintaining a tolerogenic state that increases in strength with higher continuous, steady state fVIII levels. This is supported by the fact that, in spite of chronic antigen presence, animals with lower steady state fVIII levels show increased probability of developing inhibitors after fVIII protein challenge. The bolus fVIII exposure represents a quantitatively larger variation compared to the steady state fVIII levels in those animals. Thus, tolerance likely occurs on a dynamic spectrum in the AAV-fVIII treated animals below the initial fVIII exposure immunogenicity threshold. Slow

fVIII exposure kinetics allow for establishment of tolerance, and the higher steady state fVIII levels actively maintain the tolerogenic state and suppress an immune response after any disruption. As the steady state levels decrease, this resistance becomes increasingly fragile, until the immune system has seen so little fVIII that it effectively returns to a state of ignorance much like in a naïve hemophilia A animal.

Additionally, the liver is considered an immune-privileged organ and liver organ transplants have been curative in hemophilia A patients. The contribution that the resident immune cells and the liver microenvironment makes to either establishing immune tolerance or contributing to an inhibitor response after liver-directed AAV-fVIII gene therapy needs to be investigated. While immunological contributions of the liver were not addressed in this study, abnormal liver growths were observed on multiple gene therapy treated animals at necropsy. None of the animals treated with the highest two doses of the more potent AAV-ET3 vectors that survived until the study endpoint had abnormal liver growths at time of sacrifice. It may be possible that the highest doses of more potent vectors led to increased cell death after the initial transduction, perhaps due to cellular stress induced apoptosis and/or a cytotoxic immune response, that then turned out to be protective against liver pathology. While most of the animals that had liver growths did not develop inhibitors after AAV-fVIII delivery (i.e., they were below the initial fVIII immunogenicity threshold), it was not a completely uniform result. One multiphasic response animal administered 6.67E12 vg/kg AAV-E06.TTR-ET3 and two animals administered high doses of AAV-E06.TTR-HSQ that developed inhibitors also had abnormal liver growths at necropsy.

4.2 Translational and Clinical Impact

The successful development and clinical translation of gene therapy products are forcing changes to the traditional approaches to drug development pharmacology and toxicology. New principles are in play and novel investigational approaches must be devised to assess their impact on the safety and efficacy of gene therapy drug candidates. Unfortunately, preclinical studies have been unsuccessful in predicting and preventing the occurrence of severe adverse events observed in clinical trials, such as insertional mutagenesis related to leukemogenesis and liver toxicities after administration of certain ɣ-retroviral and AAV vectors, respectively. These findings along with the inapplicability of many traditional pharmacology principles brings to light the need for development of new principles and methods to elucidate the critical parameters predictive of the safety of gene therapy product candidates. Despite concerns over potential adverse events and the gaps in knowledge regarding the effects of vector design and dosing on durability of fVIII expression, the immune response to fVIII, and the short- and long-term health of the target organ (i.e., the liver), AAV-fVIII product candidates are rapidly advancing through clinical trials in human subjects. This study presents a model platform for translational research and drug development of gene therapy products. Importantly, it employs a wide vector dose range, an immunocompetent animal model of the disease, frequent early endpoint assessments, and long-term endpoint assessments. This provides valuable information on treatment efficacy and safety as well as provided the data necessary to create a pharmacokinetic model with predictive value regarding immunological safety. With these data, a dose of any of these four vectors could be delivered to a male, E16-/- hemophilia A mouse with a predictable immunogenicity risk. These data and this model could directly inform both further translational studies in preclinical models and clinical trial protocols for testing in human subjects. All genetic medicines should be evaluated

with similar or greater rigor in order to help improve efficacy outcomes and avoid adverse events in clinical trials.

One of the major goals of fVIII gene therapy development is to be able to avoid fVIII inhibitor formation and/or induce tolerance when treating patients with established inhibitors. While preclinical studies of AAV-fVIII to immunized mice have not yielded encouraging results, the preliminary studies presented herein were not designed to formally address this question. However, taking all of the data together, it's possible that the tolerance induction outcome is largely dependent upon the magnitude of the inhibitor response and whether the source of fVIII protein intended to induce tolerance is high, sustained, and continuous enough to force tolerance. And it may also require enduring an initial humoral immune response after AAV-fVIII delivery prior to inhibitor resolution. This may be partly why current protocols of immune tolerance induction via intravenous delivery of high quantities of fVIII protein often fail: if the inhibitor titer barrier is too high and the fVIII exposure meant to induce tolerance is not high, continuous and/or steady enough, tolerance cannot be established and maintained. Therefore, AAV-fVIII should be dosed carefully in both previously untreated patients and patients who have tolerated factor replacement, as the slow exposure kinetics could be important both to avoid triggering an inhibitor response in a fVIII ignorant immune system as well as to avoid delivering an antigen disruption large enough to break tolerance in an experienced immune system. In the latter system, it may also be important to match the gene therapy fVIII sequence to that of the recombinant protein previously tolerated by the patient, as some deviations may be large enough for altered antigen presentation to trigger an inhibitor response. This opens the door for discussion of personalized medicine for gene therapy that takes into account prior exposures as well as the immune cell precursor repertoire and genetics of each patient. Whether AAV-fVIII dosed in this personalized

manner could be reliably successful at resolving existing inhibitors is an even more complicated question. Another active area of investigation remains engineering fVIII proteins with superior pharmacological properties that could be delivered to patients with anti-human fVIII inhibitors from previously used fVIII replacement proteins, and then whether these superiorly engineered fVIII proteins would make better candidates as a transgene in AAV-fVIII gene therapy to bypass the existing inhibitors and establish tolerance to the transgene product. That is the major motivation for the evaluations of ET3i presented in this project. Analyzing the pharmacological properties of ET3i in inhibitor patient plasma samples could determine the degree of anti-hfVIII antibody cross-reactivity, the extent to which ET3i is detectable or masked by the inhibitors established in the patients, and the specific activity of ET3i in the presence of those inhibitors. The results could then be cross-referenced to patient data for any correlations between patient demographics, comorbidities, other medications, etc. that influence the performance of ET3i in the context of pre-existing fVIII inhibitors. This would be valuable information to groups developing gene and cell therapies employing ET3i, or any other candidate bioengineered fVIII protein.

Special attention should be paid to the liver in the context of liver-directed gene therapy products, as some AAV gene therapy patients have shown various levels of liver toxicity. Preclinical studies have shown evidence of cellular stress resulting from strong liver-specific promoters and of AAV vector integration resulting in clonal expansion of transduced cells. Additionally, the liver is a structurally and immunologically unique organ. The contribution of the liver immune cell microenvironment to an immune response or maintenance of tolerance after gene therapy is unknown. Different regions of the liver have also been shown to proliferate differentially, which could amplify downstream consequences for gene therapy efficacy and durability, any liver-specific immune responses, or the fate of cells harboring vector integrations

depending upon whether AAV vectors transduce regions of the liver preferentially or randomly. These are among the reasons why liver biopsies are being discussed for incorporation in future clinical testing of AAV gene therapies with additional informed consent from the patient.

Another important point to consider here is that treatment of hemophilia A even with standard of care fVIII replacement protein is extremely costly. Being a chronic genetic disorder, both the requirement of regular infusions and the high cost of treatment are things that the patient will have to contend with their entire lives. Beyond this, there are populations for which the newest generation of hemophilia A treatments are either not available or too costly. As disparities remain in access to the best medical care, scientists and medical professionals have a responsibility to do their utmost in evaluation of every gene therapy product candidate for hemophilia A. Since these treatments are likely to be extremely expensive and not accessible for all populations, our best must be done to ensure the product's efficacy and safety and to make it as widely available as possible to all hemophilia A patients.

4.3 Limitations and Future Directions

As the effect of every parameter on the efficacy, safety and immunogenicity of AAV-fVIII gene therapy could not be assessed here, there are multiple limitations in this project. Firstly, only one AAV serotype (AAV8) was evaluated. Other AAV serotypes could result in differences in the humoral immune response and in the region(s) and degrees of liver cell transduction. It would also have been wise to do a more formal evaluation of liver health and events occurring on both the hepatocyte and organ levels both immediately after AAV-fVIII delivery and at the long-term study endpoint for each of the vectors at each of the doses tested. It is clear from both clinical data and

the data presented herein that the liver is an important component for AAV-fVIII efficacy, durability, immunogenicity, and patient safety. More preclinical and clinical investigations of the liver after AAV gene therapy are starting to be performed and this should continue to be rigorously investigated and monitored.

While this study produced sufficient data to suggest that fVIII, specifically ET3, antigen was still being produced in the context of an inhibitor response, antigen still cannot be reliably detected by ELISA in the presence of fVIII inhibitors. Accurate detection of fVIII antigen is essential in validating immune response hypotheses involving antigen discontinuity. Additionally, the same dose-response study should have been replicated in immunocompromised RAG-1 deficient mice, because then an accurate measure of fVIII antigen over time for each vector at each dose could have been taken and compared to the activity profiles and immune responses observed in the immunocompetent hemophilia A mice. While this study was one of the most thorough of its kind to date and provides an important platform for future study, immune response mechanisms were not thoroughly investigated. This was a long-term study, but in particular it would be very important from an immune response perspective to perform mechanistic immunology studies shortly after delivery of various doses of the different AAV-fVIII vectors for corroboration with the pharmacokinetic model and long-term data. It would also have been interesting to administer a range of doses of the vectors to pre-immunized animals in order to formally assess immune tolerance induction with AAV-fVIII. The dose response study also did not investigate the important role that genetics can play in the immune response to fVIII following AAV-fVIII gene therapy. Now that a pharmacokinetic model and immunogenicity threshold has been established, hemophilia A mice with various genetic backgrounds should be similarly investigated in order to

observe the effects genetics have on the fVIII exposure threshold and immune response mechanisms.

Another important arena for investigation is the role that vWF plays in the immune response to fVIII after AAV-fVIII delivery and whether gene therapy expression of fVIII changes the nature of the interaction between vWF and fVIII. High doses of potent AAV-fVIII vectors could rapidly lead to such high levels of plasma fVIII that the stoichiometry of vWF:fVIII binding may change. FVIII and vWF may together be components of immune complexes that participate in induction of a fVIII inhibitor response. These immune complexes may also play a role in BCR cross-linking. If vWF becomes saturated, this could also lead to a larger pool of circulating free fVIII, which could change the degree or nature of antigen presentation of fVIII despite the likely increase in fVIII clearance. Impacts of high levels of AAV transduction and fVIII production on hepatocytes could also affect the clearance of vWF and fVIII mediated by hepatocyte receptors, like the asialoglycoprotein receptor. Mechanistic investigations of the role of vWF after AAV-fVIII delivery could be undertaken in double vWF-fVIII knockout mice or using D'D3-Fc protein both *in vivo* and in biochemical assays. Regarding the pharmacokinetic model, a more detailed model could be developed that considers the multiple pools of fVIII that would likely form after high dose AAV-fVIII, like vWF-bound and free circulating fVIII. The model should also be evaluated not only in different strains of mice, but in different animal models. Preclinical data shows great variation between different fVIII gene therapies and different animal species. In order to better inform clinical trials, it is important to assess how the model and the position of the fVIII exposure immunogenicity threshold changes between species.

4.4 Conclusion

This study shines the spotlight on how critical thorough pharmacokinetic/pharmacodynamic studies and creation of new drug development methods and models are for the rapidly advancing field of genetic medicine. The current study demonstrates, for the first time, both a clear vector dose (vg/kg) and potency (transgene product expression) relationship to transgene product immunogenicity for gene therapy vectors similar to those under clinical investigation. This extensive longitudinal dose response study evaluating four different AAV-fVIII vectors made apparent the critical role that transgene expression kinetics plays in immunogenicity, where a slower rise to peak transgene product expression levels (over weeks to months) is favorable compared to a rapid rise to peak levels (over days to weeks). We have identified initial fVIII production rate (k_{fVIII}) as the strongest independent predictor of subsequent inhibitor development and used PK/PD analyses of plasma fVIII activity levels to model an immunogenicity risk threshold in fVIII-naïve hemophilia A mice. It is also vital that transgene protein product levels rise to a level that is both continuous and high enough that it can establish a state of immune tolerance. These findings suggest a treatment window with both optimal therapeutic efficacy and immunogenicity risk. Further studies are required to validate this pharmacokinetic immunogenicity model of liver-directed AAV-fVIII gene therapy in other preclinical hemophilia A models as well as for other AAV gene therapy products treating monogenic disorders. Similarly designed studies could be highly valuable in preclinical development and clinical trial design for gene therapy candidates. Once vector potency is ascertained, the dose should be adjusted accordingly to yield transgene product expression that increases as gradually as possible to a steady state level that is maintainable, efficacious, on-target, tolerated, and safe.

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