THE PREVALENCE OF ANTI-ADENO ASSOCIATED VIRUS NEUTRALISING ANTIBODIES IN SOUTH AFRICAN PEOPLE WITH HAEMOPHILIA B.

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A research report submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in Haematology.

Johannesburg, 2020.

DECLARATION

I, Nolukholo Ncete declare that this Research Report is my own, unaided work.

It is being submitted for the Degree of Master of Medicine in Haematology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

Signature of Student……. Date…….....

DEDICATION

To my loving parents Mnikeli and Nomabhaso Ncete for their unfailing support.

PRESENTATIONS ARISING FROM THIS STUDY

Nolukholo Ncete, Anna Majowicz, Floris van Waes, Sander J. van Deventer, Johnny N. Mahlangu and Valerie Ferreira.
13th Annual congress of the European association for haemophilia and allied disorders (EAHAD). The Hague, Netherlands 5-7 February 2020.

Anna Majowicz, **Nolukholo Ncete**, Floris van Waes, Sander J. van Deventer, Johnny N. Mahlangu and Valerie Ferreira.
61st American Society of Haematology (ASH) Annual Meeting & Exposition. Orlando, USA 7-10 December 2019.

Anna Majowicz, **Nolukholo Ncete**, Floris van Waes, Sander J. van Deventer, Johnny N. Mahlangu and Valerie Ferreira.
Thrombosis & hemostasis summit of North America (THSNA). Chicago, USA 23-25 April 2020.

My contribution to these presentations includes formulating the study protocol, applying for ethics clearance, obtaining consent from the participants, collecting blood samples, sample analysis, reviewing of the clinical records, data analysis, and presentation of the data in various meetings.

ABSTRACT

Adeno-associated virus (AAV) is the most common vector used in gene therapy for haemophilia B and one of the limitations of using this virus is the presence of pre-existing neutralising antibodies (NAbs) in individuals exposed to the virus in the general population. To date, there is a paucity of studies in the literature that have measured the prevalence of these NAbs in haemophilia B patients. The prevalence of anti-AAV NAbs among people with haemophilia varies with geographical location; consequently, extrapolation of prevalence data across regions is not possible. Knowledge of such prevalence is critical in the selection of participants for haemophilia B gene therapy intervention. The prevalence of anti-AAV NAbs in the South African haemophilia B population is currently unknown.

This study had several objectives which were 1.) To measure the prevalence of anti-AAV NAbs of the different serotypes, including AAV1, AAV 2, AAV 5, AAV 6 and AAV 8 in South African people with haemophilia B, 2.) To identify the most prevalent serotype, the level of titres, possible cross-reactivity and compare these findings to what is known in the literature and 3.) To determine the possible risk factors associated with the development of anti-AAV neutralising antibodies.

Serum samples were collected from 44 males with haemophilia B attending the Haemophilia Comprehensive Care Centre (HCCC) at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). Controls comprise of 44 healthy non-haemophilia B volunteers. Each haemophilia B participant and control sample were tested for NAbs against five different serotypes of AAV, which included AAV1, AAV2, AAV5, AAV6 and AAV8 using a luciferase-based transduction inhibition assay. A positive result was defined as a titre of more than 1 in 8 and the highest titre above 1 in 1030.

AAV2 had the highest seroprevalence of NAbs in participants with haemophilia B at 95%, and 39% of the participants had titres above 1030. This was followed by AAV6 with a

prevalence of 82% with only 9% of participants having titres above 1030. AAV1 had a prevalence of 77%, and 20% had titres above 1030, making it the second most prevalent serotype with titres above 1030 after AAV2. AAV5 had a prevalence of 66% and had the lowest percentage of participants in the highest titre range (> 1 in 1030) at 5%. The lowest seroprevalence of NAbs was seen with AAV8 (64%), and 32% of participants in this group had low titres ≥8-51. The frequency of all serotypes was higher in participants above 18 years of age and in those with severe haemophilia B disease. There was also a high AAV serotype co-prevalence observed at 59%, with AAV2 NAbs present in all participants that tested positive for any serotype. The seroprevalence in control participants followed a similar trend with frequencies of 75%, 100%, 61%, 80% and 64% in AAV1, AAV2, AAV5, AAV6 and AAV8, respectively.

Anti-AAV 2 neutralising antibodies are the most prevalent in the South African haemophilia B population. The AAV 5 and AAV8 vectors should be considered for use in gene therapy for our haemophilia B participants, as these two AAV serotypes had a lower prevalence with participants having lower titres.

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TABLE OF CONTENTS

LIST OF FIGURES

FIGURE PAGE NUMBER

CHAPTER 1

LIST OF TABLES

TABLE PAGE NUMBER

LIST OF ABBREVIATIONS

AAV	Adeno-associated virus
AAV-CMV-Luc	Adeno-associated virus-cytomegalovirus-luciferase
APC	Antigen-presenting cell
BCR	B cell receptor
BEV	Baculovirus expression vector
cDNA	Complementary deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
F9	Factor 9 gene
FIX	Factor IX protein
GFP	Green fluorescent protein
GOI	Gene of interest
HEK293T	Human embryonic kidney cell line
HeLa	Human cervical carcinoma cell line
HSPG	Heparan sulphate proteoglycan
ITR	Inverted terminal repeat
IU/dL	International units per decilitre
kb	Kilobases
LSEC	Liver sinusoidal endothelial cells
MHC	Major histocompatibility complex
NAbs	Neutralising antibodies

NHP	Non-human primate
pdFIX	Plasma-derived factor IX concentrate
PDGFR	Platelet-derived growth factor receptor
rAAV	Recombinant adeno-associated virus
rFVIIa	Recombinant activated factor VII
scAAV	Self-complementary adeno-associated virus.
ssAAV	Single-stranded adeno-associated virus
TI	Transduction inhibition
T regs	Regulatory T- cells

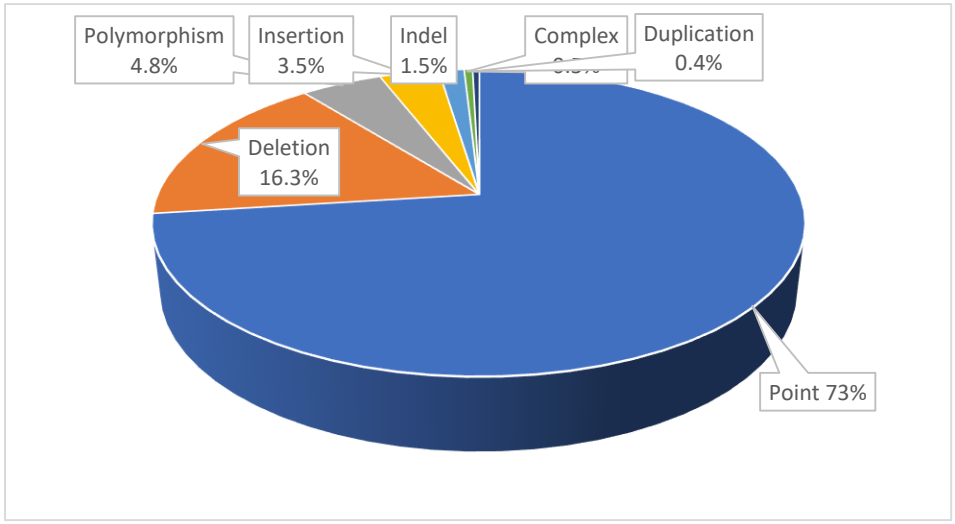
CHAPTER 1

INTRODUCTION

1.1 Haemophilia B

Haemophilia B is a bleeding disorder inherited in an X-linked recessive pattern, caused by a mutation in factor 9 (*F9*) gene with consequent dysfunction or deficiency of the factor IX (FIX) coagulation protein (1). FIX is produced in the liver by hepatocytes and requires the function of proteins that are highly expressed in hepatocytes for its activity. These proteins include vitamin K epoxide reductase and gamma-glutamyl carboxylase (2). Its role is to generate thrombin and result in clot formation (3). Thrombin is generated by activation of FIX via two mechanisms, which include cleavage of FIX by activated FVII (FVIIa) and tissue factor complex and by FXIa, and both mechanisms occur in the presence of calcium (4).

Haemophilia B characteristically presents with spontaneous or trauma-induced bleeding into joints, soft tissues and muscle (5). The prevalence of haemophilia B is around 1 in 30 000 male births (1) with the majority of participants having missense mutations (6). See Figure 1.1. The diagnosis is made by measuring FIX clotting activity and classified into mild, moderate and severe disease based on the residual factor activity in the blood (1). Mild haemophilia is defined as factor activity >5-40%, moderate 1-5% and the severe being <1% (7), with more than 50% of the patients with haemophilia B having severe disease (5).



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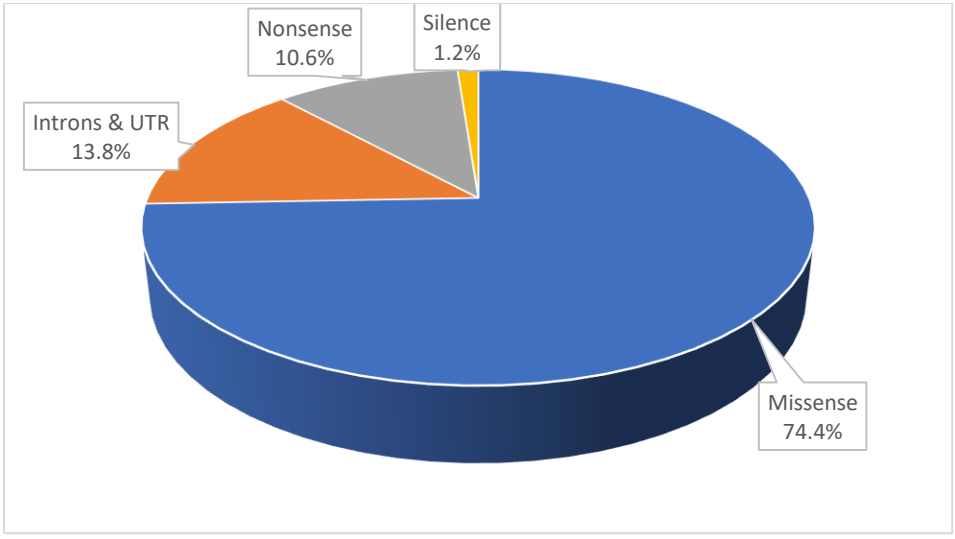

B.

Figure 1.1: The distribution of factor 9 gene mutations in haemophilia B, derived from a web-based FIX mutation database. The database includes 1113 mutations from 3721 participants. **A.** Shows all the mutations and **B** breaks down point mutations by type (6).

The current treatment of haemophilia B includes episodic or prophylactic replacement of the missing FIX for treatment or prevention of bleeding episodes (1). A factor trough level of 12-15% significantly improves joint bleeding to none. This treatment reduces mortality and has greatly improved the quality of life of people with haemophilia B (8). However, replacement therapy is associated with an increased treatment burden due to the requirement for life-long intravenous therapy (9). In a proportion of patients, replacement therapy is associated with the development of neutralising antibodies

against FIX (10). There are newer extended half-life concentrates that reduce the frequency of infusions and non-factor replacement therapies, but both these therapeutic approaches require life-long therapy (8). In addition, replacement therapy is costly and therefore not accessible to many patients in resource poor settings such as South Africa (7). The goal, therefore, has been to find a potential cure for this debilitating disease (8).

Haemophilia B is suitable for gene therapy as it affects a single well-characterised gene (5), the *F9* gene is small and fits into the commonly used viral vectors (11), and tight regulation of FIX levels is not essential as small increments in the levels will improve the phenotype of the disease (5). Lastly, response to gene therapy can be monitored by measuring FIX activity which correlates with the bleeding phenotype of the disease (12). Gene therapy in haemophilia B aims to replace the deficient or dysfunctional FIX protein with a functional F9 gene through a single intravenous infusion of the vector containing the transgene (5). The transgene compensates for the underlying mutated *F9* gene (13). This is made possible by using AAV serotypes that have tropism for the liver, where FIX is naturally produced and also utilising liver restricted promoters (12). It has been shown that transgene expression in hepatocytes induces antigen-specific immune tolerance by regulatory T cells (T-regs) and to date, no antibodies directed against the FIX transgene product have been described (14).

The cDNA of FIX is about 1.5 kilobases (kb) and fits into the recombinant AAV vector (rAAV), which can accommodate DNA sequences up to 5kb in size (7). A key component in successful gene therapy is the design of the expression cassette, including the use of codon-optimised F9 cDNA or a hyperactive FIX variant called FIX Padua (5).

1.2 Adeno-associated virus

AAV is a small replication-defective and weakly immunogenic virus and the presence of a helper virus, such as herpes or adenovirus is essential for its replication and assembly (15). The virus belongs to the Parvoviridae family and does not cause any disease in

humans (16). The AAV virus has a single-stranded DNA genome ~ 4.7 kb with inverted terminal repeats (ITR's) at each end of the gene (17). The ITR's are about 145 base pairs and their role is to package the DNA into capsids, and these are incorporated into the vector genome to allow its packaging (18). The gene consists of two coding regions, the rep and cap regions, which codes for replication-related proteins and proteins that form the viral capsid, respectively (15).

The AAV virus has been widely used as a vector (19) and is suitable for this role because of its ability to transduce non-dividing cells and drive long-term gene expression (16). The transgene is mainly expressed episomally and can be lost with subsequent cell division, which makes the use of haemophilia B gene therapy uncertain in the paediatric population with a liver that is still growing (20). There are approximately 13 serotypes identified to date, and they are differentiated based on the expression of surface antigens and differences in the capsid amino acid sequences (21). See Figure 1.2.

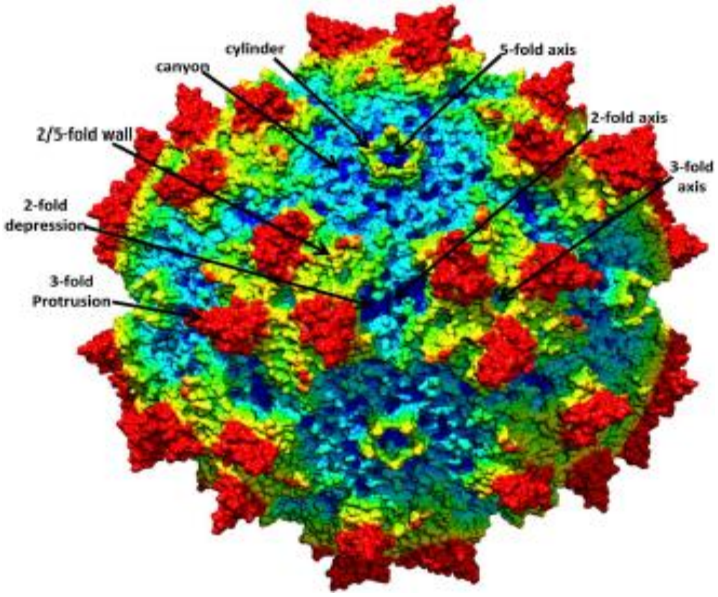

Figure 1.2: The structure of the AAV virus. AAV1 illustrates the structure of the capsid that is shared by other serotypes. The colour coding shows how the capsid is organised, the deepest areas are represented by blue and the red shows the protruding parts of the capsid (21).

1.2.1 Humoral immune response to adeno-associated virus

Humans are exposed to the wild type virus in childhood, and this exposure leads to the development of neutralising antibodies to prevent subsequent infection with the virus (22). The presence of anti-AAV antibodies against serotype 2 and 8 have been shown in new-borns as a result of vertical transmission of maternal antibodies. The prevalence declines during the first year of life, increasing after the age of one and reaching a peak at 3 years. This is suggested to be the period of exposure to natural infection and AAV2 virus is hypothesized to be the first AAV virus that humans are exposed to (22). The period in which most people do not have anti-AAV antibodies is very narrow (23). After the primary infection, the AAV genome can persist episomally for years in the host cells or become integrated into the host genome to be reactivated in the presence of a helper virus (24).

In haemophilia B gene therapy, the neutralising antibodies (NAbs) bind to the AAV vector administered intravenously and inhibit target tissue transduction (22). See Figure 1.3. This is similar to the NAbs that develop post gene transfer and prevent repeat administration using the same AAV serotype (5). Given the reduced efficacy of therapeutic gene approaches in the presence of pre-existing NAbs, screening for these antibodies will help identify those people with haemophilia B that will benefit the most from gene therapy (25).

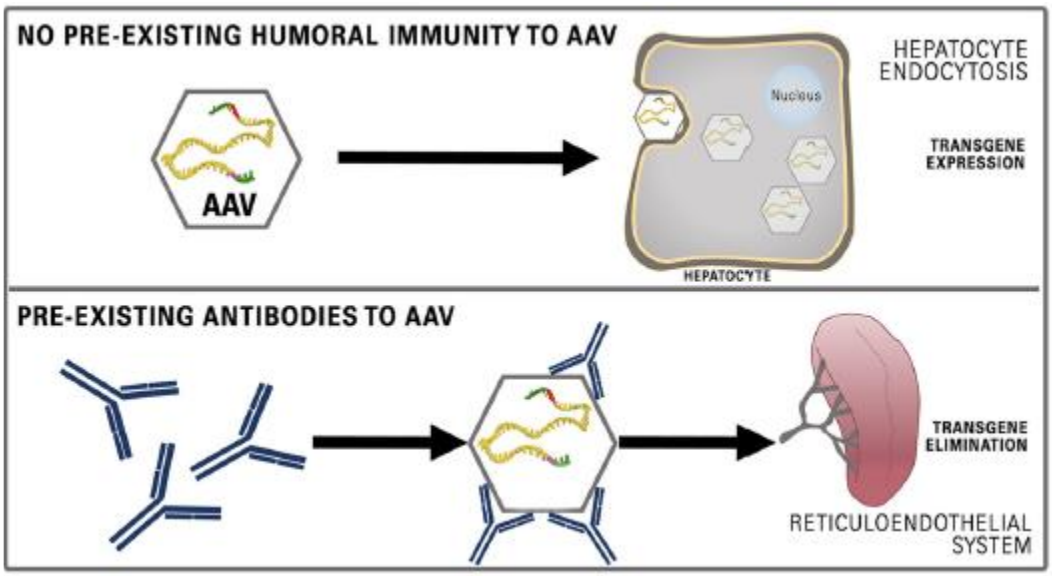

Figure 1.3: Shows successful hepatocyte transduction in the absence of Anti-AAV antibodies and increased vector and transgene elimination in the presence of antibodies (8).

1.2.2 Cellular immunity to adeno-associated virus

One of the other limitations of using these vectors is a CD8+ cytotoxic T cell (CTL) response directed against the AAV capsid (26). The vector enters the host via serotype-specific cell surface receptors, it is taken into the cell and kept in vesicles. The low pH in the endosomes, helps to remove the capsid with the release of the transgene. The transgene is transported to the nucleus where it is expressed outside the chromosomes, the capsid proteins are degraded, and the transduced hepatocytes presents the peptides of the capsid via major histocompatibility complex class I molecules (MHC I). The MHC I presentation initiates an immune response against the capsid and the CTL destroys the transduced hepatocytes with loss of the transgene (27). See Figure 1.4. A transient and self-limiting rise in transaminases develops after this immune response, and this was initially observed using AAV2 (28), especially at higher doses where memory T and B lymphocytes are activated (29).

The AAV vector is manufactured from a virus that occurs naturally and which the participants are most likely to have been exposed to, as a result, has the potential to trigger a memory response when it is now introduced as a vector to the immune system (28). The immune response is dose-dependent and there is no evidence of persistent hepatocellular damage, despite the destruction of hepatocytes, the AAV vector is still considered safe for gene therapy (9).

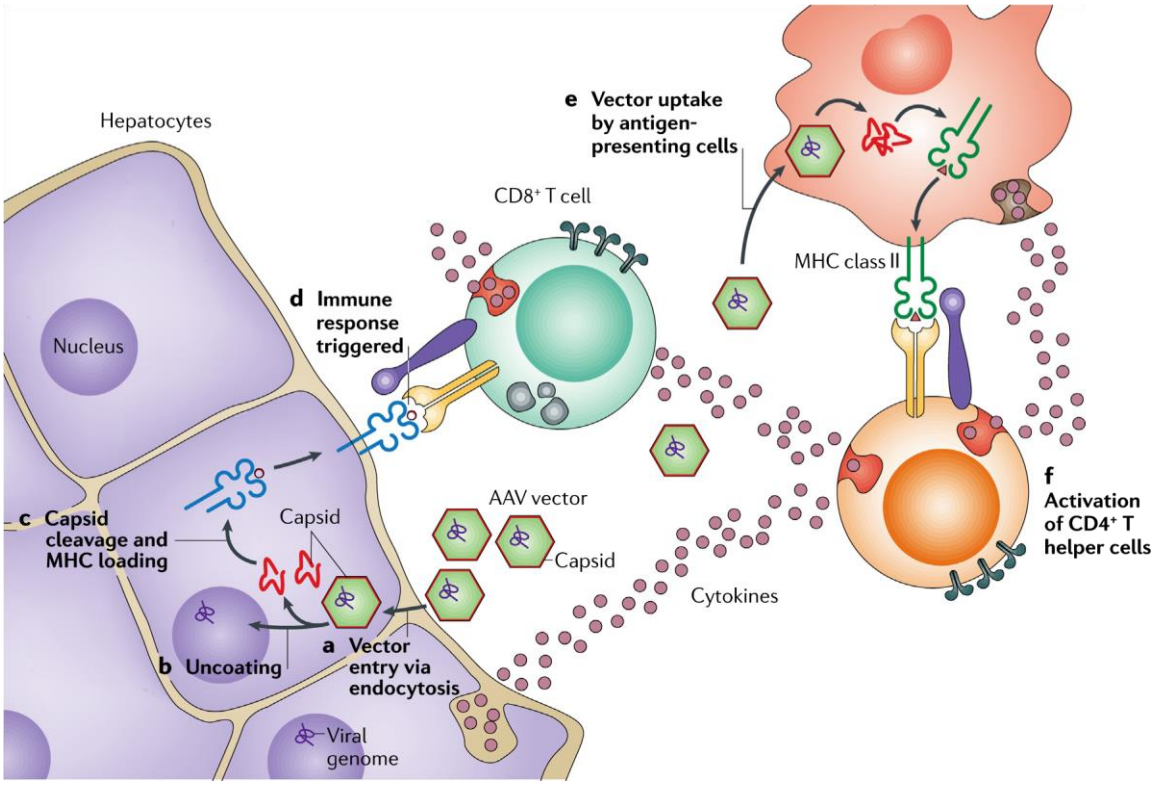

Figure 1.4 (a) shows the vector entering the cell via a surface receptor. (b) the low pH removes the capsid and releases the transgene. (c) the capsid is degraded, and the peptide presented via MHC I on transduced hepatocytes. (d) CD 8+ T cells initiate an immune response against the transduced hepatocytes. (e) APC's also take-up the vector and present the capsid to CD4+ T cells via MHC II. (f) Presentation to CD4+ T-cells results in cytokine release (30). APC, antigen-presenting cell.

1.3 Adeno-associated virus serotypes

The adeno-associated virus is divided into 6 clades, A to F, and this classification is based on whether there are any similarities in function. See Figure 1.5. Additionally, there are two AAV isolates, AAV4 and AAV5 that have significant differences compared to the other serotypes (21). The tissue tropism of each AAV serotype is determined by the difference in the capsid amino acid sequence, and the presence of a specific receptor on target cells (13). See Table 1.1. AAV5 is the most distinct serotype and shares only 58% capsid similarities with AAV2 and AAV8, whereas AAV2 shares 83% with AAV8 (21).

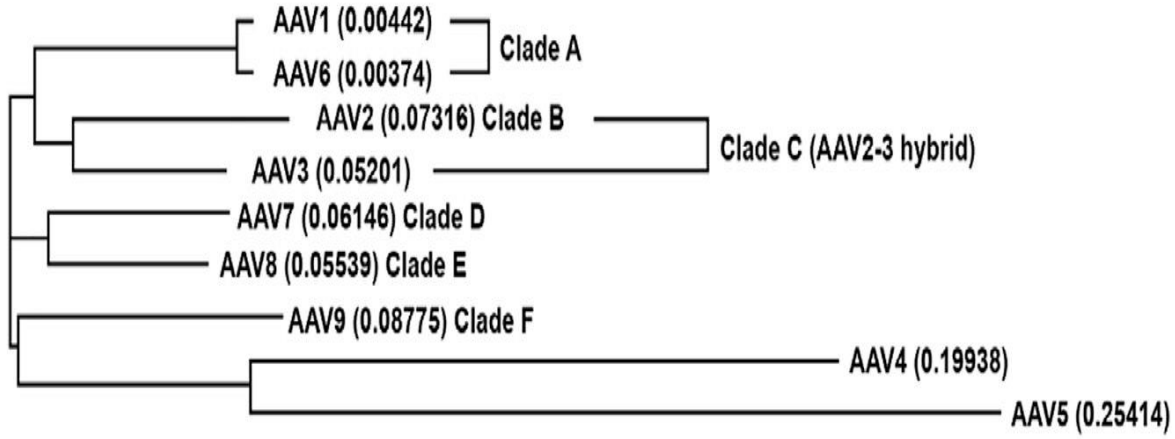

Figure 1.5. The phylogeny of AAV serotypes (21).

As the vector capsid is important in tissue tropism and efficient target tissue transduction. It is a major antigen and it's interaction with host immunity determines the vector's ability to access the target tissue (31).

Table 1.1: AAV serotypes and tissue tropism (13).

Serotype	**Source**	**Receptor**	**Coreceptor**	**Tissue tropism (relevant in haemophilia B)**
AAV1	NHP	N-linked sialic acid	Unknown	Skeletal muscle.
AAV2	Human	HSPG	PDGFR, HGFR Lam R	Liver & skeletal muscle.
AAV3	NHP	HSPG	PDGFR, HGFR, Lam R	Skeletal muscle.
AAV4	NHP	O-linked sialic acid	Unknown	Not relevant: CNS & retina.
AAV5	Human	N-linked sialic acid	PDGFR	Liver & skeletal muscle.
AAV6	Human	N-linked sialic acid, HSPG	EGFR	Skeletal muscle.
AAV7	NHP	Unknown	Unknown	Skeletal muscle.
AAV8	NHP	Unknown	Lam R	Liver & skeletal muscle.
AAV9	NHP	N-linked galactose	Lam R	Liver & skeletal muscle.

AAV-adeno-associated virus; NHP- Non-human primate; CNS-Central nervous system; HSPG- Heparan sulphate proteoglycan; PDGFR- Platelet derived growth factor receptor; HGFR- Hepatocyte growth factor receptor; EGFR- Epidermal growth factor receptor.

1.4 Adeno-associated virus vector

The recombinant AAV vector is engineered from the wild type virus, the viral genome is replaced with the transgene of choice (FIX or variant), retaining only the pair of ITR's to package the vector genome (27). The removal of the viral coding regions ensures that no viral proteins can be synthesized (8). See Figure 1.6. Pseudo-typed recombinant vectors can be created by switching capsid sequences from different serotypes, allowing the generation of an unlimited number of new vectors (31).

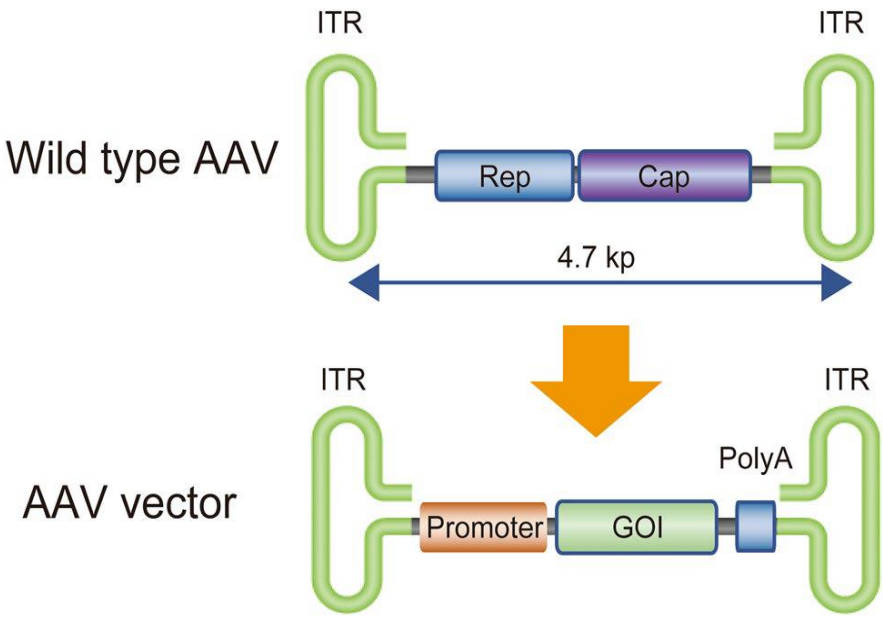

Figure 1.6: Wild type AAV and recombinant vector. The rep and cap genes code for the structural proteins of the virus. These genes are removed in the production of a vector and are replaced by the transgene of interest. (32). ITR, inverted terminal repeat; AAV, adeno-associated virus; PolyA, polyadenylation tail; GOI, gene of interest.

Vector modification to improve transgene expression include the development of self-complementary AAV vectors (scAAV) (33). The scAAV vector has two complementary copies of factor IX cDNA in the gene expression cassette and has been shown to be more efficient in gene transfer than single-stranded AAV vector (ssAAV). The latter has to be converted to a double-strand template first before DNA transcription (33). On the other hand, scAAV vector contains the coding and the complementary sequence within a single virus. DNA can therefore easily form dsDNA that is ready for transcription in the cell nucleus once released from the vector. This change can potentially result in lower and safer doses of the vector to be used in haemophilia B gene therapy (9). The disadvantage in using this type of AAV vector is the even smaller packaging capacity of ~ 2.3 kb that only accommodates FIX cDNA (1.4 kb) and its use is currently limited to haemophilia B gene therapy (7).

1.5 Immunological tolerance to the transgene product

In gene therapy directed at the liver, the development of antibodies against the transgene product is not a problem compared to other sites. Studies have shown that expression of the antigen in hepatocytes promotes antigen-specific immunological tolerance. This is because Kupffer cells, which are the resident macrophages in the liver and dendritic cells, have an immature phenotype in comparison to APC's in the periphery. This results in poor activation of T cells; additionally, Kupffer cells can secrete interleukin 10 (IL-10) which is an anti-inflammatory cytokine (13). Liver sinusoidal endothelial cells (LSEC) are also able to act as APC's, presenting antigens via MHC class II and this appears to be important in the induction of T-regs. This immunological tolerance in the liver has been shown to be dose-dependent, and high levels of antigen presentation by hepatocytes through MHC class I have been associated with an efficient way of causing CD8+ T cell exhaustion and apoptosis (13). See Figure 1.7.

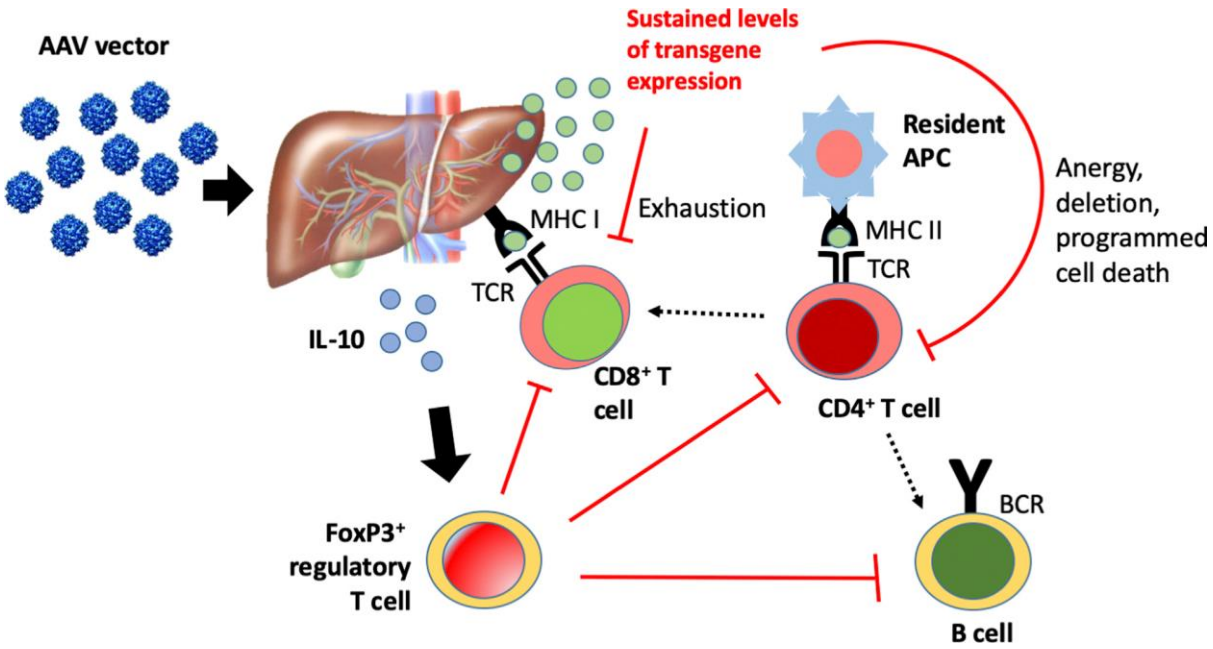

Figure 1.7: Liver induced immune tolerance against the transgene is possible due to secretion of IL-10, induction of regulatory T-cells (Tregs) and immature antigen-presenting cells with poor activation of T-cells (13). TCR, T-cell receptor; BCR, B-cell receptor; MCH1, major histocompatibility class 1; MHC 2, major histocompatibility 2; Il-10, interleukin 10.

1.6. Production of the vector

The recombinant adeno-associated virus is commonly produced in mammalian cells, usually HEK293T (human embryonic kidney) cells by triple transfection. This process involves co-transfecting cells with three plasmids that contain the transgene of interest, a packaging plasmid with AAV rep and cap genes and a plasmid encoding for the adenovirus helper genes (34). See Figure 1.8. The adenovirus enhances the expression of AAV genes and improves vector yield, as AAV requires a helper virus to replicate (14). The transfection process is the limiting step in AAV vector production and the inability to scale up vector production using these methods restricts the commercialisation of AAV vectors (35). However, rAAV vector production using baculovirus vectors and insect cell lines can achieve this goal (34).

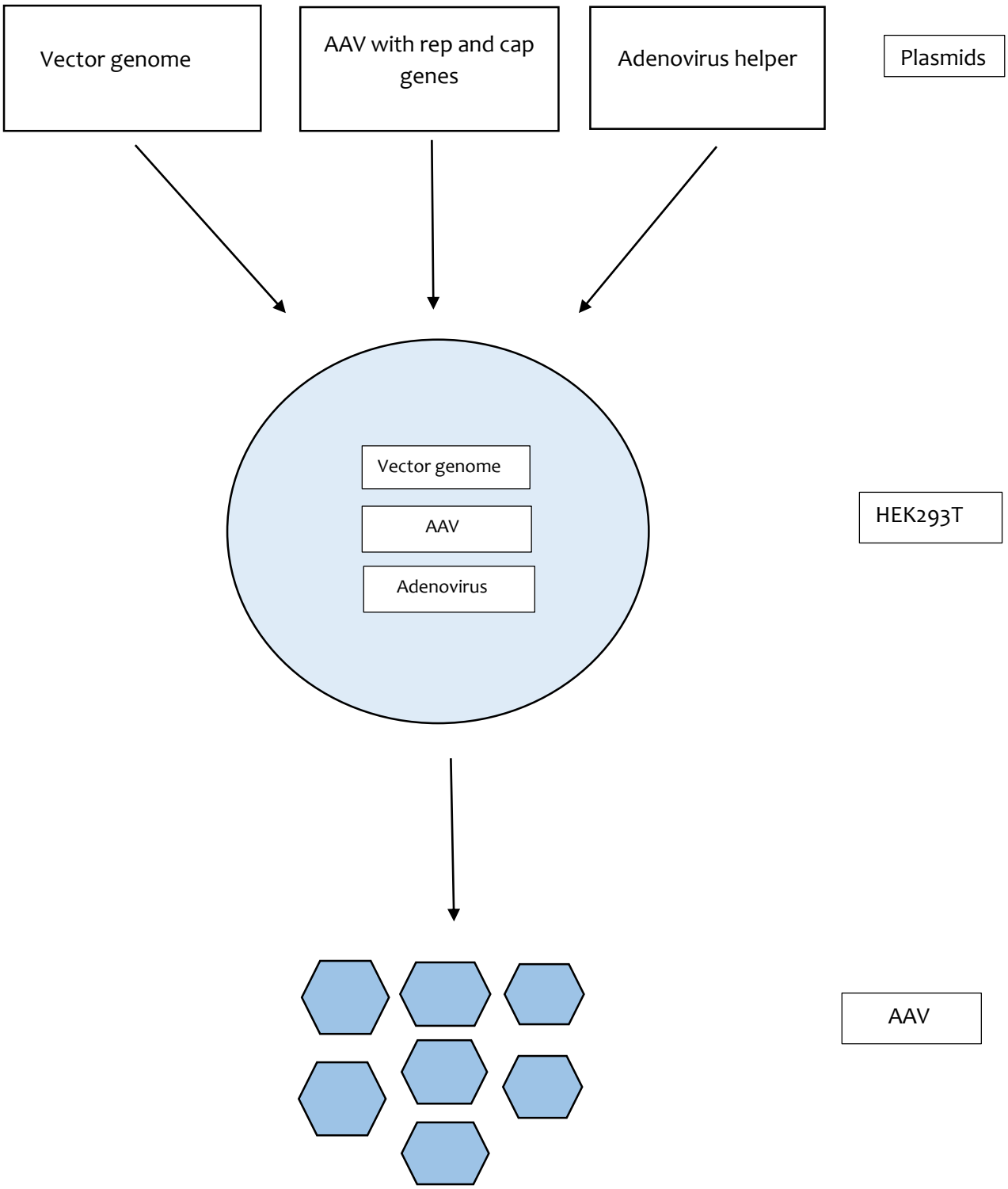

Figure 1.8. A schematic representation of the triple transfection process (34).

Baculovirus-based methods have become an alternative to conventional methods using HEK293T or HeLa (human cervical carcinoma) cells and provide a simpler and more cost-effective way of producing AAV vector (35). This system involves the use of Spodoptera frugiperda Sf9 insect cell lines that are co-infected with baculovirus expression vectors (BEV). The latter encode for the gene of interest flanked by ITR's, rep and cap genes (34). The vector produced in insect cell lines has been shown to have physical and biochemical properties similar to those of a vector that is produced in mammalian cells (35).

1.7 How the vector works

The AAV viral capsid binds to the surface of the target cell and the main receptor for AAV2 is heparan sulphate proteoglycan, there is interaction with a co-receptor to allow for internalisation of the virus. There are different binding receptors for the different serotypes of AAV. The virus is then internalised into the cell by endocytosis and is processed within the endosome (15). The processing is performed by endosomal cysteine proteases, namely cathepsins B and L which cleave serotype-specific AAV capsid peptides. The virus then travels along microtubules and microfilaments towards the nucleus with uncoating of the virus. The free AAV genome in the nucleus undergoes second strand synthesis and forms concatemers which are expressed episomally (15). The difference between the wild type virus and the AAV vector is that the genome of the latter does not undergo site-specific integration into the DNA of the host (14).

1.8 Haemophilia B gene therapy

Haemophilia B gene therapy has been in development for more than three decades (8). One of the earlier studies, the first trial in humans using parenteral AAV vector administration, involved the injection of AAV2 into muscles of participants with severe haemophilia B (36). The vector was injected at multiple sites, and gene transfer was confirmed by Southern blot performed on muscle biopsies at various time points after vector administration. Immunohistochemical stains were also performed on the biopsies

to confirm transgene expression. Although both gene transfer and transgene expression were successful locally in the muscle, the factor IX activity in the blood remained below 2%. This study however, showed that gene therapy was safe in humans at the doses that they used (36).

Subsequent studies showed that hepatocytes could be successfully transduced by parenteral administration of rAAV2. The vector was infused through the hepatic artery and therapeutic FIX activity was achieved in the higher dose group. However, this response was not sustained, with FIX levels decreasing a few weeks after vector administration (37). The drop in FIX activity was associated with an asymptomatic transaminitis, and this was hypothesised to be as a result of the destruction of the successfully transduced hepatocytes by cell-mediated immunity against antigens of the AAV capsid. This finding was not shown in pre-clinical studies and prompted the use of immunosuppressive drugs such as prednisolone in future studies (10).

The more recent trials have led to sustained increases in FIX levels with a reduction in exogenous factor usage and annual bleeding rate (38). The first successful human trial achieved this by using AAV8 with a self-complementary AAV vector and a codon-optimised F9 transgene (10), it has been more than nine years since this trial and the participants demonstrate stable FIX protein expression (39). AAV5 has also been used in combination with a liver-specific promoter and codon-optimised F9, and the highest dose group had a mean FIX activity of 6.9 IU/dL (38).

Other trials used the hyperactive FIX variant (Padua) that has an 8-fold increase in specific activity, in combination with various AAV serotypes, and the results have been impressive with higher FIX levels than in the previously mentioned studies (40). The vectors used in these trials have included AAV5 and an AAV vector with a bioengineered capsid with mean FIX levels of 31 IU/dL (41) and 33.7 IU/dL, respectively (40). See Table 1.2 for current haemophilia B gene therapy trials.

Despite the success of haemophilia B gene therapy trials, there is a common limitation to the widespread application of this therapeutic approach, and that is the presence of pre-existing NAbs to AAV (42). As a result, anti-AAV NAbs remain an exclusion criterion in the majority of haemophilia B gene therapy trials (32), as they are associated with a reduction in the efficacy of AAV mediated gene therapy (22).

Table 1.2: Summary of the haemophilia B gene therapy trials (43).

Sponsor	Vector	Transgene	Manufacturing cell line
St. Jude Children's Research Hospital/UCL*	AAV8	FIX Wild type (WT); codon-optimised	Plasmid HEK293 cell line#
Pfizer Spark Therapeutics	Spk-100 (AAV8-like)	FIX Padua; codon-optimised	Plasmid HEK293 cell line
The Shire, Takeda (Baxalta)	AAV8	FIX Padua; codon- optimised	Plasmid HEK293 cell line
UniQure Biopharma/Chiesi	AAV5	FIX WT; codon-optimised	Baculovirus Insect cell line
UniQure Biopharma/Chiesi	AAV5	FIX Padua; codon-optimised	Baculovirus Insect cell line
UniQure Biopharma/Chiesi	AAV5	FIX Padua; codon-optimised	Baculovirus Insect cell line
Dimension Therapeutics	AAVrh10	FIX WT; codon-optimized	Plasmid HEK293 cell line
UCL Royal Free Hospital- Freeline Therapeutics	AAV-LK03†	FI X Padua; codon-optimised	Plasmid HEK293 cell line
Sangamo Bioscience	AAV2/6	‡ ZN- nuclease integration of WT FIX into the albumin locus of hepatocytes.	Baculovirus Insect cell line

* University College London; # Human embryonic kidney cell line; † Synthetic liver-tropic capsid ‡Zinc finger.

1.9 Assays used in the detection of anti-AAV antibodies

The humoral immune response to natural infection or AAV vector includes the development of both binding and neutralising antibodies (22). The role of binding (non-neutralising) antibodies is unclear, but some studies suggest that these are involved in the clearance of the AAV vector by opsonisation via the Fc receptors (24). Several methods have been used to investigate anti-AAV antibodies, and earlier studies used assays that detected total binding antibodies to AAV capsid such as enzyme-linked immunosorbent assay (ELISA). However, the move recently has been on detecting serotype-specific NAbs using transduction inhibition (TI) assays (22). Transduction inhibition assays include cell-based in vitro or in vivo assays using for example mice, and the use of both screening assays (ELISA and TI) to detect total antibodies and NAbs is seen in some trials (44).

In transduction inhibition assays, the cell-based in vitro assay is more commonly used and involves a reporter AAV vector that is incubated with serially diluted test samples prior in vitro transduction of a cell line (45). The use of green fluorescent protein (GFP) reporter system was previously common and now more sensitive and high-throughput methods containing the luciferase reporter gene are used, together with a CMV promoter (44). After incubation of the vector, test samples and a cell line of choice, luminescence is measured to determine the titre of each sample. If there are NAbs present, it will cause a decrease in luminescence compared to the control (44). The neutralising titre is the dilution at which inhibition of transduction is 50%. Some of the issues raised in the literature is the non-standardisation of the assays used to measure NAbs, with different sensitivities and the different definitions of a seropositive sample (16). Other factors which contribute to the variability of the assay include the cell line used and how the reporter vector is prepared (45).

1.10 Neutralising antibodies

The prevalence of anti-AAV NAbs varies with the geographical area of the population studied and the seroprevalence of AAV2 in one study ranged from 30% in the United States to 60% in Africa. There was a higher frequency of NAbs to all AAV serotypes in samples from Africa (22). The reason for these findings is unclear as no demographic information was collected on the participants of this particular study (46). The prevalence of anti-AAV8 NAb is between 20% and 30%, but a prevalence of 94% has previously been reported. AAV5 prevalence reported in the literature ranges from 4% to 50% and is the serotype with the lowest prevalence of NAbs (47).

AAV2, the most commonly studied serotype with the highest prevalence across geographical locations, was previously widely used in gene therapy until it was demonstrated in clinical trials that even low levels of NAb titres can reduce efficacy (47). The currently preferred serotypes are AAV5 and AAV8, owing to the lower seroprevalence of NAbs against these serotypes (12). There is also the concept of cross-reactivity, and it has been previously shown that NAbs against serotypes with highly conserved capsid sequences such as AAV2 can recognise other serotypes with a similar capsid sequence. As a result, people often test positive for NAbs against several AAV serotypes (48). One study showed that in people with anti-AAV2 antibodies about 93%, 52%, 59%, 57% and 58% also had antibodies against AAV1, 5, 6, 8 and 9 with cross-reactivity of more than 50% for all serotypes (16).

There is new evidence to suggest that anti-AAV5 NAbs may not have the same clinical significance as that of anti-AAV2 or anti-AAV8. This is based on a study which showed successful factor IX transgene expression in three haemophilia B participants, that were proven retrospectively to have had pre-existing anti-AAV5 NAbs using a more sensitive luciferase-based assay (48). The titres for the initial study were measured using green fluorescent protein-based assays. On re-testing their samples these three participants now had titres of 21, 210 and 340, and the participants with titres of 210 and 340 had tested positive in the initial study for anti-AAV5 Immunoglobulin G (IgG) using an ELISA.

There was no relationship between the presence of NAbs and the efficacy of the vector, with the three participants that had titres of 21, 210 and 340 achieving factor activities of 3 IU/dL, 1.3 IU/dL and 6.8 IU/dL, respectively (48). These unexpected results are postulated to be due to the unique structure of the AAV5 capsid, as the AAV5 serotype has one of the least conserved capsid sequences and is, therefore, less likely to be cross neutralised by anti-AAV antibodies directed against other serotypes (48). Based on this advantage, Giermasz et al. (41) reported a new trial using AAV5 serotype and FIX Padua, where participants with anti-AAV5 NAbs were not excluded from the study and the results have been impressive.

The current study aimed to determine the prevalence of anti-AAV NAbs in people with haemophilia B in our setting, as most of the AAV seroprevalence studies mentioned above were done in people without haemophilia and outside South Africa. Calcedo and Wilson (22) have shown a higher prevalence in developing countries, but the reasons for their findings are largely unknown. We hope to identify the risk factors in our haemophilia B population.

CHAPTER 2

MATERIALS AND METHODS

2.1 Study design

This is a cross-sectional controlled study.

2.2 Study setting and ethics

This study was conducted at the Haemophilia Comprehensive Care Centre (HCCC) at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH). It was approved by the University of the Witwatersrand, Human Research Ethics Committee (HREC) (see appendix E) and the study was conducted according to the Declaration of Helsinki (49).

Each study participant or their legal guardian gave written informed consent, with assent where appropriate, to participate in the study.

2.3 Study Participants

The study population consists of documented haemophilia B participants attending the HCCC at CMJAH and were recruited from the HCCC patient database. The inclusion criteria were participants with haemophilia B of any severity, all races, and all age groups with a confirmed Haemophilia B diagnosis. Participants with other bleeding diatheses were excluded. Informed consent and assent where appropriate, was obtained from 44 males between the ages of one and 62 years. In addition, forty-four control participants that did not have haemophilia B were also recruited, and informed consent was obtained from 44 adult males and females.

2.4. Blood sample collection

Five millilitres (5 mL) of whole blood was collected from each participant, into a serum separator tube (yellow top) using a Vacutainer system (50). The clotted sample tube was centrifuged at 10 000 rpm at room temperature for 10 minutes and the serum aliquoted into cryovials of 1 mL each and stored in the -80 °C freezer before analysis. The samples were processed and stored at the Bleeding Disorders Research Unit at Charlotte Maxeke Johannesburg Academic Hospital.

2.5. Neutralising antibody assays

Each test sample was thawed in a water bath at 37 °C prior to processing. The lowest test serum dilution used in every assay was one in eight and samples were considered positive when the calculated anti-AAV NAb titre was ≥1 in 8. All analytical runs included appropriate positive and negative controls. (See Table 2.1).

The AAV antibody analysis was performed in collaboration with UniQure in Amsterdam, the Netherlands. The samples were shipped from Johannesburg, South Africa at -80 °C to Amsterdam where they were analysed.

Table 2.1. Protocol for the luciferase-based NAb assay.

Day 1	Cell preparation	Prepare two 96-well flat-bottom culture plates. (one black and one transparent) - 100 µL of HEK293T cells are added per well. - Incubate overnight at 37 °C in 5% CO_2 water jacket incubator.
Day 2	Preparation of serial serum dilutions and antibody controls on transparent96-well plates.	- 140 µL of a medium is added to the wells that will serve as negative controls and 70 µL are added to the wells that will contain positive controls as well as to those where test samples will be added. - Serum samples are added in triplicates except on the wells containing negative control samples. This constitutes the first dilution. - Subsequently, serial dilutions of serum are performed across the plate by transferring 70 µL from the first row with the test samples to the second one and from the second to the third until the last row. - The 70 µL pipetted from the last row is discarded. - Antibody control dilutions in the medium are prepared depending on the anti-AAV assay type i.e. AAV1, AAV2, AAV5, AAV6 and AAV8.
	Addition of *AAV-CMV-LUC to serial dilutions and antibody controls	- AAV-CMV-LUC is diluted in a medium. - 70 µL of the virus is added to the serum dilution plates except for the rows with the negative controls. - The plates are then placed onto a plate shaker for 2 minutes at 300 rpm. - The plates are then incubated for 1 hour at 1 °C.

	The mixture of serial serum dilutions and AAV-CMV-Luc is transferred onto HEK293T cells.	- The culture medium is removed from the black 96-well plates that were prepared on day 1. The serum dilutions are subsequently pipetted from the transparent plates onto the black ones that contain HEK293T cells. - These plates are incubated overnight at 37 °C in 5% CO_2 water jacket incubator.
Day 3	Cell viability read-out	This is done using the Celltiter-Fluor Cell Viability Assay. - The substrate and assay buffer are thawed in a 37 °C water bath. - These are mixed to create a reagent. - The reagent is then added to all experimental wells of the black plates and mixed by placing on the shaker for ~30 seconds. - The mixture is then incubated for at least 30 minutes at 37 °C in 5% CO_2 water jacket incubator. - The fluorescence is measured using a fluorometer.
	Luciferase activity read-out.	- 100 µL per well of reagent from ONE-Glo Luciferase Assay is added. The reagent is prepared by transferring the buffer into the bottle of the substrate, and this is mixed by inverting the bottle multiple times. - This is incubated for 3 minutes to allow for complete cell lysis. - The raw data is then analysed using Labkey software.

*****Adeno-associated virus-cytomegalovirus-luciferase

2.6 Statistical analysis

This was an exploratory study for which no power calculations were performed. The data was captured on data collection sheets and transcribed to an Excel spread sheet (Microsoft Office 2010, Redmond). Neutralising antibody frequencies were summarised by age, haemophilia phenotype and AAV serotype. Continuous variables which follow a statistically normal (Gaussian) distribution were expressed as means (SD) and categorical parameters as frequencies (%).

CHAPTER 3

RESULTS

3.1 Participants

From October 2018 until May 2019, a total of 44 haemophilia B male participants between the ages of 1 and 62 years (mean 26.7 +/- 17.6) were enrolled into the study (see Table 3.1). There are slightly more black participants (52.3%) in the cohort relative to other races. Most participants were HIV negative (93.2%) and had severe haemophilia disease (70.5%). Approximately 90.9% of the participants were on plasma-derived factor concentrates, and 72.7% were currently residing in Johannesburg.

In addition, 44 control participants that were not age or sex-matched were recruited. There were 34% males and 66% of females between the ages of 27 and 56 years (mean 36 +/-9.3).

Table 3.1. Demographic characteristics of haemophilia B participants.

Characteristic	n (%)
Age (years)	
1 - 18	21 (47.7)
>18	23 (52.3)
Gender (male)	44 (100.0)
Ethnicity	
Black	23 (52.3)
White	16 (36.4)
* Other	5 (11.3)
Disease severity	
Mild	5 (11.4)
Moderate	8 (18.2)
Severe	31 (70.5)
Type of therapy	
#rFVIIa (NovoSeven)	4 (9.1)
†pdFIX	40 (90.9)
HIV Status	
Negative	41 (93.2)
Positive	2 (4.5)
Unknown	1 (2.3)
Hepatitis C serology	
Negative	33 (75.0)
Positive	10 (22.7)
Unknown	1 (2.3)
Geographical Location	
Johannesburg	32 (72.7)
Other	12 (27.3)
Exposure to blood products	
Red cells	12 (27.3)
Fresh frozen plasma	11 (25.0)
Both	4 (9.10)
None	15 (34.1)
Unknown	2 (4.50)

* mixed race; # recombinant activated factor VII; † plasma-derived FIX

3.2 Prevalence of the different serotypes of AAV in haemophilia B participants.

The highest seroprevalence of NAbs is seen with AAV2 at 95%, with 39% of them having NAbs titres above 1030. The second most common serotype was AAV6 with a prevalence of 82%, however, compared to AAV2 only 9% of participants had titres above 1030, 36% had low titres of ≥8-50, and 30% had titres between 51-340. AAV1 had a prevalence of 77% and within this group, about 36% of participants had titres of 51-340, and 20% had the highest titres of >1030. AAV1 had the second most participants with titres above 1030, second to AAV2.

AAV5 had a prevalence of 66% with 27% of these having low titres (≥8-51), followed by 23% with titres between 51-340. AAV5 had the lowest percentage of participants in the highest titre range (>1030) at 5%. The lowest seroprevalence of NAbs was seen with AAV8 (64%), 32% of participants had low titres of ≥8-50, 18% between 51-340, 7% at 341-1030 and 7% more than 1030. AAV2 had the highest mean titre, followed by AAV1 (see Table 3.2 and Table 3.3).

Table 3.2: Prevalence of the different serotypes of AAV in haemophilia B participants.

		Titre range			
AAV serotypes	**Overall prevalence %**	**≥8-50**	**51-340**	**341-1030**	**>1030**
AAV1	77	5	36	16	20
AAV2	95	20	20	16	39
AAV5	66	27	23	11	5
AAV6	82	36	30	7	9
AAV8	64	32	18	7	7

Table 3.3: Neutralising antibody mean and median titres.

	AAV1	AAV2	AAV5	AAV6	AAV8
Positive n=	34	42	29	36	28
Median	268	494	82	55	56
Mean	1654	4156	208	407	777

3.3 Prevalence of AAV NAbs by age.

The prevalence of NAbs in haemophilia B for all serotypes, is lower in participants 18 years and younger compared to participants older than 18 years (see Table 3.4). It ranges from 25% for AAV8 to 45.5% for AAV 2, while in participants older than 18 years the frequency ranges from 38.6% (AAV5 & 8) to 50% for AAV2.

Table 3.4: The prevalence of anti-AAV NAbs by age (total n=44).

	AAV serotypes n (%)				
Age (years)	AAV 1	AAV2	AAV5	AAV6	AAV8
1 – 18	15 (34.1)	20 (45.5)	12 (27.3)	17 (38.6)	11 (25.0)
>18	19 (43.2)	22 (50.0)	17 (38.6)	19 (43.2)	17 (38.6)

3.4 Prevalence of anti-AAV NAbs by disease severity.

Approximately 70.5% of participants had severe haemophilia B, and these participants had a higher seroprevalence for all serotypes of AAV (see Table 3.5).

Table 3.5: Anti-AAV NAb frequency by disease severity.

	AAV serotypes n (%)				
Disease severity	AAV 1	AAV2	AAV5	AAV6	AAV8
Mild	5 (11.4)	5 (11.4)	5 (11.4)	5 (11.4)	4 (9.1)
Moderate	4 (9.1)	7 (15.9)	3 (6.8)	5 (11.4)	3 (6.8)
Severe	25 (56.8)	30 (68.2)	21 (47.7)	26 (59.1)	21 (47.7)

3.5 Participants with no NAbs (<8) or low titres (≤50)

4.5% participants (ages 9 and 38 years) had NAb titres less than 8 for all AAV serotypes. Six (14%) of the participants had anti AAV2 NAbs titres of 50 or less, these participants were either negative or had titres ≤50 for other serotypes. Approximately 18% (n=8) of all participants had titres ≤50 (lowest range), and most (63%) were 18 years or younger.

3.6 Anti-AAV NAb co-prevalence in haemophilia B

Six (14%) participants had positive NAbs for a single serotype, and this was AAV2 for all participants, 2 (4.5%) had double positivity and these were AAV 2 and AAV6 for both participants. Three (7%) participants had triple positivity for AAV1, AAV2 and AAV6. 5 participants were positive for four serotypes, and these were AAV1, AAV2, AAV6 and AAV8 in 2 (4.5%) participants and AAV1, AAV2, AAV5 and AAV6 in 3 (7%) participants. Fifty-nine per cent tested positive for all AAV serotypes. AAV2 was common to all participants (see Table 3.6).

Table 3.6: The co-prevalence of anti-AAV NAbs in haemophilia B.

No. of positive AAV serotypes	AAV serotype	n (%) of participants
1	AAV2	6 (14.0)
2	AAV2 & AAV6	2 (4.5)
3	AAV1, AAV2 & AAV6	3 (7.0)
4	AAV1, AAV2, AAV6 & AAV8	2 (4.5)
	AAV1, AAV2, AAV5 & AAV 6	3 (7.0)
5	AAV1, AAV2, AAV5, AAV6 & AAV8	26 (59.0)

3.7 Prevalence of the different serotypes of AAV in control participants

All the controls had NAbs against AAV2. About 29.5% had titres between 51-340 and >1030, 14% ≥8-50 and 27% had titres of 341-1030. AAV6 had the second highest prevalence at 80% with frequencies of 36%, 23%,16% and 5% for the titre ranges ≥8-50, 51-340, 341-1030 and >1030, respectively. AAV1 had a prevalence of 75%, and 27% of the participants had titres between 51-340, 14% ≥8-50, 16% 341-1030 and 18% >1030. The overall prevalence of AAV8 was lower at 64% with only a small percentage (2%) of patients with very high titres above 1030. AAV5 had the lowest seroprevalence at 61% with 27% having low titres of ≥8-50, 16% between 51-340, 11% 341-1030 and 7% above 1030 (see Table 3.7).

Table 3.7: Prevalence of the different serotypes of AAV in control participants (n=44).

		Titre range			
AAV serotypes	**Overall prevalence %**	**≥8-50**	**51-340**	**341-1030**	**>1030**
AAV1	75	14	27	16	18
AAV2	100	14	29.5	27	29.5
AAV5	61	27	16	11	7
AAV6	80	36	23	16	5
AAV8	64	21	18	23	2

CHAPTER 4

DISCUSSION

4.1 General discussion

NAbs against AAV that have developed following infection with the wild type virus are currently a major limitation to widespread applicability of haemophilia B gene therapy (16). The majority of clinical trials exclude participants with NAbs due to the reduced efficacy associated with the presence of these antibodies, except for a recent trial with an AAV5 vector and FIX Padua which has shown successful transgene expression despite the presence of anti-AAV5 NAbs (48).

This is a major challenge to AAV based gene therapy as most people are exposed to the virus in childhood and would have developed antibodies. It has been shown that the prevalence of NAbs increases with age, an alternative would be to identify the age at which these antibodies are the lowest, as this would be the ideal period for gene therapy (22). However, gene therapy for haemophilia B is currently not available to the paediatric population as there are concerns that transgene expression may be lost in the growing liver, since the AAV vector is expressed episomally and does not integrate into the host genome (15).

The other factor that further reduces the pool of participants eligible for gene therapy is that the most common serotype, AAV 2 cross-reacts with other AAV serotypes. It is, therefore, more common for a participant to have antibodies against several AAV serotypes (48). It is well known in the literature that the prevalence varies with the geographical location of the population studied and Africa has been shown to have a high prevalence and the reason for these findings are unclear (46). What further makes it difficult to extrapolate findings from previous studies is that the participants include healthy donors and in other studies participants that have haemophilia B. Additionally, except for AAV2, not all participants develop Anti-AAV Nabs and maybe there are risk factors associated with the development of these antibodies.

Our study aimed to determine the prevalence of anti AAV NAbs against several serotypes in the South African setting in people with haemophilia B. The trend in the prevalence of anti-AAV NAbs in this study is similar to that reported in the literature, with the highest prevalence (95%) seen with AAV2 across all age groups in participants with haemophilia B and 100% in healthy volunteers. A lower seroprevalence was seen with AAV5 and AAV8 with frequencies of 66% and 64%, respectively, and participants in these groups had low titres, similar to findings reported in healthy participants (51). However, although our study showed a lower seroprevalence against these two serotypes, this is still higher than the reported prevalence in other areas. A recent study in an adult United Kingdom (UK) cohort of haemophilia A participants showed a prevalence of 25% and 38% for AAV5 and AAV8, respectively (25).

4.2 AAV1

The seroprevalence of AAV1 in haemophilia B participants was 77%, higher than that observed in studies in China which showed frequencies of 69.8% in healthy donors (52), Japanese haemophilia participants (39.7%) and in an international cohort of healthy donors (27%) (47). The control participants had a prevalence of 75%, similar to that seen in haemophilia B. Our study showed a similar prevalence between controls and haemophilia B participants, which is overall higher than reported in the literature. Twenty-three per cent of our haemophilia B population would be eligible for gene therapy using this serotype.

4.3 AAV2

Ninety-five per cent of haemophilia B and 100% control participants had NAbs against AAV2 serotype. Approximately 39% of haemophilia B and 29.5% control participants had titres above 1030. The seroprevalence of AAV2 was previously shown to range from 30% to 60%, but a prevalence of 100% has also been published (16). Our study shows similar findings to those previously reported and the participants in our study have high AAV2

NAb titres. The high prevalence of antibodies against this serotype eliminates its use in gene therapy.

4.4 AAV5

The prevalence of AAV5 in the haemophilia B population is 66% with 27% of participants having lower titres at ≥8-50. This is in keeping with the trend in the literature showing a lower seroprevalence compared to AAV1 and AAV2. The control participants had a prevalence of 61% with the same percentage of participants as those with haemophilia B (27%) falling in the lowest titre range. This prevalence is similar to the global trend as it is lower compared to that of AAV2 and AAV1. However, it is higher than the reported values of 40% (47) in healthy donors, 28% in haemophilia A participants (25) and other studies reporting a prevalence of 50% (16).

These findings suggest that AAV5 might be the preferred serotype for haemophilia B gene therapy in our setting. Considering the new research in AAV5 haemophilia B gene therapy, about 47% of participants with NAb titres less than 340 would be eligible for gene therapy, compared to 34% if participants with NAbs were to be excluded. The titre of 340 is the highest titre that was observed in a recent study, associated with successful transgene expression, and subsequently led to the non-exclusion of participants with Anti-AAV5 NAbs (48).

4.5 AAV6

The overall seroprevalence of AAV6 in the haemophilia B population was 82% with 66% of participants having titres of ≤340. The control participants had a prevalence of 80% and 59% had titres of ≤340. AAV 6 was the second most common serotype in our study. In healthy donors, a prevalence of 37% has been reported (51). Our study shows an overall high prevalence, and only 18% of haemophilia B participants would be eligible for gene therapy with this serotype.

4.6 AAV8

Sixty-four per cent of haemophilia B participants had NAbs against AAV8 with 32% having titres between ≥8-50. The prevalence was the same for control participants, and 21% had titres of ≥8-50. The published prevalence ranges from 14% to 50% (16). About 36% of our haemophilia B population would qualify for gene therapy using this serotype. AAV 8 had the lowest prevalence in haemophilia B participants and the second-lowest in healthy volunteers.

4.7 Co-prevalence of AAV NAbs

Neutralising antibodies against AAV2, AAV1 and AAV6 may not be as significant in gene therapy for haemophilia B, as the most commonly used vectors are AAV5 and AAV8 (47). The relevance of AAV2 NAbs is with cross-reactivity with other serotypes, which has been shown in multiple studies. In our study, 4.5% of the haemophilia B participants that did not have AAV2 NAbs were also negative for other serotypes and approximately 14% that had low titres of AAV2 NAbs were either negative for other serotypes or had low tires (≤50).

This is similar to a study performed in paediatric patients with haemophilia that showed that NAbs against AAV5 or AAV8 were rarely seen if there were no anti-AAV2 NAbs (53). The authors postulated that NAbs to AAV5 and AAV8 were present following AAV2 exposure due to cross-reacting AAV 2 directed NAbs. The high prevalence of more than one anti-AAV NAb serotype may make switching between the serotypes for haemophilia B gene therapy difficult (47). About 59% of haemophilia B participants in this study were positive for all serotypes and in the participants with seropositivity for 1,2,3 or 4 serotypes, AAV2 was common to all groups. This is higher than a co-prevalence of AAV2, AAV5 and AAV8, which was reported in a recent study to be 40% (54).

4.8 AAV seroprevalence and age

The prevalence of NAbs against all serotypes was lower in haemophilia B participants 18 years or younger, compared to those above this age group. This finding may suggest an increase in the prevalence with increasing age, which has been previously described (19). These findings may also suggest that the target age for gene therapy should be participants 18 years or younger to benefit the most people. However, there are challenges in the paediatric population which currently limits gene therapy use.

4.9 AAV seroprevalence and haemophilia B disease severity

A higher prevalence was seen in those participants with severe haemophilia disease for all serotypes, in comparison with mild or moderate disease. The reasons for this finding are unclear, of note most (70.5%) of the participants in the study had severe disease.

4.10 Study strengths

The study was able to demonstrate that there was a similar prevalence for the different types of AAV serotypes in haemophilia B and control participants. The availability of the participant's demographic characteristics, including age, disease severity and information on therapy is a major advantage and assists in establishing associations. It is the first study of its kind in South Africa that we are aware of and can serve as the basis for more studies and not just in haemophilia B but for other conditions where AAV based gene therapy is applicable.

4.11 Study limitations

The limitations of the study include small sample size and a selection bias as most participants with haemophilia B had severe disease. The initial estimated sample size was 100 participants. However, we found that the participants with the milder disease were less likely to come to the HCCC for a check-up and were lost to follow-up.

The study was not able to identify the risk factors associated with the development of NAbs. Age and severe haemophilia B disease may be risk factors, but there are possible confounding factors such as the HLA-type (Human leukocyte antigen), the underlying genetic mutation, exposure to blood products and other infections such as hepatitis C which were not explored.

We also wanted to explore the association between the prevalence and the different geographical locations of the participants. This was not possible as many of the participants in the study resided in Johannesburg.

One of the limitations included the retrospective nature of the study, the period at which NAbs are most likely to develop and the possible exposures around that time could therefore not be established.

It was a single centre study with many of the participants residing in Johannesburg, and it may, therefore, be difficult to extrapolate these findings to other parts of the country with different exposures.

The controls used in this study were not age or sex-matched.

4.12 Future studies

Further studies are required in participants with haemophilia to confirm these finding in a larger study especially, in the more prevalent haemophilia A where it might be easier to get these numbers. Studies in other parts of the country are also needed to see if similar findings are seen.

HLA typing of haemophilia B participants is needed to establish if certain types are at risk of developing NAbs.

The higher prevalence observed in severe haemophilia B disease needs to be studied further to determine whether it is related to exposure to plasma-derived products or the presence of other infections such as hepatitis C.

CHAPTER 5

CONCLUSION

In our study, there is a higher seroprevalence of NAbs for all AAV serotypes in comparison to studies done in both healthy participants and people with haemophilia. The difference in the prevalence may be due to different locations. However, our prevalence was still higher than that shown in a previous study in samples from Cape Town, South Africa (46). The participants in this study were healthy donors, whereas our study involved participants with haemophilia B which may have possibly resulted in different outcomes. Although this is unlikely, as the current study showed a similar prevalence in both haemophilia B participants and healthy volunteers.

AAV2 is the most common serotype in the South African haemophilia B population with possible cross-reactivity with other serotypes. AAV5 and AAV8 will be a more suitable serotype for haemophilia B gene therapy as a result of the lower seroprevalence observed, although still higher than what is described in the literature. The reasons for the higher prevalence are not clear, and further studies involving a larger sample size are required. This study suggests that disease severity may be contributing and whether exposure to plasma-derived products play any role will require further investigation.

APPENDIX A: Neutralising antibody titres of Haemophilia B (HB) participants

Sample number	AAV1	AAV2	AAV5	AAV6	AAV8
South Africa HB 001	68	92	< 8	65	< 8
South Africa HB 002	253	324	121	63	24
South Africa HB 003	1087	1787	509	1015	449
South Africa HB 004	68	238	45	31	12
South Africa HB 005	< 8	31	< 8	< 8	< 8
South Africa HB 006	< 8	16	< 8	< 8	< 8
South Africa HB 007	4549	2761	1060	1155	1149
South Africa HB 008	165	2248	19	34	45
South Africa HB 009	549	2462	223	160	182
South Africa HB 010	177	372	80	42	41
South Africa HB 011	4750	12288	141	1977	430
South Africa HB 012	< 8	36	< 8	< 8	< 8
South Africa HB 013	267	1347	84	49	266
South Africa HB 014	122	263	< 8	43	11
South Africa HB 015	375	874	82	57	49
South Africa HB 016	11	44	< 8	8	< 8
South Africa HB 017	< 8	< 8	< 8	< 8	< 8
South Africa HB 018	< 8	25	< 8	< 8	< 8
South Africa HB 019	2093	2949	352	262	451
South Africa HB 020	53	78	< 8	14	< 8
South Africa HB 021	429	990	140	126	63
South Africa HB 022	5931	28418	510	1651	2518
South Africa HB 023	83	488	< 8	36	9
South Africa HB 024	106	3322	88	55	40
South Africa HB 025	269	315	33	40	< 8
South Africa HB 026	2679	20541	511	477	244
South Africa HB 027	< 8	27	< 8	< 8	< 8
South Africa HB 028	< 8	21	< 8	8	< 8
South Africa HB 029	27235	76647	1060	5634	15200
South Africa HB 030	512	752	31	54	28
South Africa HB 031	408	1499	9	707	65
South Africa HB 032	1187	3806	412	242	76
South Africa HB 033	1044	1403	266	201	237
South Africa HB 034	< 8	50	< 8	16	< 8
South Africa HB 035	< 8	< 8	< 8	< 8	< 8
South Africa HB 036	117	207	21	53	15
South Africa HB 037	416	2912	35	184	23
South Africa HB 038	39	61	23	23	< 8
South Africa HB 039	< 8	33	< 8	< 8	< 8
South Africa HB 040	252	2377	35	30	31
South Africa HB 041	144	500	31	22	18
South Africa HB 042	126	414	8	17	< 8
South Africa HB 043	207	194	43	26	20
South Africa HB 044	465	1336	57	79	66

APPENDIX B: Neutralising antibody titres of control participants

Sample number	AAV1	AAV2	AAV5(160)	AAV8	AAV6
South Africa 001	249	3976	28	74	62
South Africa 002	1739	3530	275	489	628
South Africa 003	5011	8787	1134	862	1177
South Africa 004	1970	2710	745	654	433
South Africa 005	<8	42	<8	<8	<8
South Africa 006	104	5039	18	<8	25
South Africa 007	31	314	<8	15	16
South Africa 008	<8	92	<8	<8	<8
South Africa 009	<8	36	<8	<8	<8
South Africa 010	<8	86	<8	<8	9
South Africa 011	362	848	14	46	95
South Africa 012	14	77	<8	<8	17
South Africa 013	550	617	212	353	204
South Africa 014	127	156	10	<8	27
South Africa 015	<8	56	<8	<8	<8
South Africa 016	558	336	249	231	270
South Africa 017	967	1274	174	506	390
South Africa 018	246	575	64	93	133
South Africa 019	277	409	46	81	146
South Africa 020	93	117	78	102	34
South Africa 021	1818	3786	363	682	439
South Africa 022	634	603	707	841	191
South Africa 023	26	101	<8	20	25
South Africa 024	<8	22	<8	8	<8
South Africa 025	85	653	33	12	32
South Africa 026	35	227	<8	<8	12
South Africa 027	83	265	30	97	25
South Africa 028	163	722	<8	11	44
South Africa 029	<8	799	9	<8	13
South Africa 030	404	1796	34	58	124
South Africa 031	26	1026	<8	<8	16
South Africa 032	<8	34	<8	15	<8
South Africa 033	1953	2219	515	349	368
South Africa 034	13	73	<8	<8	16
South Africa 035	8181	14848	1507	1397	1217
South Africa 036	<8	46	<8	<8	<8
South Africa 037	3860	12602	776	474	364
South Africa 038	<8	44	<8	<8	<8
South Africa 039	3388	3169	2510	629	803
South Africa 040	<8	78	<8	<8	<8
South Africa 041	930	2456	84	180	198
South Africa 042	154	364	40	39	64
South Africa 043	68	430	35	9	20
South Africa 044	164	462	14	11	17

APPENDIX C: Data collection sheet.

1. DEMOGRAPHIC INFORMATION FOR ALL PARTICIPANTS

DATE (dd/mm/yyyy): _____ **INITIALS**_____ **YEAR OF BIRTH**_____**STUDY NO**

RACE: □Black □White □Coloured □Asian **CITY IN SOUTH AFRICA**:

REQUIRES RESULTS : Yes □ No □ Contact number if yes

2. HAEMOPHILIA AND ITS TREATMENT

HAEMOPHILIA SEVERITY: □Mild □Moderate □Severe **Genotype**: _____

IMMUNOMODULATORY THERAPY EXPOSURE Yes □ No □ **Type, if yes:**

HAEMOPHILIA PRODUCT USED_____ **AGE AT FIRST EXPOSURE TO PRODUCT**(YEARS)_____

TYPE OF PRODUCT USED: □Plasma derived □Recombinant □Both plasma + recombinant products

TOTAL NUMBER OF EXPOSURES: □1-5 □6-10 □11-50 □ 51-150 □ >150

TIME SINCE TREATMENT OF LAST EXPOSURE(days) □1 □2 □3 □4 □5 □6 □ 7 □> 7 days

3. INHIBITOR HISTORY (IF PRESENT)

DOES THE PARTICIPANT HAVE INHIBITORS: Yes □ No □

INHIBITOR DIAGNOSIS DATE: (dd/mm/yyyy)_____ **PEAK INHIBITOR LEVEL** (BU)

INHIBITOR ASSAY METHOD: □Bethesda assay □Nijmegen assay □Both methods □Unknown

ITI TREATMENT GIVEN: □None □Unsuccessful □Successful Ongoing □Unknown

NUMBER OF ITI TREATMENTS: □1X □2X □3X □4X □ Unknown

ITI REGIMEN USED_____

4. BLOOD PRODUCT EXPOSURE (EXCLUDING FACTOR CONCENTRATE) HISTORY

EXPOSURE TO BLOOD PRODUCTS: □None □Red cells □ Platelets □FFP □Cryoprecipitate □Unknown

NUMBER OF EXPOSURE TO BLOOD PRODUCTS: Product type:_____□1x □2x □3x □>3x

Product type:_____□1x □2x □3x □>3x

5. HISTORY OF T R A N S F U S I O N T R A N S M I T T E D I N F E C T I O N S

HEPATITIS C: □Positive □Negative □Not tested **TEST METHOD** □Serology □PCR □Unknown

HEPATITIS BsAb: □Positive □ Negative □Not tested **ANTIBODY TITRE:**_____**DATE:**

HEPATITIS A antibody : □Positive □Negative □Not tested **ANTIBODY TITRE:**_____**DATE:**

HIV INFECTION: □Positive □Negative □Not tested **TEST METHOD** □Serology □PCR □Unknown

6. FAMILY HISTORY

IS THERE FAMILY HISTORY OF HAEMOPHILIA: □Yes □ No **RELATIONSHIP:**_____

ARE THERE FAMILY MEMBERS WITH INHIBITOR: □Yes □ No **PEAK INHIBITOR TITRE(BU):**_____**DATE:**

APPENDIX D: Mutations in haemophilia B participants.

Number	Mutation type
1	Insertion of 289 bp* nucleotides.
2	Large deletion of the *F9* gene.
1	Frameshift mutation which results in a truncated protein. C.1176InsA (p.346 +A) mutation in exon 8.
3	Missense mutation in exon 2. c.127c > T (p.-4R>W).
1	Donor splice site mutation at the intron 4/exon 5 junction. c.392-1G>A.
1	IVS4+2delTA in intron 4.
1	Missense mutation: H221R (829G>A) in exon 7.
1	One bp* deletion at position 10436, causes a frameshift after serine 61 and a stop codon at leucine 84 in first growth factor domain.
2	A missense mutation: c.722A >G in exon 6, predicted to result in p.Gin 241 Arg.
1	A missense mutation: p.C23Y (c.206G>A).
1	A nonsense mutation in exon 5, resulting in premature truncation of the protein. R116x (513C>T).
1	A missense mutation: c.1295 G>A (G386D) mutation in exon 8.
28	Genotype testing was not performed.

*base pair

APPENDIX E: Human research ethics committee clearance certificate.

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr Nolukholo Ncete

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M180408

NAME: (Principal Investigator)	Dr Nolukholo Ncete
DEPARTMENT:	Molecular Medicine and Haematology Charlotte Maxeke Johannesburg Academic Hospital National Health Laboratory Service
PROJECT TITLE:	The prevalence of anti-adeno associated virus neutralizing antibodies in South African people with Haemophillia B
DATE CONSIDERED:	04/05/2018
DECISION:	Approved Unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Johnny Mahlangu
APPROVED BY:	Professor CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	30/07/2018

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in **April** and will therefore be due in the month of **April** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature Date

APPENDIX F: Charlotte Maxeke Johannesburg Academic Hospital CEO'S permission letter.

GAUTENG PROVINCE
HEALTH
REPUBLIC OF SOUTH AFRICA

CHARLOTTE MAXEKE JOHANNESBURG ACADEMIC HOSPITAL

Enquiries:
Ms. N. Mzila
Office of the Clinical Director
Email:Nolwazi.Mzila@gauteng.gov.za
Tell: (011): 488-4812
21 June 2018

Dear Dr. N. Ncete

STUDY TITTLE: Prevalence of Anti-Adeno Associated Virus Neutralizing Antibodies in South African People with Haemophilia B.

Permission to conduct the above mentioned study is provisional approved. Your study can only commence once Ethics approval is obtained. Please forward a copy of your Ethics Clearance Certificate as soon as the study is approved by the Ethics Committee for the CEO's office to give you the final approval to conduct the study.

Supported / ~~not supported~~

Dr. M.I. Mofokeng
Clinical Director
DATE: 21/06/2018

Approved / not approved

Ms. G. Bogoshi
Chief Executive Officer
DATE: 25.06.2018

REFERENCES

1. Peyvandi F, Garagiola I, Young G. The past and future of haemophilia: diagnosis, treatments, and its complications. LANCET. 2016;388:187–97.

2. Doering CB, Spencer HT. Replacing bad (F)actors: hemophilia. Hematology Am Soc Hematol Educ Program. 2014;(1):461–7.

3. Hartmann J, Croteau SE. 2017 Clinical trials update: Innovations in hemophilia therapy. Am J Hematol. 2016;91(12):1252–60.

4. Rallapalli PM, Kemball-Cook G, Tuddenham ED, Gomez K, Perkins SJ. An interactive mutation database for human coagulation factor IX provides novel insights into the phenotypes and genetics of hemophilia B. J Thromb Haemost. 2013 July;11(7):1329-40.

5. Perrin GQ, Herzog RW, Markusic DM. Update on clinical gene therapy for hemophilia. BLOOD. 2019 Jan 31;133(5):407–14.

6. Goodeve AC. Hemophilia B: molecular pathogenesis and mutation analysis. 2015 July;13:1184–95.

7. George LA, Fogarty PF. Gene therapy for hemophilia: past, present and future. Semin Hematol. 2016 Jan;53(1):46–54.

8. Chapin JC, Monahan PE. Gene Therapy for Hemophilia: Progress to Date. BioDrugs. 2018 Feb;32(1):9–25.

9. Lheriteau E, Davidoff AM, Nathwani AC. Haemophilia gene therapy: Progress and challenges. Blood Rev. 2015 Sep;29(5):321–8.

10. Nathwani AC, Tuddenham GD, Rangarajan S, Rosales C, McIntosh J, Linch DC et al. Adenovirus-Associated Virus Vector–Mediated Gene Transfer in Hemophilia B. N ENGL J MED. 2011 Dec 22;365(25):2357–65.

11. Tuddenham E. Gene therapy for haemophilia B. Haemophilia. 2012;18:13–7.

12. High KA, Anguela XM. Adeno-associated viral vectors for the treatment of hemophilia. Hum Mol Gen. 2016;25(R1):R36–41.

13. Verdera HC, Kuranda K, Mingozzi F. AAV Vector Immunogenicity in Humans: A Long Journey to Successful Gene Transfer. 2020 Mar;28(3):1–24.

14. Colella P, Ronzitti G, Mingozzi F. Emerging Issues in AAV-Mediated In Vivo Gene Therapy. Mol Ther Methods Clin Dev. 2018 Mar;8:87–104.

15. Schultz BR, Chamberlain. Recombinant adeno-associated virus transduction and integration. Mol Ther. 2008 Jul;16(7):189–99.

16. Jeune LV, Joergensen JA, Hajjar RJ, Weber T. Pre-existing anti-adeno-associated virus antibodies as a challenge in AAV gene therapy. Hum Gene Ther Methods. 2013 Apr;24(2):59–67.

17. Lisowski L, Tay S, Alexander IE. Adeno-associated virus serotypes for gene therapeutics. Curr Opin Pharmacol. 2015;24:59–67.

18. Nienhuis AW, Nathwani AC, Davidoff AM. Gene therapy for hemophilia. Mol Ther. 2017 May;25(5):1163–7.

19. Calcedo R, Morizono H, Wang L, McCarter R, He J, Jones D, et al. Adeno-associated virus antibody profiles in newborns, children, and adolescents. Clin Vaccine Immunol. 2011 Sep;18(9):1586–8.

20. Arruda VR, Samelson-Jones BJ. Obstacles and future of gene therapy for hemophilia. Expert Opin Orphan Drugs. 2015;3(9):997–1010.

21. Pipe S, Leebeek WG, Ferreira V, Sawyer EK, Pasi J. Clinical Considerations for Capsid Choice in the Development of Liver-Targeted AAV-Based Gene Transfer. Mol Ther Methods Clin Dev. 2019 Dec;15:170–8.

22. Calcedo R, Wilson JM. Humoral Immune Response to AAV. Front Immunol. 2013 Oct 18;4:341–341.

23. Mingozzi F, High KA. Immune responses to AAV vectors: overcoming barriers to successful gene therapy. Blood. 2013 Jul 4;122(1):23–36.

24. Vandamme C, Adjali O, Mingozzi F. Unraveling the Complex Story of Immune Responses to AAV Vectors Trial After Trial. Hum Gene Ther. 2017 Nov 1;28(11):1061–74.

25. Stanford S, Pink R, Creagh D, Clark A, Lowe G, Curry N et al. Adenovirus-associated antibodies in UK cohort of hemophilia patients: A seroprevalence study of the presence of adenovirus-associated virus vector–serotypes AAV5 and AAV8 neutralizing activity and antibodies in patients with hemophilia A. Res Pract Thromb Haemost. 2019 Apr;3(2):261–7.

26. Herzog RW. Complexity of immune responses to AAV transgene products – Example of factor IX. Cell Immunol. 2017;342:1–8.

27. Swystun LL, Lillicrap D. Gene Therapy for Coagulation Disorders. Circ Res. 2016 Apr;118(9):1443–52.

28. High KA. The gene therapy journey for hemophilia: are we there yet? BLOOD. 2012 Nov 29;120(23):4482–7.

29. Monahan PE, Gui T. Gene therapy for hemophilia: advancing beyond the first clinical success. Curr Opin in Hematol. 2013 Sep;20(5):410–6.

30. Mingozzi F, High KA. Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. Nat Rev Genet. 2011;12(5):341–55.

31. Arruda VR, Xiao W. It's all about the clothing: capsid domination in the adeno-associated viral vector world. J Thromb Haemost. 2007;5:12–5.

32. Ohmori T. Advances in gene therapy for hemophilia: basis, current status, and future perspectives. Int J Hematol. 2018 Aug 6;111:3-41.

33. Monahan PE. Emerging genetic and pharmacologic therapies for controlling hemostasis: beyond recombinant clotting factors. Hematology Am Soc Hematol Educ Program. 2015. 2015;1:33–40.

34. Ayuso E, Mingozzi F, Bosch F. Production, purification and characterization of Adeno-associated vectors. Curr Gene Ther. 2010 Dec;10(6):423–36.

35. Urabe M, Ding C, Kotin RM. Insect Cells as a Factory to Produce Adeno-Associated Virus Type 2 Vectors. Hum Gene Ther. 2002 Nov 1;13(16):1935–43.

36. Manno CS, Chew AJ, Hutchison S, Larson PJ, Herzog RW, Arruda VR, et al. AAV-mediated factor IX gene transfer to skeletal muscle in patients with severe hemophilia B. BLOOD. 2003 Apr 15;101(8):2963–72.

37. Manno CS, Pierce GF, Arruda VR, Glader B, Ragni M, Rasko JE, et al. Successful transduction of liver in hemophilia by AAV-Factor IX and limitations imposed by the host immune response. Nat Med. 2006 Mar;12(3):342–7.

38. Miesbach W, Meijer K, Coppens M, Kampmann P, Klamroth R, Schutgens R, et al. Gene therapy with adeno-associated virus vector 5–human factor IX in adults with hemophilia B. BLOOD. 2018 Mar 1;131(9):1022–31.

39. Nathwani AC, Reiss UM, Tuddenham EGD, Rosales C, Chowdary P, McIntosh J. Long-Term Safety and Efficacy of Factor IX Gene Therapy in Hemophilia B. N ENGL J MED. 2014 Nov 20;371(20):1994–2004.

40. George LA, Sullivan SK, Giermasz A, Rasko EJ, Samelson-Jones BJ, Ducore J et al. Hemophilia B Gene Therapy with a High-Specific-Activity Factor IX Variant. N ENGL J MED. 2017 Dec 7;377(23):2215–27.

41. Giermasz A, Von Drygalski A, Castaman G, Key NS, Lattimore S, Leebeek WG, et al. AMT-061 (AAV5-Padua hFIX variant) an Enhanced Vector for Gene Transfer in Adults with Severe or Moderate-Severe Hemophilia B: Follow-up up to 9 Months in a Phase 2b Trial. Presented at: ISTH 2019 Congress; July 6-10, 2019; Melbourne, Australia. Abstract OC 01.1.

42. Batty P, Lillicrap D. Advances and challenges for hemophilia gene therapy. Hum Mol Genet. 2019;28(R1):R95-101.

43. Peyvandi F, Garagiola I. Clinical advances in gene therapy updates on clinical trials of gene therapy in haemophilia. Haemophilia. 2019 Sep;25(5):738–46.

44. Falese L, Sandza K, Yates B, Triffault S, Gangar S, Long B et al. Strategy to detect pre-existing immunity to AAV gene therapy. Gene Ther. 2017;24:768–78.

45. Meliani A, Leborgne C, Triffault S, Jeanson-Leh L, Veron P, Mingozzi F. Determination of Anti-Adeno-Associated Virus Vector Neutralizing Antibody Titer with an In Vitro Reporter System. Hum Gene Ther Methods. 2015 Apr;26:45–53.

46. Calcedo R, Vandenberghe LH, Gao G, Lin J, Wilson JM. Worldwide Epidemiology of Neutralizing Antibodies to Adeno-Associated Viruses. J Infect Dis. 2009 Feb 1;199(3):381–90.

47. Kruzik A, Fetahagic D, Hartlieb B, Dorn S, Koppensteiner H, Horling FM, et al. Prevalence of anti-adeno associated virus immune responses in international cohorts of healthy donors. Mol Ther Methods Clin Dev. 2019 Sep;14:126–33.

48. Majowicz A, Nijmeijer B, Lampen MH, Spronck L, de Haan M, Petry H.et.al. Therapeutic hFIX Activity Achieved after Single AAV5-hFIX Treatment in Hemophilia B Patients and NHPs with Pre-existing Anti-AAV5 NABs. Mol Ther Methods Clin Dev. 2019 Sep 14;14:27–36.

49. Shrestha BM. The Declaration of Helsinki in relation to medical research: historical and current perspectives. J Nepal Health Res Counc. 2012;10(22):254–7.

50. Cox SR, Dages JH, Jarjoura D, Hazelett S. Blood samples drawn from IV catheters have less hemolysis when 5 mL (vs 10 mL) collection tubes are used. J Emerg Nurs. 2004;30:529–33.

51. Boutin S, Monteilhet V, Veron P, Leborgne C, Benveniste O, Montus M et al. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. Hum Gene Ther. 2010 Jun;21(6):704–12.

52. Liu Q, Huang W, Zhao C. Zhang L, Meng S, Gao D et al. The prevalence of neutralizing antibodies against serotype 1 in healthy subjects in China: implications for gene therapy and vaccines using AAV1 vector. J Med Virol. 2013;85(9):1550–6.

53. Li C, Narkbunnam N, Samulski RJ, Asokan A, Hu G, Jacobson LJ, et al. Neutralizing antibodies against adeno-associated virus examined prospectively in pediatric patients with hemophilia. Gene Ther. 2012 Mar;19(3):288–94.

54. Rajavel K, Ayash-Rashkovsky M, Tang Y, Gangadharan B, de la Rosa M, Ewenstein B. Co-Prevalence of Pre-Existing Immunity to Different Serotypes of Adeno-Associated Virus (AAV) in Adults with Hemophilia. Presented at the 61st American Society of Haematology (ASH) Annual Meeting & Exposition, 7-10 December 2019, Orlando, USA.