Advances in designing Adeno-associated viral vectors for development of anti-HBV gene therapeutics

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Abstract
Despite the five decades having passed since discovery of the hepatitis B virus (HBV), together with development of an effective anti-HBV vaccine, infection with the virus remains a serious public health problem and results in nearly 900,000 annual deaths worldwide. Current therapies do not eliminate the virus and viral replication typically reacti- vates after treatment withdrawal. Hence, current endeavours are aimed at developing novel therapies to achieve a functional cure. Nucleic acid-based therapeutic approaches are promising, with several candidates showing excellent potencies in preclinical and early stages of clinical development. However, this class of therapeutics is yet to become part of standard anti-HBV treatment regimens. Obstacles delaying development of gene-based therapies include lack of clinically relevant delivery methods and a paucity of good animal models for preclinical characterisation. Recent studies have demonstrated safety and efficiency of Adeno-associated viral vectors (AAVs) in gene therapy. However, AAVs do have flaws and this has prompted research aimed at improving design of novel and artificially synthesised AAVs. Main goals are to improve liver transduction efficiencies and avoiding immune clearance. Application of AAVs to model HBV replication in vivo is also useful for characterising anti-HBV gene therapeutics. This review summarises recent advances in AAV engineering and their contributions to progress with anti-HBV gene therapy development.

Keywords: AAV, Hepatitis B virus, Capsid engineering, Genome engineering, HBV replication models

Background
Hepatitis B virus (HBV) infection is a major public health burden. Approximately 257 million individuals worldwide are chronically infected with the virus and therefore predisposed to cirrhosis, hepatocellular carcinoma (HCC) and liver failure [1]. HBV has a partially double-stranded relaxed circular DNA (rcDNA) genome of approximately 3.2 kilobases (kb) in length. The genome has four overlapping open reading frames (ORFs), namely the polymerase (P), core (C), surface (S) and the X ORFs. Expression of HBV genes is controlled by four separate promoters: the basic core, preS1, preS2 and X promoters. The cis enhancer elements are enhancer I, located upstream of the X promoter, and enhancer II which is located upstream of the basic core promoter. These reg- ulatory elements are responsible for liver-specific viral gene expression. The P ORF encodes a DNA polymerase with priming, reverse transcriptase and RNase H activity. The C ORF comprises precore and core regions, collectively termed precore/core. The core region encodes HBV core antigen (HBcAg) which forms the viral capsid, whereas the precore region encodes the HBV e antigen (HBeAg), an immune suppressor and an indicator of active viral replication. The S ORF has three initiation codons: preS1, preS2 and S, which respectively initiate translation of large, middle, and small surface proteins.

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The X ORF encodes the regulatory HBx protein, which is essential for viral replication [2].

HBV infection is initiated by low affinity interaction of the infectious Dane particle with glycosaminoglycans located on the hepatocyte surface [3]. Enhanced by the presence of epidermal growth factor, the high affinity binding of myristylated large surface antigen to the sodium taurocholate co-transporting polypeptide (NTCP) receptor facilitates entry of the nucleocapsid. Mediated by endocytosis, the nucleocapsid is then transferred to the nucleus via the microtubules [4–7]. This is followed by nuclear release of rcDNA, which is then repaired to form covalently closed circular DNA (cccDNA). The cccDNA then serves as the template for transcription of pregenomic RNA (pgRNA) and viral protein-encoding mRNAs (reviewed in [8]). HBx binds the DDB domain of ubiquitin ligase 1 to render structural maintenance of chromosomes protein 5/6 unstable and thereby facilitates HBV gene expression [9–11]. Translation of the precore/core RNA producesHBcAg. Encapsidation of the pgRNA is followed by its reverse transcription. The mature nucleocapsid is then transported to the nucleus to maintain cccDNA pools or acquires a surface antigen-containing envelope to form intact virions (Dane particles) following secretion via the endoplasmic reticulum.

Current treatment of HBV infection requires long-term therapy and reduces severe complications and death, but rarely eliminates the virus. This is as a result of inability to clear the stable cccDNA episome from infected hepatocytes and antigenemia-mediated exhaustion of HBV-specific CD8+ T cells and B cells [12, 13]. Hence developing a cure for HBV infection is a priority. Recently, gene-based and combinatorial strategies targeting multiple steps in the HBV replication cycle have shown promise. The potential of gene-based strategies for eliminating cccDNA and reducing antigenemia has been demonstrated in preclinical studies (reviewed in [14]). These gene-based approaches include gene editing and gene silencing. Strategies have applied technology based on clustered regulatory interspaced short palindromic repeats (CRISPR)/CRISPR associated (Cas) systems, transcription activator-like effector nucleases (TALENs) and RNA interference (RNAi) to inhibit HBV gene expression (reviewed in [15]). The current challenges of anti-HBV gene therapeutics include difficulties with obtaining a clinically relevant vector for hepatic transgene delivery and the paucity of suitable animal models that simulate natural HBV infection.

Recent US Food and Drug Administration (FDA) approval of AAV-based therapies Zolgensma and Luxturna reinforces a well-established biosafety profile and efficacy of AAVs for human application [16–18]. AAV mediation of hepatic transgene expression is also now well established [19–21]. Moreover, HBV infection enhances AAV transduction of hepatocytes [22]. Despite these appealing features, use of AAVs has not been without its challenges. These include low packaging capacity, reduced transduction efficiencies in specific tissues, induction of CD8+ T cell responses and clearance by pre-existing immunity. This review focuses on recent progress with modifying AAVs and their contribution to advancing anti-HBV gene therapy.

Biology of Adeno-associated viruses

AAVs are small viruses that cannot replicate on their own, but depend on co-infection with other viruses such as adenoviruses or herpes simplex virus or vaccinia virus or human papilloma virus (reviewed in [23]). Twelve AAV serotypes have been identified to date. The non-enveloped viral capsid, comprising VP1, VP2 and VP3, has conserved eight-stranded β-barrel motifs, an α-helix and nine variable regions that confer AAV tropism diversity [24]. Multiple viral surface sites have been mapped and characterised as T-cell epitopes, immunogenic motifs and monoclonal antibody docking sites [25–27].

The AAV capsid encases the genome that comprises linear single-stranded DNA (ssDNA) of about 4.7 kb. The genome consists of Cap and Rep open reading frames (ORFs) flanked by 145 bp T-shaped hairpin inverted terminal repeats (ITRs). The ITRs are made up of three complementary palindromes (A–A′, B–B′ & C–C′), a single non palindromic region (D) and cis-acting elements, which are the Rep protein binding element (RBE) and terminal resolution site (trs). Upon host cell entry, AAVs enter latency. In the presence of a helper virus AAVs express their genes from a trio of promoters (p40, p5 and p15) and becomes lytic (reviewed in [23]). Transcription from all the three promoters is terminated by a common poly-adenylation signal. Expression from the p40 promoter produces the three capsid proteins (VP1, VP2 and VP3) and an assembly activating protein (AAP). The capsid proteins assemble in 1:1:10 ratio of VP1:VP2:VP3 to form an icosahedral capsid while AAP mediates capsid assembly. Rep gene expression is driven from p5 & p19 promoters to produce two large (Rep78 and Rep68) and two small (Rep52 and Rep40) Rep proteins (reviewed in [28, 29]).

AAV2 infection is initiated by binding to the heparin sulphate proteoglycan receptor and a co-receptor fibroblast growth factor receptor. Endocytosis through clathrin-coated vesicles then mediates viral entry [30, 31]. In the absence of a helper virus, AAV gene expression is limited and the genome mainly persists episomally. Less frequently, while various integration sites exist for AAV such as AAVS2 or AAVS3, AAV DNA integrates
preferentially into the AAVS1 site of the host genome [32, 33]. In the presence of a helper virus, expression from the Rep ORF is activated and enables AAV genome rescue. DNA polymerase mediates second strand synthesis using one ITR as a primer to produce a double stranded DNA (dsDNA). Together with Rep78/68 and several host factors, DNA polymerase uses the dsDNA as a template for re-initiation and polymerisation from one end to generate a double stranded full-length genome and displace a single stranded full-length genome. The double stranded genome serves as a template for further rounds of replication, while the rep proteins mediate ssDNA loading into the capsid. An active release pathway of AAV particles following viral assembly remains to be described (reviewed in [34, 35]).

**Engineering AAV genome for delivery of anti-HBV sequences**

For AAV vector production, the ITR sequences are retained but the promoter sequences, rep and cap genes are removed to accommodate transgene cassettes (Fig. 1A). This generally allows insertion of a maximum of about 5 kb sequences into the vectors. Although this is enough for smaller anti-HBV effectors, such as RNAi activators, it precludes delivery of larger anti-HBV effectors, such as HBV CRISPR/Cas and TALEN sequences. Early studies described successful production of oversized AAV vectors and packaging of genomes larger than 5 kb (Fig. 1B) [36–40]. However, during viral production AAVs with heterogeneous genome sizes with truncations are often produced from these oversized genomes. Upon transduction, large AAV genomes may be reconstituted by concatenation, but reconstitution using the truncated genomes is highly inefficient and not desirable for clinical application.

Based on ability of AAV genomes to concatenate and serve as substrates for homologous recombination, another strategy to increase transgene capacity entails use of dual AAV vectors [40, 41]. The most commonly used are dual overlapping vectors, dual trans-splicing vectors and dual hybrid vectors (Fig. 1C–E). The design is to split the expression cassette into two parts, each contained in an AAV, and the intact transgene is reconstituted in a cell after transduction by homologous recombination or concatenation. As with oversized vectors, reconstitution of dual vectors is inefficient. This results in poor transduction efficacies and a requirement for high vector doses to achieve therapeutically relevant effects [37, 42–44].

Recent studies have taken advantage of CRISPR/Cas systems being made up of two components, i.e. the nuclease and the single guide RNA (sgRNA) (Fig. 1F). These may be expressed on separate AAVs and in combination effect DNA cleavage upon transduction of a cell by the two vectors [45]. With recent availability of smaller nucleases, a single AAV can now be used to deliver both the nuclease- and sgRNA-encoding sequences to effect cccDNA cleavage [46–49]. TALEN activity requires two subunits, each encoded by DNA of at least 3 kb, to effect dsDNA cleavage. Although the evidence is scant, two component vector systems should be applicable to delivering sequences that together constitute complete anti-HBV TALENS.

A requirement to convert an ssDNA AAV genome to a dsDNA before transgene expression is a limiting step of AAV-based gene transfer. For quicker transgene expression, the trs site may be mutated to inhibit terminal resolution. This results in AAVs bearing a long hairpin loop molecule with complementary duplex strands referred to as self-complementary AAVs (scAAVs, Fig. 1G). Although this reduces packaging capacity by half, scAAVs bypass the requirement for second strand synthesis with consequent faster and higher transgene expression [35, 50].

AAVs are known to infect a diverse range of tissues, which might lead to undesirable off-target transgene expression [51]. Hepatic tissue-specific expression of anti-HBV gene therapies can be achieved by placing transgene expression under control of liver-specific promoters, such as Transthyterin (TTR) or mouse Transthyretin (mTTR) (Fig. 1) [19, 20]. Liver-specific regulatory elements derived from core domains of human apolipoprotein hepatic control region, human α-1-antitrypsin and hybrid liver promoters successfully drive factor IX expression in the liver [52, 53] and may be applicable to anti-HBV gene therapy. When in silico identified evolutionary conserved hepatocyte-specific
Fig. 1 (See legend on previous page.)
cis-regulatory modules (CRMs) were incorporated into scAAVs, up to 100-fold higher transgene expression was achieved when compared to scAAVs cassettes containing the TTR promoter (Fig. 1) [54].

**AAV capsid engineering for improved transduction and evasion of pre-existing immunity**

The requirement for a high AAV dose to achieve therapeutic effects in non-human primates has been reported to result in death [55]. Hence, production of AAV capsids that achieve high transduction efficiencies at low dose is an important goal of the field. AAV capsid structural properties determine vector tropism, immune detection and transduction efficiency. Hence, manipulation of capsid architecture is central to enhancing the vectors’ therapeutic efficacy. High prevalence of pre-existing AAV-specific antibodies in humans, which limits AAV-mediated gene transfer, is another major reason for investigating utility of AAV capsid modification [56, 57]. In addition, proteasomal degradation, breakdown of capsids following endosomal escape and MHC1 presentation of AAV peptides with cell-mediated elimination of infected hepatocytes result in poor transgene expression [58–61]. Approaches have mainly involved rational design or directed evolution to modify AAV capsids. The former relies on prior knowledge of capsid architecture and intracellular trafficking of AAVs. By contrast, directed evolution utilises stringent selection methods to concentrate and confer advantageous and beneficial traits on a vector.

**Rational designs of capsids**

Several AAV variants with desirable features have been developed by using different rational design strategies. Some of the AAV strategies discussed below were used to develop variants for transduction of non-liver-derived cells. However, these approaches can be applied to improve efficiency of liver-targeting vectors. Docking sites of monoclonal antibodies (mAbs) or capsid antigenic motifs (CAMs) located in capsid variable regions (VR) serve as targets of capsid modification to avoid neutralising antibody (NAb) recognition. When these CAMs were mutated to produce libraries of novel AAV capsid variants (AAV-CAMs) followed by iterative rounds of selection in endothelial cells, antigenically advanced capsids were identified [62, 63] (Fig. 2).

Using a method called barcoded rational AAV vector evolution (BRAVE), rational design and screening for AAV variants that ably transduce cells of the central nervous system (CNS) were identified. To build an AAV library, proteins with synaptic affinity were identified using bioinformatics, and their peptide fragments inserted at a specific position to produce mutant capsids. AAV genomes bearing unique nucleotide barcode sequences were packaged into the mutant capsids to enable identification of individual capsid structures [64, 65]. Another method designated Cre-recombination-based AAV targeted evolution (CREATE) was used to generate vectors capable of transducing the CNS. The 7-mer random PCR-generated fragments were inserted between sequences encoding amino acid residues 588 and 589 of the capsid gene. The downstream poly A signal was flanked by lox P sites. AAV library administration in a cell type specific cre transgenic mice allowed inversion of the poly A signal, creating a sequence that could be amplified using pre-designed PCR primers. This led to the isolation of capsids that were capable of infecting cre-expressing cells (Fig. 2) [66].

Phosphorylation of AAV tyrosine or lysine residues by host cellular machinery leads to AAV capsid degradation by the ubiquitin–proteasome pathway [61, 67]. Other post-translational modifications such as glycosylation, SUMOylation, and neddylation also impact on viral transduction. Glycosylation facilitates viral cell entry, trafficking to the nucleus, virulence and immune evasion (reviewed in [68]). As with ubiquitination, neddylation and SUMOylation form reversible covalent attachments at lysine residues. These modifications affect protein stability, subcellular localisation, structure and function to inhibit AAV transduction of cells [69, 70]. Mutation or chemical modification of lysine residues in AAV2 or AAV8 capsids where glycosylation, neddylation or SUMOylation occurs resulted in higher transgene expression and decreased interaction of the AAV with NAbs (Fig. 2) [71–74].

**Directed evolution designs of novel capsids**

DNA shuffling of capsid-encoding sequences from multiple AAV serotypes has also been used to generate libraries. This approach has been used to identify capsids with improved hepatocyte transduction capabilities (Fig. 3) [75–77]. Libraries of AAVs may also be generated by random capsid sequence mutagenesis. Stringent selection of these mutant libraries in vitro and in chimeric murine livers identified variants with improved transduction efficiencies (Fig. 3) [78, 79]. Another strategy employed phylogenetic techniques to predict ancestral AAV capsid sequences that mediated higher transgene expression than natural AAV serotypes [80] (Fig. 3). Studies carried out in vivo on small and large animals identified another antigenically distinct and antibody-evading ancestral AAV vector that efficiently transduced a variety of cells including the liver [81–86].
How has anti-HBV gene therapy designs benefited from the advances in AAV vector developments?

**Application of improved AAVs to deliver anti-HBV gene therapeutics**

The higher liver transgene expression from AAV8-pseudotyped scAAVs enables use of low vector doses. Also, the dsDNA nature of the scAAV genome makes it more stable [52, 87–89]. These properties are favourable for targeting chronic viral infections such as are caused by HBV. The feasibility of delivering anti-HBV RNAi activators using scAAVs is well documented [90, 91]. In mice, following a single dose of AAV8-pseudotyped scAAVs a reduction of HBV replication markers was observed over 10 months [19]. Targeting both HBV and the host factors that mediate fibrosis with scAAVs improves therapeutic efficacy [92]. Supporting this combinatorial approach is the observation that scAAVs used to co-deliver an RNAi effector against HBV and Argonaut2, the rate limiting host factor in the RNAi pathway or a RNAi activator sense strand targeted decoy, improves safety, specificity, and efficacy [20, 93, 94].

Because of the limiting packaging capacity of scAAVs, several studies have used ssAAVs for delivery of a smaller *Staphylococcus aureus* Cas9 (SaCas9) with single or combination of guides targeting several coordinates in the cccDNA. These studies showed significant decline in markers of HBV replication in cultures and in mice [46–48]. A recent study has illustrated that using ssAAVs engineered to express saCas9 from a chimeric...
liver-specific promoter resulted in preferential liver expression and superior suppression of HBV replication in mice [95]. One study that used scAAVs to deliver anti-HBV gene editors co-transduced cells with two scAAVs, each carrying one ZFN monomer against HBV Pol, C or X genes. scAAVs against Pol resulted in near undetectable levels of HBV replication makers [96].

The anti-HBV gene therapy field has not yet fully capitalized on the availability of modified or synthetic AAV capsids described above. However, these developments, especially the genetically modified and synthetic AAV capsids that shows high liver transduction efficiencies, are alluring and promise to bring positive outcomes to HBV treatment.

**Application of AAVs to model chronic HBV infection**

The plethora of AAV serotypes, either extant or artificially synthesized and recent discovery of various receptors and co-receptors that facilitate AAV binding, are valuable tools to model HBV replication [97, 98]. These models are key to evaluating novel anti-HBV therapeutic interventions before clinical translation. Despite impressive progress with understanding the molecular biology of HBV, an easily accessible model that can recapitulate all stages of HBV remains elusive. Although chimpanzees are susceptible to HBV infection, and their immune responses are similar to those observed in humans, high cost and ethical concerns limit use of these animals in research (reviewed in [99]). Models using species-specific hepatitis strains such as duck hepatitis B virus and woodchuck hepatitis virus are limited by infection mechanisms and disease manifestations in these models that differ from natural HBV infection (Table 1) [100, 101].

Mouse models to simulate HBV replication remain the most accessible and commonly used. Chimeric mice with livers engrafted with human hepatocytes are valuable, but use of these animals is limited by their extreme immuno-deficiency and difficulties with maintaining hepatocyte function over long periods of time [102–105]. Transgenic mice with integrated DNA comprising greater-than-genome-length HBV sequences mimic chronic HBV replication [106–108]. However, transgenic mice show inter-individual variability, variation of HBV
replication markers over time and high markers of HBV replication that often exceed those of natural HBV infection (Table 1) [19, 109].

Use of recombinant adenoviruses and AAVs to deliver greater-than-genome-length HBV sequences has also been explored to simulate HBV replication in vivo. Adenoviral vectors efficiently induce HBV replication in mice [110–112]. However, transduction results in a strong immune response to the adenoviral vectors and early clearance of transduced hepatocytes. Three studies using an AAV8-carrying replication-competent greater-than-genome-length HBV genotype D (AAV-HBV model) demonstrated the potential of these vectors for simulation of chronic HBV infection [113–115]. HBsAg, HBeAg and HbcAg expression, accompanied by hepatitis B virion production over a period of up to 16 months, were observed. Production of anti-HBcAg antibodies, but not anti-HBsAg and HBeAg antibodies which is a phenomenon observed in HBV chronic carriers, was also demonstrated. Interestingly, between 12 and 16 months, mice developed features of HCC and elevated markers of liver injury. Liver fibrosis, chronic liver injury and minimal or no acute inflammatory responses were observed in these mice. Given that T cell exhaustion is a well-documented feature of chronic HBV infection, the lack of a significant immune response in these mice is perhaps not surprising (Table 1) [116, 117]. Although the mechanism of HCC, fibrosis and chronic liver damage in AAV-HBV murine models remains to be established, expression of HBV antigens such as HBx and HBSAg, together with AAV-mediated HBV DNA integration may be the contributing factors. Interestingly, recent studies showed that the AAV-HBV model based on either HBV serotype A, B, C or D may result in formation of cccDNA in murine hepatocytes [118, 119]. Although the replication intermediate was lost over time in transduced livers, the sequence and functionality was not distinguishable from cccDNA derived from natural HBV infection. The mechanism of its formation is not clear, however the cccDNA is HBV replication-independent and originates from intramolecular recombination of the HBV genome ends [119].

Another model recently established in non-human primates used AAV8 vectors expressing human NTCP (AAV8-hNTCP) [120, 121]. The study also employed helper-dependent adenoviral vectors (HDAds) to deliver sequences encoding hNTCP. Rhesus macaques, naturally not infectable by HBV, were injected with hNTCP-expressing vectors and then infected with HBV. HBV gene expression and HBV replication intermediates were detected over a period of 42 weeks. Moreover, markers of liver injury and T cell responses to HBV antigens were reported. Importantly the essential replication intermediate comprising cccDNA could also be detected in these macaques. Although a higher AAV vector dose was required and HDAd vectors promoted superior hNTCP expression and HBV infection, adenoviral vectors are limited by their high immune stimulation and the resultant short-term transgene expression. Hence, administration of immunosuppressants before injecting HDAds was required to prolong HBV gene expression. This makes HDAd-based models less favourable for mimicking chronic HBV infection.

### Conclusion

Studies described here show that progress with the design of improved AAV vectors will assist with addressing challenges facing development of anti-HBV gene therapy. Improved liver transduction will make it possible to administer lower vector doses to achieve clinically relevant therapeutic outcomes. Engineering AAVs to produce vectors that can evade systemic and cellular trafficking hurdles to deliver anti-HBV payloads to target cells expands the toolbox of gene therapy for the viral infection. Harnessing AAVs’ liver tropism to model HBV replication also shows potential.

| Animal model | Immune response | HBV antigen expression | Infection/replication | cccDNA formation | Fibrosis | Liver injury | References |
|--------------|----------------|------------------------|-----------------------|-----------------|----------|-------------|------------|
| Chimpanzee   | Immuno-competent | Yes                    | Infection             | Yes             | Yes      | Yes         | [99]       |
| Woodchuck    | Immuno-competent | Yes                    | Infection             | Yes             | Yes      | Yes         | [100, 101] |
| Duck-HBV     | Immuno-competent | Yes                    | Infection             | Yes             | Yes      | Yes         | [101]      |
| Human liver Chimeric mouse model | Immuno-deficient | Yes                      | Infection             | Yes             | Yes      | Yes         | [103–105] |
| HBV Transgenic mouse model | Immuno-competent | Yes                    | Replication           | No              | No       | No          | [19, 122] |
| AAV-HBV mouse model | Immuno-competent | Yes                    | Replication           | No              | Yes      | Yes         | [113–115] |
| hNTCP expressing Rhesus macaques | Immuno-competent | Yes                    | Infection             | Yes             | –        | Yes         | [121, 123] |

Key: – not known
murine models remain the most accessible and simple, demonstration that AAVs may be used to make non-human primates susceptible to HBV infection is significant. Collectively these developments will facilitate clinical translation of AAV-based, as well as other potentially curative therapies, to eliminate chronic HBV infection.

Abbreviations
AAV: Adeno-associated virus; BCP: Basic core promoter; bp: Base pairs; C: Core; Cas: CRISPR associated; cccDNA: Covalently closed circular DNA; CRISPR: Clustered regulatory interspaced short palindromic repeats; HBCAg: Hepatitis B core antigen; HBsAg: Hepatitis B surface antigen; HBV: Hepatitis B virus; Hbc: Hepatitis B X protein; HCC: Hepatocellular carcinoma; ITR: Inverted terminal repeats; Kbp: Kilobases; NTCp: Sodium taurocholate polypeptide; Orfs: Open reading frame; P: Polymerase; Rep: Rep binding element; rcDNA: Relaxed circular DNA; RNAi: RNA interference; S: Surface; TALENS: Transcription activator-like effector nucleases; Trs: Terminal replication site; VR: Capsid variable regions.

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Authors’ contributions
NM wrote all the sections on AAV genome and capsid modification. SWL wrote the section on AAV application in modelling HBV infection. MBM conceptualised the idea, wrote the abstract, introduction and conclusion, supervised the section on AAV application in modelling HBV infection. MBM revised the section on AAV application in modelling HBV infection. MBM supervised the writing process and was responsible for putting the different sections together, final editing and submission of the manuscript. PA contributed to the final editing and proof reading of the manuscript. All authors read and approved the final manuscript.

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