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Review

Immune responses to central nervous system directed adeno-associated virus gene therapy: Does direct CNS delivery make a difference?

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ABSTRACT

Adeno-associated virus (AAV) mediated gene therapy is a leading gene delivery platform with potential to transform the landscape of treatment for neurological disorders. While AAV is deemed non-immunogenic compared to other viral vectors, adverse immune reactions have been observed in the clinic, raising concerns. As the central nervous system (CNS) has a tightly regulated immune system, characterized by a degree of tolerance, it has been considered a unique target for AAV gene therapy. AAV vectors have shown promising results for the treatment of several CNS disorders including Spinal Muscular Atrophy, Giant Axonal Neuropathy, Amyotrophic Lateral Sclerosis, Tay Sachs Disease, Parkinson's Disease, and others, demonstrating safety and success. The Food and Drug Administration (FDA) approval of Zolgensma and European Medicines Agency (EMA) approval of Upstaza, for Spinal Muscular Atrophy (SMA) and Aromatic L-amino acid decarboxylase deficiency (AADC) respectively, represent this success, all while highlighting significant differences in immune responses to AAV, particularly with regards to therapeutic administration route. AAV therapies like Upstaza that are injected directly into the immune-specialized brain have been characterized by mild immune response profiles and minor adverse events, whereas therapies like Zolgensma that are injected systemically demonstrate more robust immune stimulation and off-target toxicities. Despite these contrasting parallels, these therapeutics and others in the clinic have demonstrated clinical benefit for patients, warranting further exploration of immune responses to CNS-directed AAV clinical trials. Thus, in this review, we discuss effects of different routes of AAV administration on eliciting local and peripheral immune responses specifically observed in CNS-targeted trials.

Introduction

The central nervous system (CNS) has long been described as immune privileged, however this definition has recently been refined, owing to data demonstrating the intricate interplay between immune cells of the CNS and the periphery. The unique environment in the CNS is protected by the immune system, both by resident cells, and cells that traffic to the CNS during injury or disease. However, immune responses are also restricted in the CNS due to the presence of the blood-brain barrier (BBB), low levels of major histocompatibility complex (MHC), and an abundance of anti-inflammatory factors [1]. Because of this distinctive environment, the CNS is an attractive target for the administration of

Abbreviations: AAV, Adeno-associated virus; BBB, Blood-brain barrier; CNS, central nervous system; MHC, major histocompatibility complex; EMA, European Medicines Agency; FDA, Food and Drug Administration; AADC, Aromatic I-Amino acid decarboxylase; SMA, Spinal Muscular Atrophy; US, United States; ALS, Amyotrophic Lateral Sclerosis; PAMPs, pathogen-associated molecular pattern molecules; TMA, thrombotic microangiopathy; NHPs, non-human primates; NAbs, neutralizing antibodies; TAbs, total antibodies; Tregs, T-regulatory cells; IP, intraparenchymal; CSF, cerebrospinal fluid; PMBCs, peripheral blood mononuclear cells; ELISPOT, enzyme-linked immunosorbent spot; SMN1, survival motor neuron 1; MPS IIIB, mucopolysaccharidosis type IIIB Syndrome; GAN, giant axonal neuropathy; BCSFB, blood-CSF barrier; ICM, intra-cisterna magna; ICV, intra-cerebroventricular; IT, intrathecal; DRG, dorsal root ganglion; NF-L, neurofilament light chain; BLA, biologics license application.

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therapeutics like adeno-associated virus (AAV). The European Medicines Agency (EMA) and Food and Drug Administration (FDA) have approved AAV gene therapies like Upstaza for Aromatic L-amino acid decarboxylase (AADC) deficiency, and Zolgensma for Spinal Muscular Atrophy (SMA), propelling AAV into the spotlight as a leading candidate for the treatment of neurological disorders. Although both are therapies for CNS disorders, the dosage, capsid variant, and route of delivery are quite different, which greatly impacts the immune response to each therapy. Upstaza is a direct injection into the brain parenchyma, specifically bilaterally in the putamen, whereas Zolgensma is an intravenous (IV) infusion. In the United States (US), there are a total of five approved gene therapies including Zolgensma, indicating AAV to be considered a relatively safe and effective gene delivery vector. However, adverse reactions have been observed in clinical trials and further evaluation is necessary to characterize the immune response to AAV when targeting such a unique immunological space. Nonetheless, this unique environment also makes measuring immune responses to AAV and the effects on gene delivery and therapeutic benefit more difficult to evaluate. With an increasing number of studies using AAV, especially for CNS targets, it is essential that we better understand the immune response to this vector and the role it plays in both safety and therapeutic outcomes. In this review, we will summarize current knowledge about the immune response to AAV and dissect the differences between responses to different delivery routes targeting the CNS.

Immune Response to AAV

Although AAV is considered relatively safe, particularly when compared to other viral vectors, immune responses to AAV may hinder both the efficacy and safety of AAV-mediated gene therapy. Broadly speaking, the immune system has two arms: the innate immune response, which acts very quickly yet less specifically, and the adaptive immune response, which is slower but has antigen-specific recognition and action that work together to ameliorate infections [2]. The two arms of the immune system are tightly intertwined, whereby innate immune responses are needed to generate adaptive immune responses. The slower adaptive immune system then generates memory T cells and B cells, that in the future, upon re-exposure to the respective antigen, generate a faster and more robust immune response [3]. Memory cells can persist for an extended period, and while they are beneficial for clearing unwanted pathogens, they can be detrimental to the success of genetic therapies utilizing viral vectors for delivery, like AAV.

The innate immune system is responsible for immune surveillance and responds broadly to pathogen-associated molecular patterns (PAMPs). Although AAV does not robustly stimulate the innate immune system, unmethylated CpG sequences within the AAV genome can stimulate TLR9 [4], as well as capsids by TLR2 [5]. AAV has also been shown to interact with MDA5 and RIG-I, which can lead to downstream inflammatory cytokine production [6,7]. Complement responses have additionally been observed in some clinical trials, particularly in incidences where high doses of AAV were delivered systemically, resulting in thrombotic microangiopathies (TMA) with low platelet counts, leading to kidney damage [8,9]. Recently, a novel finding of microvascular injury without TMA or complement activation, but rather capillary leakage has been described in both a patient [10] and in nonhuman primates (NHPs) [11]. Innate immune responses to AAV have been summarized here [12].

The innate immune response is responsible for activating the adaptive immune response, which produces antigen-specific responses. Innate immune cells like dendritic cells and macrophages are needed to present antigens to B and T cells for specific antigen recognition [13,14]. B cells, in response to soluble or presented antigen, can produce antibodies against either AAV capsids or transgenes in the gene therapy context [15–18]. The development of neutralizing antibodies (NAbs) which block viral cell transduction are an important evolutionary defense against viral pathogens. However, once NAbs are developed, either from prior gene therapy or natural AAV infection, which is common in the global population, the effectiveness of gene transfer will be low [19]. In most clinical trials, particularly in those delivered IV, seropositive patients are excluded, limiting the widespread application of AAV gene therapy. The production of antibodies against AAV capsid in gene therapy trials does not appear to limit therapeutic effect for most therapies, however, does limit the ability to re-dose virus. Nonetheless, host antibody responses to transgene can limit therapeutic efficacy, particularly for secreted transgenes [20]. Cytotoxic capsid-specific or transgene-specific $CD8^+$ T cells can also be induced after AAV gene therapy. These immune responses have been associated with elevated transaminases and loss of transgene expression, particularly after systemic administration, corresponding to loss of transduced cells in the liver and also potentially muscle [21]. Immunosuppressive cells, such as T-regulatory cells (Tregs) can also be induced to AAV capsid or transgene [22,23]. Induction of these cells is associated with long term transgene expression [24].

Initial trials using AAV gene therapy used limited or no immunosuppression, however, since clinical findings of gene therapy related immune responses, broad immunosuppression is regularly used prophylactically in clinical trials [25–27]. Immunosuppression can be used to control immune responses, and differing strategies have been tested including the use of drugs to block both innate and adaptive immune responses such as Eculizumab (C5 blocker), rituximab (B cell depletion), sirolimus/rapamycin (mTOR inhibitor) and corticosteroids [15,25,28]. Immune responses to AAV gene therapy have been reviewed at more depth elsewhere [29,30].

Immune Responses in the CNS and Privilege

Due to the protection conferred by the BBB, it has long been thought that the CNS is "immune privileged". However, the idea that the CNS is protected from immune responses has been challenged, and the term has further been refined, owing to more recent data demonstrating the tightly intertwined relationship of the CNS and the immune system [31]. Compared to other tissues, the CNS has its own unique immune microenvironment which is strongly influenced by its distinctive resident cells. Glia are broadly considered to be the resident immune cells of the CNS, particularly microglia [32]. One of the main responsibilities of glia is to protect neurons from damage and degeneration, which is one of the key characteristics associated with CNS disorders [33]. Upon infection or injurious stimuli, glia undergo an inflammatory transformation, releasing proinflammatory molecules, like cytokines, to restore the CNS to homeostasis. When reactive gliosis goes unchecked, excess inflammation can contribute further to neuronal damage, which is why it is so important that immune responses to AAV in the CNS are properly regulated [34]. Microglia, the resident macrophages in the CNS, phagocytose and eliminate particles that may pose a threat to the CNS. Once the danger has been eradicated, microglia return to a homeostatic state to continue to survey the brain for new insults [35]. Interestingly, targeting glia, particularly microglia with AAV, has been a significant challenge in the field, however advancements have been made [36–39]. Another important immune-like cell in the CNS is the astrocyte, which is more often transduced with AAV alongside neurons [36,40–42]. Astrocytes are responsible for upholding the blockade at the BBB and preventing the passage of injurious molecules or cells from the periphery to the CNS [43,44]. Dysregulation of astrocytes, whether due to disease or inflammation, can lead to disproportionate BBB leakage, potentially allowing more detrimental molecules or cells to enter the normally well-protected nervous system [45].

In addition to CNS-specific cells like glia, there is also a very small CNS-resident population of T cells, B cells, NK cells, and dendritic cells that can be activated and expand upon inflammatory insults [46–49]. Should inflammation persist, evidence has shown that inflammatory molecules released by resident cells can recruit an influx of immune cells from the periphery or stimulate the production of immune cells from the bone marrow in the skull [50] to help put out the inflammatory fire [51]. In certain clinical trials, particularly with CSF administration routes,

cellular infiltration has been observed in the CSF of patients in response to AAV treatment [25,52]. In addition, the CNS has a functional lymphatic system in the meninges that allows immune cells to drain from the CNS into the deep cervical lymph nodes, further demonstrating the tight, albeit separate, interaction with the periphery [53]. Despite the robust cross-talk between the periphery and the CNS, the BBB imparts specific immune privileges to the CNS, including blocking antibodies from crossing into the CNS [43]. As mentioned previously, the development of NAbs from gene therapy or prior natural AAV infection can limit the effectiveness of gene transfer. However, AAV injection directly into the brains of patients with pre-existing immunity has been successful, indicating that it is possible to evade pre-existing immunity with the correct administration route. These characteristics of the CNS may explain differences in responses to AAV gene therapy after direct vs. systemic administration.

CNS-Directed AAV Clinical Trials

For disorders with an affected CNS, there are multiple routes of administration to consider, including intraparenchymal (IP), intracerebrospinal fluid (CSF), and systemic or intravenous (IV). The administration route will have a profound effect not only on the efficacy of AAV gene therapy, but also the immune response and safety profile.

Many factors influence the immune response to CNS-directed AAV gene therapy and comparing differing administration routes is difficult when so many different factors are at play. Major differences between direct-CNS injections (parenchyma and CSF) and systemic include dose, the influence of pre-existing immunity, and biodistribution (Fig. 1). Despite the breadth of preclinical work testing the efficacy and safety of these AAV therapies prior to progression into clinical trials, immune responses are either not observed, or not investigated and characterized, leading to unforeseen reactions in human participants [28,54–60]. Thus, the following sections will focus on and carefully dissect the immune responses to CNS-directed AAV therapies across different anatomical administration routes.

Immune responses to intraparenchymal delivery

Pre-existing immunity is a major challenge that AAV clinical trials face when enrolling patients. The assays used to measure pre-existing and acquired humoral responses are total antibody assays (TAbs: anti-AAV, anti-transgene) or neutralizing antibody assays (NAbs) which measure antibodies that can specifically block viral entry into cells. Patients with pre-existing immunity to AAV who would traditionally be excluded from clinical trials as the antibodies would prevent transduction, often are not excluded from clinical trials with intraparenchymal delivery, owing to the fact that antibodies cannot cross the BBB and therefore cannot block transduction in the brain [61]. In particular, an AAV2-GAD therapy in Parkinson's Disease patients demonstrated that although 2 patients had anti-AAV2 immunity prior to administration, levels of antibodies did not appear to change post-administration, even without an immunosuppressive regimen [62]. However, some intraparenchymal studies still choose to exclude patients with pre-existing immunity, which is a more conservative route when trying to avoid immune responses that may affect the success of the therapeutic [63,64]. Still, the cutoff for exclusion is rather high, with two studies only excluding patients with baseline NAb titers of 1:1200 or higher [63,64]. In contrast, in an IV-delivered AAV clinical trial for Hemophilia, transgene expression was attenuated in a patient with AAV2 NAb titers of 1:17 [65]. Thus, a unique advantage to delivering into the parenchyma is the ability to circumvent pre-existing immunity and

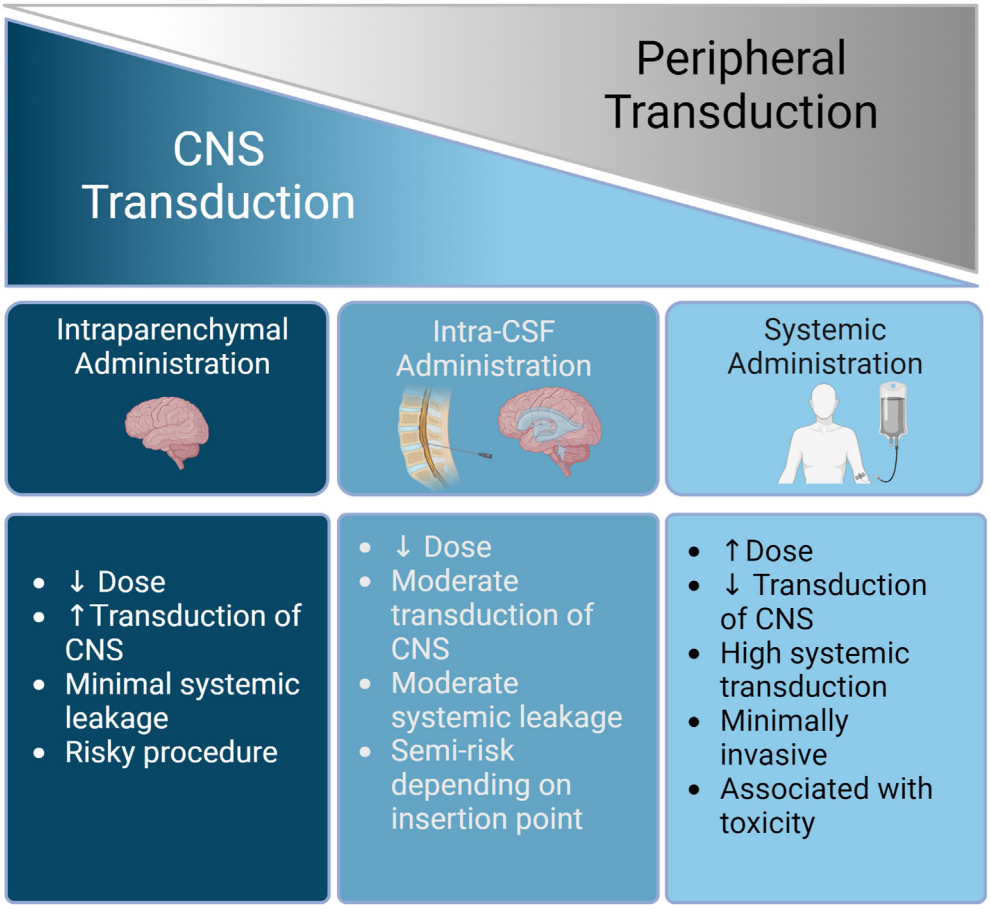

Fig. 1. Comparison of different AAV administration routes to target the CNS.

treat a larger patient pool. While some trials mentioned above tested for NAbs prior to AAV administration [63,64], others have not tested for pre-existing immunity prior to direct brain administration [18,62,66–80]. Although pre-existing immunity may not interfere with viral transduction when delivering to the CNS like it would the periphery, it is not totally clear if patients with pre-existing immunity may have a differing immune response than patients who have no previous exposure. It is important to have data on a patient's antibody levels pre- and post-gene delivery when evaluating therapeutic benefit to further understand how pre-existing immunity may affect gene therapy long term. In addition, while both total and neutralizing antibody assays provide valuable information regarding immune responses to AAV therapy, standardization of assays used to measure both pre-existing and acquired B cell responses would allow for more direct comparisons across trials [81]. For example, Zolgensma (FDA-approved IV delivery for SMA) has exclusion criteria based on total antibodies, whereas Upstaza (EMA-approved IP delivery for AADC, FDA accepted biologics license application (BLA) and has granted priority review for the end of 2024) has exclusion criteria based on NAbs. Thus, even at the level of FDA-approval, there are inconsistencies in the assessment of humoral responses.

Although intraparenchymal delivery can bypass some antibody responses, acquired B cell responses often occur after gene therapy. The large majority of these trials report that most patients develop mild levels of anti-AAV TAbs or NAbs in the serum following administration [18,64,71–73,82,83]. In the few instances where CSF is available for analysis post-administration, no detectable levels of NAbs are reported [18]. In some trials, patients exhibited no changes in anti-AAV TAbs or NAbs in the serum throughout the study [62,77,80]. These results suggest that the intraparenchymal route may allow for re-dosing, although that has not been well characterized [84]. In other trials, high levels of anti-AAV antibodies have been observed in patients after AAV treatment, although it appears unrelated to pre-existing immunity [75]. One possible explanation is that there may be a dose-dependent humoral response. For example, an AAV2-NGF trial for Alzheimer's Disease (AD) reported no anti-AAV2 or anti-NGF IgG after 1.2×10^{10} and 1.2×10^{11} administration [80], while another reported that the higher dose 2×10^{11} resulted in 5 patients with anti-AAV2 Abs [82]. However, this doesn't explain differences in response between patients that receive the same dose in the same trial [64,74,83]. There also does not appear to be a difference in response between patients who receive [26] or do not receive [18,62,64,71,73,80,82] immunosuppressive drugs. Though it should be highlighted that the majority of trials for intraparenchymal delivery do not include immunosuppressive drugs yet still see mild immune reactions. Regardless of the degree of humoral response to AAV administration, clinical outcome is not affected [18,62,71–73,82,85]. While it may still be important to assess total antibody levels and NAbs following administration, they do not appear to be strong indicators of clinical outcome.

In trials where T cell responses are investigated, it appears that some patients have at least a mild, spontaneous, capsid-specific, T cell response [86], while others have a transgene-specific T cell response [87]. In a clinical trial for Batten disease, patients received intraparenchymal AAVrh10-CLN2 and spontaneous, mild, capsid-specific T cell responses were observed in peripheral blood mononuclear cells (PBMCs) as measured by interferon-γ enzyme-linked immunosorbent assays (ELISpot) [86]. Although this study did not detect transgene-specific T cell responses, another study using AAV5-NAGLU to treat mucopolysaccharidosis type IIIB syndrome (MPS IIIB) demonstrated circulating CD4 and CD8 T cells secreting IFN-γ or TNF-α in response to NAGLU or NAGLU-derived peptides at 48 and 66 months [26,87]. This difference could be due to the nature of the transgene, as it is possible that patients with MPS IIIB have less tolerance to NAGLU peptides. In dogs, intracerebral therapy resulted in an immune response against NAGLU due to the lack of immune education to NAGLU during development and prior to treatment [88]. This rejection of NAGLU-expressing cells in pre-clinical trials resulted in the immunosuppression of patients in the MPS IIIB trial with oral tacrolimus and oral mycophenolate starting 14 days before AAV, and prednisolone 1 day before to 10 days following therapy. Despite this robust immunosuppressive regimen, these patients still developed inflammatory T cells responding to NAGLU as described above. Whereas, in the Batten trial, patients were not immunosuppressed and developed mild capsid-specific responses. These studies used two different methods for evaluating T cell responses and more uniform monitoring of cellular responses to AAV gene therapy moving forward would allow for more direct comparisons between trials. Despite these differences, both studies reported that these T cell responses had limited effects on transgene expression and the clinical success of the therapy [86,87]. More studies are necessary to understand the role of capsid and transgene specific T cell responses on transgene expression, particularly since these T cell immune responses are measured from peripheral samples.

Few trials look at the local immune response in the CNS, which is not entirely the fault of the studies, but rather the inability and invasiveness of collecting CNS tissue and CSF. In studies where CSF was isolated, there was a noted absence of abnormal cells, protein, or electrolytes, and no signs of inflammation [18,85,87]. In the Batten trial, baseline levels of certain inflammatory cytokines and chemokines were found at high concentrations, likely due to disease-mediated neuroinflammation, however, only one of four patients saw increases in IFN-γ and CXCL10 in the first year post-AAV, indicating that the therapy did not influence neuroinflammation in the majority of patients [87]. MRI findings have indicated no signs of oedema, inflammation, or signs of local necrosis [26]. Thus, it appears that injection of AAV directly into the brain does not induce any excess migration of immune cells or the release of inflammatory markers into the CSF. However, other studies have noted MRI findings of unknown significance causing some concerns over safety of this route [86,89].

While directly injecting AAV into the brain appears to have a very attractive immune profile, based on evading pre-existing immunity, which is prevalent in most patient populations, limited seroconversion, mild and occasional T cell responses to capsid or transgene, and the lack of inflammation and cell recruitment in the CNS, one obstacle to intraparenchymal delivery is the route itself. Although brain structures are interconnected, broadly transducing the CNS is challenging with intraparenchymal injections, even when injecting AAV bilaterally or at multiple anatomical sites [26,90]. Across studies, patients reported intracranial hemorrhages and headaches due to the surgical procedure [66,71,74,75,83]. However, even this was variable, with multiple studies showing that hemorrhaging was not apparent post administration and patients did not exhibit any notable adverse events related to the surgery [67,72,80]. Although intraparenchymal delivery is quite invasive, if we focus solely on the immune response, both systemically and locally in response to direct brain injection, it is clear that this injection route is favorable. Further supporting this argument is the limited use of immunosuppressive drugs in many of these trials. Even without immunosuppression, which can contribute to dangerous off-target side effects, these patients mount minor immune responses that do not appear to directly contribute to the efficacy or safety of the therapeutic. It is important however to note that there have been brain MRI findings of unknown significance after IP injections of AAV [86,89]. It appears that these findings did not affect clinical outcomes, however, further investigation is warranted to determine if this administration route has any other unforeseen drawbacks. There are many other active AAV clinical trials utilizing intraparenchymal delivery for neurological disorders including for different types of MPS [91], Alzheimer's Disease and frontotemporal dementia (NCT05040217; NCT06064890), and Huntington's Disease [92](NCT04120493). Immune responses to these therapeutics have yet to be reported.

Immune responses to cerebrospinal fluid delivery

While IP delivery of AAV is beneficial for disorders that affect certain brain regions, it is not as effective at transducing the hindbrain and spinal cord. CSF injection on the other hand may result in more global CNS transduction [54]. Three predominant routes of administration into the CSF include intracerebroventricular (ICV), intra cisterna magna (ICM), and intrathecal (IT) delivery. It is important to note however, that the blood-CSF barrier (BCSFB) is more permeable when compared to the blood-brain barrier. Thus, the CSF injection route can result in a stronger systemic immune response, similar to what is observed in IV delivery trials.

Although the BCSFB may be more permeable compared to the BBB, patients with pre-existing immunity to AAV have still been treated with AAV gene therapy in the CSF [25,52]. In trials for both Amyotrophic Lateral Sclerosis (ALS) and Giant Axonal Neuropathy (GAN), patients, with or without positive NAb titers at baseline were treated with either intrathecal AAVrh10-miR-SOD1 or intrathecal AAV9-JeT-GAN respectively [25,52]. In the GAN trial, participants who were baseline AAV9 seropositive had higher anti-AAV9 NAb titers at 3 weeks in the serum compared to those who were seronegative, indicating that seropositive individuals may be prone to stronger humoral responses after CSF-AAV administration [52]. Additionally, vector clearance occurred faster in participants who were originally seropositive [52]. This is in stark comparison to patients who received gene delivery through intraparenchymal delivery, where minor to no changes in B cell responses were observed, regardless of baseline anti-AAV titers or immunosuppressive regimen. In the ALS trial, two patients were treated and the one with pre-existing immunity had a very different immune response to AAV therapy. However, the patients received very different immune suppression which may have had an influence. Patient 1 lacked serum AAVrh10 NAbs and total capsid antibodies at baseline [25]. However, by 8 months, their NAb titer peaked at over 1:150,000 suggesting robust acquired B cell immunity. This is in contrast to patient 2 who had a NAb titer of 1:160 at the outset, but had blunted generation of NAbs compared to patient 1 (approximately 1/16th of the increase in titer in patient 1) [25]. While this would indicate that baseline NAbs were not responsible for the spike following administration, a confounding factor is the administration of additional immunosuppressive drugs in patient 2. Patient 1 received an IV dose of methylprednisolone at the time of treatment, followed by another dose of methylprednisolone the following day and oral prednisone daily thereafter, but developed meningoradiculitis, pain syndrome, and potentially dorsal root ganglion (DRG) toxicity, indicating that intrathecal infusion of this vector may lead to an adverse inflammatory response. In an attempt to prevent an adverse immune reaction in patient 2, rituximab and sirolimus were administered prior to treatment with AAV-miR-SOD1, and sirolimus and prednisone were continued post-treatment [25]. The blunted NAb response in patient 2 is likely attributed to the addition of rituximab, which is an antibody that specifically depletes B cells. It is however, important to note that although the NAb response was significantly lower in patient 2 compared to patient 1, the addition of rituximab and sirolimus did not completely abolish the anti-AAV NAb response, as their titers still peaked at 1:10, 240 at ~45 weeks post-treatment [25].

While few intraparenchymal trials investigated T cell responses to AAV, both the ALS and GAN trials reported capsid-specific ELISpot data. In the ALS trial, both patients were reactive to AAVrh10 peptide pools, however patient 1 had a significant spike in positivity 10 weeks post-AAV, which correlated with a rise in ALT, that subsequently tapered [25]. It is likely that the lowering of the ALT and ELISPOT response in the following weeks could be attributed to the increase in prednisone that patient 1 began receiving ~5 weeks post administration in response to pain syndrome described above that began around 4 weeks. Patient 2's stable, yet positive ELISpot responses and lack of transaminase elevation may also be attributed to the more robust immunosuppressive regimen that they received [25]. Similarly, 12 out of 13 patients in the GAN trial had increased capsid-specific IFN-γ ELISpot responses [52]. Interestingly, patients that received rapamycin and tacrolimus had decreased IFN-γ responses to capsid, but the response was not completely diminished, indicating that although powerful, increased immunosuppression cannot completely eradicate capsid-specific T cells [52]. This data, in conjunction with the data from the ALS trial, supports the use of immunosuppression to dampen unwanted immune responses. Compared to intraparenchymal delivery, trials with CSF delivery often had more robust immunosuppressive regimens. However, even in trials where immune suppression was not used for intraparenchymal delivery, they had fewer and milder T cell responses when compared to those who received AAV in the CSF. While the different immunosuppressive regimens make comparing the responses directly across delivery routes challenging, we can deduce that T cell responses in patients who receive CSF gene therapy are more frequent, more robust, and likely require enhanced immune suppressive drugs, like rituximab and sirolimus, to control the response.

In addition to immune responses observed in peripheral samples, samples collected from the CNS (either from spinal taps or autopsy) from patients who received AAV gene therapy in the CSF show cellular infiltration and inflammation [25,52]. Post-mortem analysis for patient 1 in the ALS trial (15.6 months after treatment) revealed gliosis throughout the spinal cord and motor cortex, as well as T cell infiltrates in proximal nerve roots [25]. However, neuroinflammation is common in ALS patients even without therapy, thus it is hard to determine if this is disease specific or treatment related. All 13 patients in the GAN trial exhibited lymphocyte infiltration in the CSF between 3 and 6 months post-administration, though they were asymptomatic and resolved within a year [52]. Together, T cell infiltration is observed in the CSF of patients who received CSF delivered AAV but was not frequently observed in patients that received AAV intraparenchymal.

Some studies have further combined these routes, intraparenchymal and CSF, to take advantage of targeting both deep brain structures as well as more broad distribution. In a compassionate use trial for Tay-Sach's disease [93], patients received AAVrh8-HEXA/AAVrh8-HEXB via combination intraparenchymal delivery to the thalamus and CSF delivery. Similar to intraparenchymal and other CSF delivery routes, patients were not screened for pre-existing antibodies, and 1 out of 2 patients developed elevations in NAbs following administration. Despite robust immunosuppression with rituximab, methylprednisolone, sirolimus, and prednisone, these patients both developed mild, occasional, capsid-specific, but not transgene-specific ELISpot responses. Preliminary data presented at ASGCT from a phase I dose escalation trial using the AAVrh8-HEXA/AAVrh8-HEXB vector showed that all patients developed NAb elevations and IFN-γ positive ELISpot responses post-AAV delivery [94]. It is possible that the increase in response could be dose dependent, as the patient treated with the lowest dose, similar to the compassionate use trial, had a delay in ELISpot response compared to other patients who were treated at higher doses. Positive ELISpots were commonly accompanied by elevations in liver transaminases, indicating cytotoxic T cell responses and were treated with additional steroids. However, several patients at varying timepoints had increases in circulating capsid-specific Tregs as measured by flow cytometry. Despite T and B cell responses following therapy, patients in the Tay Sach's trial still showed clinical benefit, potentially being attributed to the immunosuppressive regimen, the induction of capsid-specific Tregs, or both. Tregs have not been investigated in other CNS-directed clinical trials, but have been observed after intramuscular AAV delivery [23], and due to the tolerogenic nature of the CNS, it is possible that targeting the CNS may induce tolerance. Future studies investigating tolerance in the CNS could be incredibly beneficial for long-term transgene expression and success of AAV therapy.

Overall, the CSF is an attractive delivery route for more broad transduction of the CNS when compared to more direct, targeted intraparenchymal delivery. While injecting into the CSF is still more targeted than systemic delivery, the blood-CSF barrier is much leakier than the

blood-brain barrier, which has led to more systemic immune activation [25,52]. Compared to intraparenchymal delivery, CSF delivery leads to elevated NAb titers, consistent capsid-specific T cell responses, and T cell infiltration into the CNS [25,52]. Further, these immune responses are observed despite robust immune suppression, whereas intraparenchymal delivery was rarely accompanied by immunosuppressive drugs, and yet fewer inflammatory responses were observed. Despite the increase in immune responses in CSF trials compared to intraparenchymal, CSF delivery still avoids liver and heart toxicities that are common in systemic delivered therapies. Other clinical trials employing the intra-CSF route include ICV injection of AAV targeting Oligodendrocytes in Canavan Disease (NCT04833907), a dose escalation study in Tay Sachs or Sandhoff Disease (NCT04669535), IT injection for adrenomyeloneuropathy (NCT05394064), and Apolipoprotein E2 via CSF for Alzheimer's Disease (NCT03634007). Additionally, trials employing dual CSF/IV administration have been conducted, with few immune responses recorded [15]. In conjunction with the current knowledge, understanding the safety and immune responses to AAV in these ongoing trials will help inform decisions regarding administration route and immunosuppressive regimens for AAV gene therapy moving forward.

Immune responses to intravenous delivery

In order to protect the central nervous system, the BBB limits the entry of most systemically administered drugs. If the systemic administration route is desired, it is necessary to select an AAV capsid variant that can traverse the BBB. The discovery that AAV9, when administered by IV route is able to traverse the BBB, opened up therapeutic opportunities to target the CNS [42]. Currently, the most widely used AAV capsids for CNS delivery are AAV9 and AAV-rh10 because of their ability to cross the BBB [95]. With that said, both capsids still have limited capacity for crossing the BBB and transducing the CNS when delivered systemically, also potentially transducing a large number of off-target tissues. In addition, high titers of IV AAV are generally required to transduce sufficient cells in the CNS and this can cause significant toxicities associated with the heart and the liver [96,97]. Rapid developments in engineering AAV capsids has resulted in CNS tropic AAV capsids using approaches such as directed evolution, rational design, and in silico design [98–101]. These strategies leverage either natural or de novo receptor interactions to tailor capsid tropism and avoid toxicities associated with systemic delivery. Recently, an AAV capsid, BI-hTFR1, was engineered to engage the highly abundant CNS cell surface protein, human transferrin receptor, to cross the BBB and deliver transgenes to neurons and glia [98]. Many additional CNS-tropic AAV capsids have been engineered in recent years and have been reviewed elsewhere [102]. In spite of these advancements, many of these engineered AAVs have not yet been translated into the clinic but could be a promising tool for systemic AAV delivery to the CNS [42]. Despite these limitations, systemic delivery of AAV is optimal for targeting the brain as it is minimally invasive and effective in global transduction of the CNS.

While many clinical trials are ongoing for CNS diseases using IV delivery (Canavan Disease (CANaspire, NCT04998396), GM1 Gangliosidosis (NCT03952637), and Alzheimer's Disease (NCT04133454)), there is a multitude of data that has been published for AAV gene therapy in SMA. SMA is a progressive genetic motor neuron disease caused by a deletion or mutation in the survival motor neuron 1 (SMN1) gene which leads to failure in voluntary motor functions and can lead to fatality by 2 years of age. AAV9's ability to bypass the BBB has been shown to be a successful therapy for delivering a functional SMN1 gene to motor neurons in SMA disease. Thus, in 2019 the FDA approved a single dose IV administration of AAV9-SMN, Zolgensma, for treatment of SMA in pediatric patients [103]. The first AAV clinical trial for SMA (START) enrolled 15 patients with SMA type 1, where they received a single IV dose of AAV9 carrying SMN gene [27]. While SMA predominantly affects the CNS, it also affects other systems like the cardiovascular system [104], making IV delivery a favorable route for targeting multiple organs. While clinical outcome data for these trials is widely available, immune responses have been more limited.

Systemic delivery is hindered by the presence of NAbs unlike delivery strategies discussed above, therefore, exclusion criteria for Zolgensma is outlined that patients must have an anti-AAV9 antibody titer less than 1:50 [27,105–108]. While not surprising, this criterion may exclude significantly more patients than trials using intraparenchymal or intra-CSF administration routes. As seen in previous AAV clinical trials for disorders such as Hemophilia, enrolling patients into studies with pre-existing immunity may inhibit transduction of cells and attenuate transgene expression, limiting the success of the therapy [65]. Patients enrolled in the START trial who had post-treatment assessments had elevated anti-AAV9 antibodies in response to IV delivery [109], which has been seen in other administration routes for CNS-directed therapies, although to lesser degrees with CSF and intraparenchymal delivery. Elevated anti-AAV9 antibodies were also reported in the STR1VE-US clinical trial to varying degrees between patients, with the highest titer being 1:12,800 [105].

Pre-clinical safety testing of AAV9-SMN in non-human primates reported no T cell immune response as tested by ELISpot at 6 months [110]. However, in the START trial, subject 1 had a sudden ELISpot response to the AAV9 capsid, accompanied by a spike in AST and ALT 30 days post-dosing [27]. This ELISpot response and elevations in liver enzymes in 3 other patients in cohort 2, were attenuated by prednisolone [27]. This urged an amendment in treatment plans for upcoming patients to be placed on prophylactic prednisolone treatment and for 30 days following AAV delivery. If AST and ALT exceeded 120 IU/L and T cell responses were above 100 SFUs per 10^6 PBMCs after 30 days, this amendment outlined guidelines for maintaining prednisolone until these responses fell below these levels [27]. Running ELISpots in a timely manner better informs clinical decisions and trials can adjust immune suppression as needed in response to cytotoxic T cell responses. Data from the STR1VE-US trial indicates that cellular responses were observed, however the lack of pre-screening samples limited interpretation of post-administration responses [105]. Running timely cellular response assays provides a significant benefit to patients and the success of the therapy, allowing clinicians to respond to immune responses in real time.

As mentioned, elevations in liver enzymes have been reported in multiple trials for IV delivery of AAV9-SMN for SMA [27,105–108]. Elevations in liver enzymes are indicative of hepatotoxicity, which is a major drawback of IV administration. In clinical trials, 90% of patients saw post-dosing elevations in liver function tests, and post marketing data shows that there were 337 cases of isolated liver function test elevations [96]. Further there were multiple cases of acute liver failure.

Another adverse event observed in the IV delivery of AAV for SMA that was not observed via other administration routes is thrombocytopenia and TMA. In clinical trials, two patients had thrombocytopenia, with one patient presenting in combination with multiorgan failure and sepsis. Post marketing analysis reported there have been 134 cases of thrombocytopenia, including 4 patients who developed TMA, and 2 of the patients after recent infections [96]. Incidences of TMA showed the presence of complement activation by the alternate pathway in some cases [97]. This indicates activation of an innate immune response associated with AAV in these trials [97]. TMA had not previously been observed in the clinical trials.

Further, high dose AAV therapy may be unavoidable for IV delivery of gene therapy to the brain, as high titers may be required to cross the BBB and transduce a sufficient quantity of cells in the CNS. However, no ganglionopathies were observed in any clinical or post-licensure studies [96]. The STR1VE-EU trial confirmed previously observed safety risks, including hepatotoxicity, transient thrombocytopenia, and cardiac adverse events [106]. While a profound number of patients that receive systemic AAV therapy have significant elevations in liver enzymes that require steroid intervention, only one patient that received intraparenchymal AAVrh10-CLN2 for Batten disease had transient elevations in liver enzymes that resolved spontaneously and without treatment

[86]. Post-licensure, Novartis reported that the death of 2 patients treated with Zolgensma occurred due to acute liver failure. This incident prompted Novartis and the FDA to update their prescription label with a warning of acute liver failure as a known side effect for the treatment [111]. Thus, we can conclude that delivering AAV intravenously comes with incredibly high risk of hepatotoxicity.

Despite these immune responses, Zolgensma has been approved by the FDA for the treatment of SMA, due to impressive clinical outcomes [27,105–108]. IV delivery remains an attractive delivery route because it is minimally invasive and can globally transduce cells throughout the body, including the brain. However, there are significant toxicity risks associated with this route, including but not limited to hepatotoxicity, cardiac toxicity, and TMA. High-doses of AAV are required to transduce the brain when delivered IV, and these high doses may directly contribute to these toxicities. The future implementation of more CNS-tropic AAV vectors, delivered at lower doses, could potentially reduce immune responses and create safer IV-delivered therapies.

With 2 approved therapies targeting the CNS, one in the US and one the EU, and many additional trials ongoing with potentially promising clinical results, AAV remains an attractive option for the treatment of neurological disorders. Yet, adverse reactions have been observed in clinical trials, particularly at high doses, and unexpected findings have been observed in the CNS prompting the need to further characterize the immune response to AAV. However, characterizing immune response to AAV in the CNS, as well as measuring the effects of the immune response on therapeutic benefit remains difficult.

Multiple factors influence the immune response to CNS-directed AAV gene therapy and comparing different administration routes is challenging when so many different factors are at play. Major differences between direct-CNS and systemic injections include dose, the influence of pre-existing immunity, immunosuppressive regimen, and biodistribution. The benefits of direct injection into the CNS include lower dose and more direct cell transduction, fewer off-target toxicities associated with heart and liver, and the potential to circumvent systemically circulating NAbs. In many intraparenchymal clinical trials, antibody conversion was not observed suggesting that very little if any vector leaked out of the CNS [18,68]. However, it is also important to consider that different trials are using different B cell assays, either binding or neutralizing, to evaluate responses, and sensitivity and standardization may vary making it more difficult to compare results across trials. Further, samples cannot be obtained from CNS tissue except for post-mortem, making it harder to understand what is going on locally in the CNS. Additionally, directly injecting into the CNS results in a more limited transduction area, and it is a more invasive procedure that can lead to adverse events. However, many studies, outlined in Table 1, show limited to no immune suppression was used in intraparenchymal injection strategies and with little to no indication of adverse immune responses. Nonetheless, it is likely that different brain structures may be more tolerant to both surgical procedures as well as immune toxicities. The environment in which the immune system first comes into contact with AAV and pre-existing immunity status, will have a substantial impact on the immune response to the AAV gene therapy. Differing delivery routes will certainly influence immune response if the AAV is encountered in the periphery versus in the CNS. It is also important to remember that substantial neuroinflammation is present in almost all of the diseases that CNS-directed AAV therapies are trying to treat, which will also influence the immune response [112]. This is incredibly difficult to model in animal models as most rodent models do not accurately reflect immune responses observed in clinical trials, and most large animal modeling is done on healthy, non-diseased animals, nor do they have similar immune responses to AAV as humans. However, overall, the CNS is considerably more tolerant than other tissues, and has physical constraints of the skull that prevents significant cellular infiltration and swelling. Likely the unique environment of the CNS as well as the unique disease state of the CNS defines different immune responses in individual patients.

Table 1
Immune responses to AAV gene therapies for CNS disorders.

Trial	Route	AAV	Dose	Immunosuppression	Post AAV-antibodies	Post AAV T-cell responses	Citation
Canavan	IP-frontal periventricular occipital lobes	AAV2-ASPA	Cohort 1: 8.0E11 vg Cohort 2: 1.0E12 vg	None	3/10 patients had anti-rAAV 2 NAbs	Not measured	18
Canavan	IV and ICV	AAV9-ASPA	IV: 4.5E14vg/kg ICV 5.E13vg	Rituximab Sirolimus Solumedrol and prednisolone to day 5	0/1 No changes in anti-AAV9 IgG levels compared to baseline Slight increase in anti-AAV9 IgM levels on day 14	Not measured	15
PD	IP-subthalamic	AAV2-GAD	Cohort 1: 1.0E11 vg/mL Cohort 2: 3.0E11 vg/mL Cohort 3: 1.0E12 vg/mL	None	2/12 patients showed high titers of pre-existing anti-AAV2 TAbs and NAbs which remained unchanged post delivery	Not measured	62
PD	IP-putamen	AAV2-AADC	Cohort 1: 9.0E10 vg Cohort 2: 3.0E11 vg	None	Not measured	Not measured	66
PD -Japan version	IP-putamen	AAV2-hAADC	3e11 vg	None	6/6 patients had mildly increased anti-AAV2 NAbs titers at 6 months which decreased toward baseline by 1 year	Not measured	71
PD -Taiwan version	IP-putamen	AAV2-hAADC	1.6E11 vg	None	Phase 1: 2/4 developed slightly increased titers of anti-AAV2 NAbs Phase I/II:10/10 showed increased titers of anti-AAV2	Not measured	72

(continued on next page)

Table 1 (*continued*)

Trial	Route	AAV	Dose	Immunosuppression	Post AAV-antibodies	Post AAV T-cell responses	Citation
					antibodies post vector infusion, which declined by 6-months		
PD -Upstanza	CED to substantia nigra and ventral tegmental area	AAV2-AADC	Cohort 1: 1.3E11 vg Cohort 2: 4.2E11 vg	None	Not measured	Not measured	67
PD	IP-putamin	AAV2-NTRN	5.4E11 vg	None	10/58 Increased titer of anti-AAV2 in serum was noted	Not measured	74
PD	IP-Nucleus basalis of Neynert	AAV2-NGF	Phase I Cohort 1: 1.2E10 vg Cohort 2: 5.8E10 vg Cohort 3: 1.2E11 vg	None	Phase I: 0/10 Phase II: 5/49	Not measured	80
Batten	12 white matter sites	AAV2-CLN2	1.8 to 3.2E12 vg	None	4/10 (2 patients developed anti-AAV2 NAbs at 1 month and other 2 at 6 months post AAV delivery	Not measured	85
Batten	IP	AAVrh.10h-CLN2	2.85E11 to 9.0E11 vg	None	11/13 patients developed AAVrh.10 NAbs, 2 of which had higher titers whereas the rest had mildly detectable NAbs in the serum	2/13 patients develop positive IFNy ELISpot response against AAVrh.10 capsid peptide pools Elevated ALT and AST 1 patient at 6 months	86
Tay-Sachs	IP thalamus plus intrathecal	AAVrh8-HEXA/AAVrh8-HEXB	1e14 or 4.2E13 vg	Rituximab, methylprednisolone, sirolimus, prednisone for 90 days and sirolimus 180 days followed by taper.	0/2 Increase in anti-AArh8 NAbs occurred but they corresponded to timepoints when IVIG was administered	IFNy ELISPOT assay positive: Intrathecally injected patient showed mild positive IFNy ELISpot whereas, patient injected by IP and IT route showed positive response until 1 month	93
MPS IIIB	IP into cerebellum	rAAV2/5-NAGLU	Phase 1/2 4.0E12 vg	Tacrolimus, Mycophenolate mofetil	0/4 No NAb detected in patients	No proliferation of CD4 or CD8 T lymphocytes detected upon stimulation with NAGLU in an ex-vivo T cell proliferation assay	26
ALS	IT	AAVrh.10-mir-SOD1	4.2E14 vg	Pt1: IV methylprednisolone Pt2: Rituximab, methylprednisolone, sirolimus, prednisone for 90 days and sirolimus 180 days followed by taper	2/2 patients developed anti-AAVrh.10 capsid IgG 2/2 patients developed NAb titer, of which one had higher titers	IFNy ELISpot was positive in both patients, plus post-mortem cellular infiltration in patient 2	25
GAN	IT	AAV9-JeT-GAN	Cohort 1: 3.5e13 vg Cohort 2: 1.2e14 vg Cohort 3: 1.8e14 vg Cohort 4: 3.5e14 vg	1st 6 patients: IV methylprednisolone day 0 followed by prednisone for 4 weeks Pts 7–14: Above regimen but prednisone out to 4 months CRIM-negative patients: Above regimen plus tacrolimus and rapamycin	All patients in CSF and serum NAbs	12/14 patients saw elevations in IFNγ as seen by ELISPOT Patients that received T cell immunosuppression exhibited attenuated responses	52
SMA (START trial)	IV	AAV9-SMN	Cohort 1: 6.7E13 vg/kg Cohort 2: 2.0E14 vg/kg	Oral prednisolone, starting 1 day prior to vector administration until 30 days	Not measured post AAV administration	4/15 patients had elevated AST levels in serum	109
SMA (STR1VE trial - US)	IV	AAV9-scSMA	1.1.E14 vg/kg	Prophylactic prednisolone - tapered depending on liver function tests	Not measured post AAV administration	Transient increases in aminotransferases	96
SMA (STR1VE trial - EU)	IV	AAV9-scSMA	1.1E14 vg/kg	All patients received prednisolone one day prior to AAV administration and continued for 48 h post-infusion, lowered dose daily until 30 days	Not measured post AAV administration	9 patients exhibited increased alanine aminotransferase	106

Immune responses to CNS-directed AAV therapies are measured by B and T cell assays, which inform us on peripheral immune reactions, but do not provide insight to CNS-local responses. Although understanding of immune responses in the periphery is important, understanding if immune responses leading to some loss of transgene and cell death in the parenchyma, or conversely inducing tolerance is important. Some studies have evaluated cytokines/chemokines and antibody titers in the CSF [52,87,94,113], but repeated CSF collection is fairly invasive and additionally does not necessarily provide insight into the parenchyma. Some insight has been gained from samples of brain parenchyma from autopsies, but they have been fairly limited. Importantly, those that were obtained did show signs of DRG loss [25,52]. Identification of markers that suggest immune responses or toxicities in the CNS are necessary to better understand CNS-specific immune responses. It is generally well recognized that elevations of liver transaminases are indicative of potential cytotoxic T cell responses, however these elevations will only provide insight on peripheral immune clearance, particularly the liver and to some extent muscle, but are not indicators of clearance in the CNS [114]. Neurofilament light chain (NF-L) has recently emerged as a potential biomarker for DRG toxicity and should be considered in future clinical trials, however most neurological diseases already have elevations in NF-L so it may be difficult to distinguish disease from toxicity [115]. Additional biomarkers need to be identified to help determine CNS specific toxicities.

Taken together there is still a considerable number of unknowns about how the immune system recognizes and responds to AAV gene therapies targeted to the CNS, despite the approval of different gene therapies. Yet, CNS-directed gene therapies have been some of the most successful and safest therapies to date. Further development of validated and universally adopted assays are necessary, as well as the development of new assays and biomarkers which can be indicators of immune responses in the CNS but evaluated by minimally invasive techniques. Development of novel AAV capsids which are more CNS tropic and specific may additionally allow for less invasive methods to be utilized for delivery while still benefiting from some immunological advantages. Overall, the unique environment of the CNS provides some hurdles and some benefits, but it remains an important target for AAV therapeutic delivery.

Author contributions

Conceptualization, A.L.H. and A.M.K.; writing—original draft preparation: A.L.H. and A.M.K.; writing—review and editing, A.L.H., P.P.A. and A.M.K.; visualization, A.L.H., P.P.A. and A.M.K. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

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