

## **Title page**

### **Delivering efficient liver-directed AAV-mediated gene therapy**

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## **Manuscript**

Adeno-associated virus vectors (AAV) have become the leading technology for liver-directed gene therapy.<sup>1</sup> After the pioneering trials using AAV2<sup>2</sup> and AAV8<sup>3</sup> to treat haemophilia B, D'Avola *et al* recently reported the first-in-human clinical trial of adeno-associated virus vector serotype 5 (AAV5) in acute intermittent porphyria (AIP).<sup>4</sup> Treatment was reported as safe but the main surrogate biomarkers of AIP, porphobilinogen (PBG) and delta-aminolevulinate (ALA), were unchanged. This lack of efficacy contrasts with results from the haemophilia B trial using AAV8 capsid by Nathwani *et al.*, which showed a significant and long-lasting improvement of the clinical phenotype.<sup>3</sup> Haemophilia B is an amenable target for successful gene therapy as raising expression of plasma factor IX (FIX) level above 1% can modify the phenotype from severe to moderate.<sup>3</sup> Development of a variety of capsids for clinical application is useful to overcome pre-existing neutralising antibodies. The differences in cell-specific transduction by different AAV serotypes are primarily due to specificities in cellular uptake or post cell-entry processing. Indeed AAV5 presents several theoretical advantages as an alternative capsid to AAV8 for liver-directed gene therapy: suitable liver tropism, less off-target biodistribution,<sup>5</sup> low seroprevalence in humans and minimal cross-reactivity with other serotypes.<sup>6</sup>

### **Reliability of animal models in capsid testing**

The reliability of the available animal models for comparison of transduction of the liver by different AAV serotypes has been questioned<sup>7</sup>. In the AIP trial<sup>4</sup>, the high-dose group received  $1.8 \times 10^{13}$  vg/kg, which is equivalent to the therapeutic threshold needed to achieve a correction of the murine phenotype ( $1.25 \times 10^{13}$  vg/kg)<sup>8</sup> but lower than that required for supra-physiological enzymatic activity in Rhesus macaques ( $5 \times 10^{13}$  vg/kg)<sup>5</sup>. AAV5 is currently used

in a clinical trial for haemophilia B with the same transgene cassette used by Nathwani *et al.*<sup>9</sup> Nine months post-infusion, the low-dose group, who received  $5 \times 10^{12}$  vg/kg, showed a plasma FIX of 5.4% (range 3.1%-6.7%; n=5)<sup>9</sup> which is similar to the level observed in the high-dose group of the AAV8 trial receiving  $2 \times 10^{12}$  vg/kg (plasma FIX of 5.1%, range 2.9%-7.1%; n=6) 4 months post-infusion.<sup>3</sup> These results suggest that, to obtain similar plasma FIX levels to those achieved in AAV8 trial, administration of 2.5-fold more AAV5 vector is necessary.

Although this assumption is made on the basis of a small number of treated subjects, and confounded by different methods of production, titration and purification, it supports data obtained after intravenous injection in different animal models:

i) In murine models of AIP, AAV5 resulted in ten-fold less liver transduction compared to AAV8.<sup>8</sup>

ii) In Gunn rats, AAV5 vector was inefficient at restoring metabolic activity and achieved 3 times lower copy number compared to AAV8.<sup>6</sup>

iii) In Rhesus macaques, AAV5 vector produced slightly lower plasma FIX in adult animals with slower kinetics compared to AAV8,<sup>10</sup> lower hepatocyte transduction after fetal intrahepatic venous injection and higher plasma FIX 2 months post-injection ( $<1 \mu\text{g/mL}$  (n=3) versus  $5 \mu\text{g/mL}$  (n=1)).<sup>11</sup>

iv) In *Fah<sup>-/-</sup>/Rag2<sup>-/-</sup>/Il2rg<sup>-/-</sup>* (FRG) mice, AAV5 achieved transduction of ten-fold fewer of human hepatocytes than AAV8 (0.1% versus 1.1% respectively).<sup>12</sup>

Further results from larger human trials will provide further information on the reliability of animal data, which will accelerate the development of liver-directed gene therapy.

### **Episomal versus endogenous gene expression**

D'Avola *et al* are the first to report data from human liver biopsies after AAV treatment.<sup>4</sup>

Interestingly, the liver vector copy number one year post-injection did not correlate with the

escalating doses of vector received. This finding is in contrast with the studied tissues from animal models<sup>5, 8</sup> or plasma FIX levels in haemophilia B trial.<sup>3</sup> In liver biopsies with high vector copy number of the transgene codon-optimised PBG deaminase (*coPBGD*) (Patients 2, 5, 7), *coPBGD* mRNA expression compared to endogenous *PBGD* (normalised by DNA copy number) was lower by 45%, 76% and 36% respectively.<sup>4</sup> In AAV-mediated gene therapy, most of the transgene DNA copies persist as non-integrated episomes. Different episomal expression compared to the endogenous gene of interest underpins results observed in an ornithine transcarbamylase<sup>13</sup> deficient *Spf<sup>ash</sup>* mouse model. Untreated *Spf<sup>ash</sup>* mice with a 5-7% wild type residual OTC activity become hyperammonaemic after a shRNA-mediated knockdown of the endogenous OTC activity to 0-2.5%. In shRNA-injected *Spf<sup>ash</sup>* mice, the level of AAV-encoded OTC activity required to normalise ammoniaemia was threefold higher than the residual OTC activity in untreated *Spf<sup>ash</sup>* mice.<sup>14</sup> An AAV pattern of transduction not reproducing the physiological metabolic zonation of the liver might have played an additional role. Although these findings rely on a small cohort and require caution in interpretation, various explanations might account for a different episomal expression such as inadequate chromatinisation, incomplete circularisation of the AAV genome altering the constitution of the open reading frame for transgene expression, or inverted terminal repeats (ITR) recombination. The exact mechanism for this phenomenon is yet to be identified.

### **Functional metabolic assays as efficacy endpoints in clinical trials**

Finally, the use of metabolite levels as primary endpoint for trials in metabolic diseases can be questioned. These surrogate markers often reflect a static picture and remain indirect assessments of the metabolic flux and its environmental or epigenetic regulation. Indeed, heme biosynthesis is mainly regulated by heme-mediated inhibitory feedback of the transcription of ALA-synthetase but other parameters can exert an influence such as glucose

intake, stress, drugs, circadian rhythm<sup>15</sup> and may potentially affect ALA and PBG results. Thus whenever feasible, stable isotope studies would be better indicators of the *in vivo* dynamics of the pathway. For example, oral administration of N<sup>15</sup> labelled glycine can monitor the biosynthesis of heme and its intermediate compounds in physiology and patients with inherited porphyrias.<sup>16</sup> This approach has been successfully used in other metabolic pathways like the urea cycle to assess ureagenesis utilising either N<sup>15</sup> labelled urea in animal models after AAV-mediated gene therapy<sup>17,18</sup> or C<sup>13</sup> labelled acetate in humans for accurately stratifying the disease severity in OTC deficiency.<sup>19</sup> Furthermore, the use of clinically relevant endpoints would not only provide better assessment of the effect of therapy but may be viewed more favourably by regulatory bodies.

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