IgG-cleaving endopeptidase enables *in vivo* gene therapy in the presence of anti-AAV neutralizing antibodies

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69 **Abstract**

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71 Neutralizing antibodies (NAbs) to adeno-associated virus (AAV) vectors are highly
72 prevalent in humans^{1,2}, block liver transduction³⁻⁵ and vector readministration⁶, thus
73 representing a major limitation to *in vivo* gene therapy. Strategies aimed at overcoming
74 anti-AAV antibodies are being studied⁷, which often involve immunosuppression and
75 are not efficient in removing pre-existing antibodies. Imlifidase (IdeS) is an
76 endopeptidase able to degrade circulating IgG that is currently being tested in
77 transplant patients⁸. Here we studied if IdeS can eliminate anti-AAV antibodies in the
78 context of gene therapy. We showed efficient cleavage of pooled human IgG (IVIg) *in*
79 *vitro* upon endopeptidase treatment. In mice passively immunized with IVIg, IdeS
80 administration decreased anti-AAV antibodies and enabled efficient liver gene transfer.
81 The approach was scaled up to non-human primates, a natural host for wild type AAV.
82 IdeS treatment prior to AAV vector infusion was safe and resulted in enhanced liver
83 transduction, even in the setting of vector readministration. Finally, IdeS reduced anti-
84 AAV antibody levels from human plasma samples *in vitro*, including plasma from
85 prospective gene therapy trial participants. These results provide a potential solution to
86 overcome pre-existing antibodies to AAV-based gene therapy.

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89 **Main**

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91 Gene therapy can treat a variety of human diseases through systemic administration of adeno-
92 associated virus (AAV) vectors⁹⁻¹². Yet, pre-treatment neutralizing antibodies (NAbs) directed
93 against the AAV capsid pose a major limitation to AAV gene transfer, as they block target
94 tissue transduction even at relatively low titers³⁻⁵.

95 Here, we explored the use of the IgG-degrading enzyme derived from *Streptococcus pyogenes*
96 Imlifidase (IdeS)¹³ as a strategy to overcome the limitation of NAbs to AAV. IdeS is an
97 endopeptidase that cleaves human IgG into F(ab')₂ and Fc fragments, which was successfully
98 tested in clinical trials in the setting of antibody-mediated graft rejection⁸ and preclinically
99 heparin-induced thrombocytopenia¹⁴. Commercially sourced (IdeS-C) or lab-made (IdeS)
100 endopeptidases showed similar cleavage activity of purified human IgG (intravenous
101 immunoglobulin, IVIg) *in vitro*, resulting in a complete degradation of IgG into F(ab')₂ and
102 Fc at 24 hours (Fig. 1a) and a significant decrease in anti-AAV serotype 8 (AAV8) IgG
103 concentration (Fig. 1b), and NAb titer¹⁵ (Extended Data Fig. 1a). No effect of IdeS on AAV8
104 transduction of HEK293 cells *in vitro* was noted (Extended Data Fig. 1b). Because IdeS does
105 not cleave efficiently mouse IgG¹⁶, a first *in vivo* experiment was performed using a passive
106 immunization model of gene transfer¹⁷, in which C57BL/6 mice were infused with
107 neutralizing amounts of IVIg and then treated with IdeS prior to the administration of an
108 AAV8 vector expressing Gaussia luciferase (AAV8-GLuc, Fig. 1c). Consistent with human
109 studies^{18,19}, IdeS was cleared from the circulation within 24-48 hours from the infusion in
110 mice (Fig. 1d) and non-human primates (Extended Data Fig. 1c). Cleavage of IgG resulted in
111 detection of residual levels of single-chain IgG (scIgG) along with F(ab')₂ and Fc fragments
112 (Fig. 1d). Anti-AAV8 IgG and NAb titers were significantly decreased in IdeS-treated
113 animals (IVIg/IdeS cohort, Fig. 1e,f) and, accordingly, GLuc expression levels were in the

114 range of those of control animals receiving PBS followed by IdeS (PBS/IdeS cohort, Fig. 1g).
115 Conversely, animals treated with IVIg followed by PBS showed no transgene expression
116 (IVIg/PBS cohort, Fig. 1g). Vector genomes in liver were undetectable in IVIg/PBS animals
117 (Extended Data Fig. 1d). IVIg/IdeS-treated mice showed a partially decreased vector genome
118 copy number (VGCN) compared to PBS/IdeS controls (Extended Data Fig. 1d), likely due to
119 some degree of vector neutralization mediated by residual circulating IgG fragments which
120 were not detected in the NAb assay (Fig. 1d). The same experimental setting was also applied
121 to a therapeutically relevant mouse model of hemophilia B (HB), in which animals received
122 an AAV8 vector expressing human coagulation factor IX (hFIX) following passive
123 immunization with IVIg (Fig. 1c). In this setting, treatment with IdeS also resulted in
124 elimination of anti-AAV8 IgG and NAb (Extended Data Fig. 1e,f), with complete rescue of
125 transgene expression (Fig. 1h) and liver transduction (Extended Data Fig. 1g), and correction
126 of the bleeding diathesis (Fig. 1i).

127 We next tested IdeS in non-human primates (NHPs, *Macaca fascicularis*). NHPs are natural
128 hosts for AAV²⁰, and therefore carry anti-AAV NAb. Two male animals, NHP3 and NHP5
129 with similar anti-AAV8 NAb titers (1:17.2, Supplementary Table 1), were infused with
130 2×10^{12} vector genomes (vg)/kg of an AAV8-hFIX vector. Prior to vector infusion, NHP5
131 received two infusions of IdeS (Fig. 2a). Following IdeS administration, total circulating IgG
132 in NHP5 decreased significantly (Fig. 2b). IgG cleavage was confirmed by Western blot,
133 although a larger proportion of IgG and scIgG remained detectable (Fig. 2c) compared to
134 human IgG in mouse plasma (Fig. 1d). Despite the partial cleavage, anti-AAV8 IgG and NAb
135 titers in NHP5 decreased following IdeS treatment, while they remained unchanged in the
136 control animal NHP3 (Fig. 2d,e, horizontal dashed line). Accordingly, following vector
137 administration, hFIX transgene expression peaked and plateaued³ at significantly higher hFIX
138 levels in NHP5 compared to the control animal NHP3 (Fig. 2f). Consistently, VGCN analysis

139 in liver at sacrifice showed higher transduction levels in NHP5 compared to NHP3 (Fig. 2g).
140 The presence of anti-IdeS antibodies before treatment in NHP5 (Supplementary Table 1) did
141 not prevent rescue of liver transduction with IdeS. After vector administration, the IdeS-
142 treated animal NHP5 developed lower anti-AAV8 IgG and NAb titers than the control animal
143 NHP3 (Fig. 2h,i).

144 Two additional NHPs with anti-AAV8 NAb titers of 1:3.16 were then studied (NHP2 and
145 NHP6, Supplementary Table 1), to test the efficacy of IdeS at lower pre-existing antibody
146 titers. IdeS treatment resulted in digestion of total IgGs (Extended Data Fig. 2a), reduction of
147 anti-AAV8 IgG and NAbs (Extended Data Fig. 2b,c), and enhanced transgene expression and
148 liver transduction in NHP6 (Extended Data Fig. 2d,e). No change in anti-AAV8 IgG and
149 NAbs was observed in the control animal NHP2 (Extended Data Fig. 2b,c, horizontal dashed
150 line), which had lower transgene expression and VGCN than NHP6 (Extended Data Fig.
151 2d,e). Following vector administration, both animals had an increase in anti-AAV8 IgG
152 (Extended Data Fig. 2f) and NAb titers (Extended Data Fig. 2g), although in NHP6, the IdeS-
153 treated animal, the increase was mostly transient. Administration of vector alone or in
154 combination with IdeS was not associated with evidence of toxicities (Supplementary Table
155 2). Together, these results indicate that IdeS treatment reduces natural humoral immunity to
156 AAV and enhances AAV vector transduction upon systemic delivery in NHPs.

157
158 We next tested the IdeS technology in the context of AAV vector re-administration. A pilot
159 study was conducted in two animals, NHP1 and NHP4 (Extended Data Fig. 3a), with pre-
160 existing NAbs to AAV8 (1:31.6, Supplementary Table 1). NHP4 received IdeS prior to vector
161 administration, resulting in a significant decrease of anti-AAV8 IgG (Extended Data Fig. 3b).
162 No change in anti-AAV8 IgG was observed in control animal NHP1 (Extended Data Fig. 3b,
163 horizontal dashed line). Following the first vector administration, the IdeS-treated animal,

NHP4, showed higher circulating hFIX levels compared to the control animal, NHP1 (Extended Data Fig. 3c). Both animals developed anti-AAV8 IgG and NAbs following vector infusion, although the IdeS-pretreated animal, NHP4, developed significantly lower anti-AAV8 IgG (Extended Data Fig. 3d). At day 82 post-vector infusion, both animals received two doses of IdeS (500 µg/kg, one day apart, Extended Data Fig. 3a). In both animals, IdeS treatment resulted in the digestion of circulating IgG, although large amounts of scIgG and, to a lesser extent, IgG remained detectable (Extended Data Fig. 3e). Accordingly, anti-AAV8 IgG as well as NAbs titers decreased in both animals (Extended Data Fig. 3f,g). Following the second vector administration, an increase in hFIX circulating levels was observed in NHP4 (Extended Data Fig. 4a). NHP1 developed anti-human FIX antibodies prior to vector re-administration (Extended Data Fig. 4b and Supplementary Table 1), a phenomenon commonly seen in NHPs^{21,22}, thus preventing the measurement of hFIX circulating levels. Administration of IdeS in NHP1 resulted in a transient decrease in anti-hFIX antibody levels (Extended Data Fig. 4b), which did not result in detection of circulating hFIX (Extended Data Fig. 4a). VGCN measured in liver showed a higher transduction of hepatocytes in NHP4 compared to NHP1 (Extended Data Fig. 4c), reflecting the fact that NHP1 had residual antibodies to AAV8 at the time of both the first and the second vector infusions (Extended Data Fig. 3b,f,g). Following vector re-administration, NHP4, the animal that received IdeS at days -1, 82 and 83 (Extended Data Fig. 3a), did not develop anti-AAV8 IgG (Extended Data Fig. 4d) or NAbs (Extended Data Fig. 4e), and had a lower IgM response compared to NHP1 (Extended Data Fig. 4f).

A study in a large cohort of NHPs (*Chlorocebus sabaeus*) was then conducted. Eight animals were immunized with an AAV vector serotype AAV-LK03²³ at a dose of 2×10^{12} vg/kg at day 0, and then at day 210 randomly assigned to a PBS control group (n=3) or a IdeS treatment group (n=5) (Fig. 3a). Vector administration at day 0 resulted in development of a broad

range of anti-AAV-LK03 IgG and NAb titers (Fig. 3b-e). A single IdeS treatment resulted in a decrease of anti-AAV-LK03 IgG (Fig. 3b,c) and NAb (Fig. 3d,e) titers in all animals, while no significant change in titers was observed in the PBS treatment group. Accordingly, administration of an AAV-LK03 vector expressing human factor VIII (AAV-LK03-hFVIII) on day 211 resulted in significantly higher expression of the hFVIII transgene in the IdeS treated animals compared to controls (Fig. 3f-h). Following the second vector infusion, all animals developed anti-capsid IgM (Extended Data Fig. 5a,b) and IgG (Extended Data Fig. 5c,d). As previously described²⁴, hFVIII expression was transiently detected because of the formation of antibodies to hFVIII (Extended Data Fig. 5e,f).

Analysis of aggregated data from 6 NHPs, NHP2 and NHP3 having received also a double IdeS injection at day 35, showed, as observed in humans⁸, a good safety profile (Supplementary Tables 2-5) and a transient degradation of total IgG in all animals after IdeS administration (Extended Data Fig. 6a). All animals dosed with IdeS developed binding antibodies to the enzyme after treatment (Extended Data Fig. 6b).

Next, to study the effect of IdeS on anti-AAV IgG in humans, we treated plasma from healthy donors and from a cohort of Crigler-Najjar (CN) patients²⁵, a rare liver disease for which an AAV gene therapy trial is ongoing (NCT03466463). Following *in vitro* incubation with IdeS, anti-AAV8 IgG titers were significantly decreased in all subjects tested (Fig. 4a). The presence of anti-IdeS antibodies was then evaluated in sera from 52 healthy donors (Extended Data Fig. 6c), confirming the previously reported seroprevalence data¹⁸. Anti-IdeS antibodies did not affect the cleavage of IgG specific to the enzyme (Fig. 4b), although a less efficient degradation of IgG was observed in serum from NHPs after immunization against IdeS (Extended Data Fig. 7a-d). Finally, to further bridge the results obtained in NHPs to humans, Western blot analysis of IgG after IdeS digestion *in vitro* was performed. Complete cleavage of human IgG was shown, while residual levels of scIgG, which can retain partial

neutralization activity, were found only in NHPs (Fig. 4c). Combined with the *in vitro* IgG digestion data (Fig. 4a) and published results in humans⁸, these results suggest that IdeS might more efficiently remove anti-AAV NABs in humans compared to NHPs.

Anti-capsid antibodies are a major limitation to AAV vector-mediated gene transfer. Several strategies have been attempted to circumvent anti-AAV NABs⁶, although to date seropositive individuals are mostly declared ineligible to receive AAV-based treatments when the vector is administered systemically. Here we showed that a one-time *in vivo* administration of IdeS results in a decrease in anti-AAV antibody titers and allows the successful transduction of the liver, in the setting of both pre-existing natural immunity to AAV and, possibly, vector readministration. The procedure appeared to be safe, consistent with studies in healthy individuals¹⁸ and in patients undergoing graft transplantation⁸, which showed that transient digestion of circulating IgG was not associated with severe adverse events and did not appear to increase the risk of opportunistic infections.

Natural humoral immunity to IdeS, deriving from exposure to *Streptococcus pyogenes*, may represent a limitation to the use of the technology. While this is a potential concern, our results would argue that IdeS is active even in the presence of antibodies against the enzyme itself. Repeated administrations of IdeS can eventually result in neutralizing antibodies to the enzyme, although it has been shown that humoral immune responses triggered by IdeS in humans are only transient¹⁸.

In summary, results presented here indicate that IdeS can efficiently cleave anti-AAV antibodies thereby enhancing IgG clearance and reducing their AAV neutralizing activity in blood to levels compatible with transduction of hepatocytes. Pre-treatment with IdeS at the time of AAV vector infusion is a potential strategy to enable efficient systemic gene transfer

in the presence of low to moderate pre-existing anti-capsid antibodies, which are commonly found in humans¹⁷, and will possibly allow for repeated vector administration. This work provides key proof-of-concept of data on the use of IdeS technology to address a major limitation of *in vivo* gene transfer with AAV vectors. Future studies will help translating these findings to human trials.

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Author contributions

C.L., E.B., J.M.A., S.D., H.H., F.C., S.M., D.L., J.S., K.H., L.v.W., B.M., A.M., A.F., V.D., D.M.C., and H.B. performed the experiments. C.L., G.R., S.M.A., S.L.-D., and F.M. analyzed the results and wrote the manuscript. X.M.A., S.M.A., S.L.-D., and F.M. conceived and directed the studies.

Competing interests

The authors declare the following competing interests.

Employment. J.M.A., H.H., S.M., D.L., J.S., K.H., D.M.C., H.B., X.M.A., S.M.A., and F.M. are employees of Spark Therapeutics, Inc. a Roche company.

Patent. F.M., S.L.D., S.M.A. and C.L. are inventors on a patent related to this work. Patent applicants: INSERM (Institut National de la Santé et de la Recherche Médicale), Genethon, Sorbonne Université, Université Paris Descartes, Université Paris Diderot, Spark Therapeutics, Inc; inventors: Sebastien Lacroix-Desmazes, Federico Mingozi, Christian

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271 application request filed. Specific aspects of manuscript covered in the patent application: use
272 of IdeS to decrease pre-existing antibody immunity to AAV; use of IdeS to allow for vector
273 readministration.

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Figures legends

Figure 1 IdeS degrades anti-AAV antibodies and allows for successful liver transduction in mice passively immunized with IVIg. **a**, Western blot analysis of IVIg incubated 5 minutes and 24 hours with commercial (IdeS-C) or lab-made (IdeS) endopeptidase, or with PBS. The predicted molecular weight of intact IgG, single-chain IgG (scIgG), F(ab')₂ and Fc fragments is shown. One representative out of two independent experiments shown. **b**, Anti-AAV8 IgG concentration in IVIg measured after a 24-hour incubation with PBS, IdeS-C, or IdeS (n=1 per condition tested in duplicate, one representative out of two independent experiments shown). **c**, Protocol outline. C57BL/6 (n=6 mice per group, one representative out of two independent experiments shown) or hemophilia B (HB, n=4 mice per group, one experiment) mice were passively immunized with IVIg or received PBS as control. Thirty minutes later, mice were injected with either IdeS or PBS one day prior to the delivery of AAV8 -GLuc or -hFIX vectors. **d**, Western blot analysis of human IgG in serum of passively immunized C57BL/6 mice before or 30 minutes and 24 hours after IdeS treatment. One representative out of two independent experiments shown. **e,f**, Effect of IdeS on **e**, anti-AAV8 IgG and **f**, NAb titers measured 24 hours after treatment. Bars represent the group mean $\pm$ s.d. (n=6 mice per group). **g**, GLuc activity in serum of passively immunized mice treated with IdeS (IVIg/IdeS) or PBS (IVIg/PBS). Naïve mice receiving IdeS were used as controls (PBS/IdeS). RLU, Relative Light Units. **h,i**, Rescue of transgene expression in passively immunized HB mice (n=4) treated with IdeS (IVIg/IdeS) or PBS (IVIg/PBS) prior to AAV8-hFIX delivery. Naïve HB mice receiving IdeS were used as controls (PBS/IdeS). **h**, hFIX in plasma measured by ELISA over time. **i**, bleeding time following tail clip assay. All data are shown as mean $\pm$ s.d. Statistical analyses were performed by **b**, repeated measures one-way ANOVA with Dunnett's multiple comparisons test; **e**, one-way ANOVA with Tukey's multiple comparisons

test; **f**, one-way ANOVA, non-parametric with Dunn's multiple comparisons test; **g**, two-way ANOVA with Dunnett's multiple comparisons test (vs. IVIg/IdeS and PBS/IdeS); **h**, two-way ANOVA with Dunnett's multiple comparisons test (vs. IVIg/IdeS and PBS/IdeS); **i**, one-way ANOVA with Tukey's multiple comparisons test.

Figure 2 IdeS treatment enhances transduction in NHPs in the presence of pre-existing anti-AAV NAb. **a**, Experimental protocol. Two male Cynomolgus macaques with an anti-AAV8 NAb titer of 1:17.2 were infused with either saline (NHP3, control), or twice with 500 $\mu\text{g kg}^{-1}$ of IdeS (NHP5, treated) before the administration of an AAV8-hFIX vector. **b**, Total IgG over time in NHP5 (n=1 animal, samples tested in duplicate). **c**, Western blot showing IgG degradation over time in NHP5. One representative out of two independent experiments shown. **d**, Effect of IdeS administration on anti-AAV8 IgG and **e**, NAb (n=1 monkey per treatment group, triplicate independent measurements of each sample). The horizontal dotted lines represent the levels of anti-AAV8 IgG and NAb in the control animal NHP3, see also Supplementary Table 1. **f**, hFIX transgene levels in plasma over time (n=1 monkey per treatment group, triplicate independent measurements of each sample). **g**, vector genome copy number (VGCN) in liver at sacrifice (n=1 monkey per treatment group, average VGCN in each of the four main liver sections). **h**, Anti-AAV8 IgG and **i**, anti-AAV8 NAb at baseline and post AAV8-hFIX vector administration (n=1 monkey per treatment group, duplicate independent measurements of each sample). All data are shown as mean $\pm$ s.d. Statistical analyses were performed by **b,d,e**, repeated measures one-way ANOVA with Dunnett's multiple comparisons test (vs. Day -2); **f,h,i**, repeated measures two-way ANOVA with Sidak's multiple comparisons test (vs. NHP3); **g**, two-tailed unpaired t-test.

Figure 3 IdeS treatment allows for AAV-LK03 vector readministration in NHPs. **a**, Experimental protocol. Eight African green monkeys received 2×10^{12} vg kg⁻¹ of AAV-LK03-GAA at Day 0. At Day 210, animals were given one dose of 1 mg kg⁻¹ IdeS (IdeS, n=5) or vehicle control (PBS, n=3), followed by infusion of 2×10^{12} vg kg⁻¹ of AAV-LK03-hFVIII at Day 211. **b,c**, Anti-AAV-LK03 IgG levels as a function of time in animals treated with either PBS (**b**) or IdeS (**c**). **d,e**, Anti-LK03 NAb titers before (day 210) and after (day 211) treatment with PBS (**d**) or IdeS (**e**). **f,g**, hFVIII antigen levels in plasma as a function of time in animals treated either with PBS (**f**) or IdeS (**g**). **h**, Aggregate hFVIII expression was quantitated using area under the curve (AUC) from day 211 to day 239 in PBS- and IdeS-treated animals (bars and whisker indicate the mean and s.d.). Statistical analyses performed by **d,e**, two-tailed ratio paired t-test. **h**, two-tailed Mann-Whitney U test.

Figure 4 Efficient cleavage of human IgG in plasma in the presence of anti-IdeS antibodies. Serum samples from 10 healthy donors and 12 subjects affected by Crigler-Najjar syndrome were incubated *in vitro* with IdeS or PBS for 24 hours. **a**, Anti-AAV8 IgG and **b**, and anti-IdeS IgG were then assessed by ELISA. **c**, Western blot analysis of cleavage of IgG by IdeS in sera from 4 healthy donors (HD1-4) and 5 monkeys (NHP1, 2, 4, 5, 6) incubated with IdeS (+) or PBS (-) for 24 hours. All data are shown as mean $\pm$ s.d. Statistical analyses were performed by two-tailed, paired Wilcoxon test.

Methods

Care and use of animals

Mouse studies were performed according to the French and European legislation on animal care and experimentation (2010/63/EU and CE12-037) and approved by the local institutional ethics committee (Protocols n 2017-014-B). All procedures using cynomolgus macaques (*Macaca fascicularis*) were approved by the institutional animal care and use committee (protocol number 2018120409565637, APAFIS 18092) and were conducted at Nantes veterinary school (Oniris, Nantes, France). All procedures using African green monkeys (*Chlorocebus sabaeus*) were conducted under IACUC approved protocols (# AC18175) at the Saint Kitts Biomedical Research Foundation/Virscio (Saint Kitts and Nevis).

Cloning and production of IdeS endopeptidase

The IdeS coding sequence was cloned into a pEX-N-His-tagged cloning vector (OriGene Technologies, Inc., Rockville MD), which was transformed into BL21 competent *E. coli* (NEB, Ipswich, MA) following manufacturer's instructions. Protein expression was induced at 37°C for 4 hrs by adding IPTG 0.5 mM to the bacterial culture. Detection and purification of the protein were performed using the anti-FabRICATOR antibody A3-AF1-010 (Genovis, Lund, Sweden) and a nickel affinity column (GE Healthcare, Chicago, IL), respectively. Endotoxin removal was performed using poly(ε-lysine) resin spin columns (Thermo Scientific, Waltham, MA) and residual endotoxin concentration was assessed by LAL test with Endosafe Cartridge Technology (Charles River Laboratories, Wilmington, MA), according to manufacturer's instructions. IdeS endopeptidase was diluted in the injectable solution buffer, composed of 50 mM sodium phosphate, 150 mM NaCl, 0.25% human serum albumin, pH 6.6.

AAV vectors

AAV vectors were prepared as previously described²⁶ in roller bottles following a triple transfection protocol with subsequent cesium chloride gradient purification. The AAV vector titration was performed by real time PCR (qPCR) performed in ABI PRISM 7900 HT Sequence Detector using Absolute ROX mix (Taqman, Thermo Fisher Scientific, Waltham, MA), except for empty capsids that were titrated by SDS-PAGE followed by silver staining and quantification by densitometry against an AAV capsid used as a standard. For the *in vitro* neutralization assay, an AAV8 or an AAV-LK03 vector encoding Firefly Luciferase (AAV8-Luc or AAV-LK03-Luc) was used as reporter as previously described¹⁵. The AAV8 vectors used in the *in vivo* experiments expressed human factor IX (hFIX) or Gaussia Luciferase (GLuc), under the control of a liver-specific promoter^{4,17}. The AAV-LK03 vectors used for the *in vivo* studies expressed the human alpha-acid glucosidase (AAV-LK03-GAA) or the human factor VIII (AAV-LK03-hFVIII) transgene under the control of liver specific promoters. These vectors were purified by a triple transfection as above followed by column purification.

Human serum samples

Commercially available serum samples from deidentified healthy volunteers were obtained from the Etablissement Français du Sang (EFS). Serum samples from patients with the Crigler-Najjar (CN) syndrome were collected in the context of an observational international multicenter study (NCT02302690) to study AAV seroprevalence in this patient population. Anti-AAV antibodies in CN serum were assessed at Genethon (Evry, France). The study was reviewed and approved by the independent ethics committees of the five participating sites (Hôpital Antoine Bécclère, Clamart, France; Academic Medical Center, Amsterdam, the

Netherlands; Azienda Ospedaliera Papa Giovanni XXIII, Bergamo, Italy; Federico II University Hospital of Naples, Naples, Italy; Hannover Medical School, Hannover, Germany) and was carried out in compliance with the Good Clinical Practice guidelines and according to the principles of the declaration of Helsinki. All participants gave written informed consent prior to study enrollment.

Cell culture

The 2V6.11 cells (ATCC CRL-2784) were maintained under 37°C, 5% CO₂ condition in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% FBS, 2 mM GlutaMAX (Invitrogen Life Technology, Carlsbad, CA) and were used for the AAV neutralization assay¹⁵.

Capsid antibody ELISA

Anti-AAV8 Ig capture assay was performed as previously described¹⁷. Briefly, recombinant AAV8 empty capsids were diluted in coating buffer (35 mM bicarbonate, 13 mM carbonate, pH> 9.2) to a final concentration of 2×10^{10} vector particles per ml. Fifty microliters were added to each well in a 96-well Nunc Maxisorp Immunoplate (Thermofisher, Waltham, USA). A standard curve made from purified human IgG (IVIg, Tégéline, LFB, Les Ulis, France) was added directly to the plates. Plates were coated overnight at 4°C. The next day, plates were washed three times with wash buffer (PBS, 0.05% Tween-20) then blocked with blocking buffer (PBS, 6% fat-free milk) for 2 hrs at room temperature. Plates were again washed three times with wash buffer then incubated with serum diluted in blocking buffer for 1 hr at 37°C. After 3 washes, horseradish peroxidase (HRP)-conjugated antibodies specific for the heavy chain of human IgG (Southern Biotech, Birmingham, US-AL) were incubated for 1 hr at 37°C. Plates were then washed three times with wash buffer and revealed with o-

phenylenediaminedihydrochloride (OPD) substrate (Sigma, Saint Louis, USA). Optical density (OD) was measured at 492 nm using a microplate reader (ENSPIRE™, Perkin Elmer, Waltham, USA). Anti-AAV8 IgG concentration was determined against the specific standard curve using 4-parameters regression and results were expressed as µg/ml of IgG.

For NHP samples, a purified monkey IgG or IgM for the standard curves and an HRP-conjugated antibody specific for gamma chain of monkey IgG or IgM (Fitzgerald Industries, Acton, MA) for the revelation, were used.

The anti-AAV-LK03 IgG and IgM capture assay was performed as described above with the following modifications in the protocol. Plates were coated with AAV-LK03 empty capsid at 1.57×10^{11} capsid particles per ml (50 µl total) in coating buffer. Detection of bound IgG was performed with an HRP-conjugated goat anti-monkey IgG, Fc-specific polyclonal antibody (GAMon/Ig(Fc)/PO, Nordic MUBio, Susteren, The Netherlands) at a 1:5,000 dilution. Detection of bound IgM was performed with an HRP-conjugated polyclonal goat anti-monkey IgM (43R-IG074HRP, Fitzgerald Industries International, Acton, MA) at a 1:10,000 dilution. For IgG, 7-point standard curve was generated by passively absorbing purified monkey IgG (Rockland Immunochemicals, Limerick, PA) onto duplicate wells in place of the AAV coating step at concentrations ranging from 2 µg/ml to 31.25 ng/ml. For IgM, purified monkey IgM (Fitzgerald Industries International) were used at concentrations ranging from 250 to 1.95 ng/ml.

***In vitro* AAV8 neutralization assay**

The NAb assay was performed as previously described¹⁵. Briefly, on day 1, 96-well plates were seeded with 2×10^4 2V6.11 cells/well for 24 hrs in the presence of ponasterone A (Life Technologies, Carlsbad, USA). An AAV8-Luc or an AAV-LK03-GLuc vector was then

diluted in serum-free DMEM (Life Technologies, Carlsbad, USA) and incubated with semi-log-fold serial dilutions of the serum samples for 1 hr at 37°C. Subsequently, the serum-vector mixtures were added to the cells at an multiplicity of infection (MOI) of 200. After 24 hrs, cells were lysed and the luciferase activity was measured on a luminometer (ENSPIRETM, Perkin Elmer, Waltham, MA). For the AAV-LK03-GLuc vector, cells were not lysed and luciferase activity measured on cell supernatant. Luciferase expression was measured as Relative Light Unit (RLU) per second. The neutralizing titer was reported as the highest serum dilution that inhibited AAV transduction by $\geq 50\%$ compared to the 100% transduction control.

***In vitro* incubation of IgG and AAV vector with IdeS**

Human sera from AAV8-seropositive healthy donors or CN subjects²⁵ were diluted at 1:3 in PBS and incubated with or without IdeS (200 IU, 4 μ g) at 37°C for 24 hrs. Intact anti-AAV8 IgG and anti-IdeS IgG were assessed by ELISA or by Western Blot as described.

For the *in vitro* incubation of AAV8 vectors with IdeS, 1.5×10^{11} vg of AAV8-CMV-Luc were mixed with increasing amounts of IdeS (0, 0.6, 3, or 6 μ g) in a total volume of 50 μ l and incubated at 37°C during for 24h. An untreated AAV8-CMV-Luc vector without incubation at 37°C was used as control to check the effect of a prolonged incubation at 37°C on transduction efficiency. After incubation, vectors were added to HEK 293 cells (seeded in 96 well plates at a concentration of 2×10^4 cells/well) at an MOI of 100,000, 10,000, or 1,000. After 30 hours, cells were lysed and the luciferase activity was measured on a luminometer. Luciferase expression was measured as Relative Light Unit (RLU) per second.

Mouse studies

Wild-type male C57BL/6 mice or factor IX (FIX)-deficient hemophilia B (HB) male and female mice, all aged 6-8 weeks, were used in these studies. In all experiments, animals were passively immunized by intravenous injection of 9 mg of IVIg (Privigen, CSL-Behring, King-of-Prussia, PA USA) in a final volume of 100 μ l. After 30 min, mice received 25 μ g of IdeS by retro-orbital injection. Twenty-four hours later, mice were injected with an AAV8 vector at the dose of 1×10^{12} vg/kg (2×10^{10} vg/mouse) by tail vein route. Blood samples were collected via mandibular puncture or from the retro-orbital plexus in heparin coated capillary tubes (Sarstedt, Nümbrecht, Germany). At euthanasia, whole livers were collected and snap-frozen for subsequent assessment of vector genome copy numbers (VGCN).

Tail clip bleeding assay

HB mice were anesthetized with a mixture of ketamine and xylazine delivered intraperitoneally and placed in prone position. A distal 3 mm segment of the tail was amputated with a scalpel. The tail was immediately immersed in a 50 ml tube containing isotonic saline pre-warmed at 37°C. The position of the tail was vertical with the tip positioned about 2 cm below the body horizon. Each animal was monitored for 20 minutes even if bleeding ceased, in order to detect any re-bleeding. Bleeding time was determined using a stop clock. If bleeding on/off cycles occurred, the sum of bleeding times within the 20-minute period was used. At the end of the experiment, animals were sacrificed for liver VGCN analysis.

NHP studies

Cynomolgus monkeys:

Six Cynomolgus monkeys (*Macaca fascicularis*, 2-3 years old), were selected based on their anti-AAV8 neutralizing antibody titer (between 1:3.16 to 1:31.6). The study plan was divided

into 3 sub-studies and monkeys were distributed as treated and untreated groups, each group having similar anti-AAV8 NAb titers.

The injection protocol was performed as follows. IdeS was administered via slow intravenous infusion at the dose of 500 µg/kg. Thirty minutes before IdeS injection, an antihistamine drug (Polaramine, 4mg/kg) was administered. The AAV8-hFIX vector was infused 24 hrs after the last IdeS injection. Blood samples were collected before and immediately after IdeS treatment, and before AAV administration to assess the efficiency of degradation of IgG. Other blood samples were collected throughout the protocol in order to follow up the anti-AAV8 humoral response, transgene expression as well as to monitor hematology and clinical chemistry parameters. At sacrifice, 4 wedge biopsies were taken at different sites from the 4 liver lobes, right, left, caudate and papillary process, to assess VGCN in different areas of the liver. Animals were also submitted to full necropsy including a macroscopic examination and liver histopathological evaluation.

African green monkeys:

Eight adult male African green monkeys (*Chlorocebus sabaeus*), confirmed seronegative for AAV-LK03 neutralizing antibodies, were enrolled in the study. At day 0, animals were immunized with an AAV-LK03 vector encoding for alpha-acid glucosidase (AAV-LK03-GAA)²⁷. At study day 210, 5 animals were randomly assigned to receive a single infusion of IdeS delivered via infusion pump a dose of 1 mg/kg, while 3 animals received vehicle control (saline). Approximately 15-30 minutes prior to infusion of IdeS or PBS animals were treated with diphenhydramine (5 mg/kg, injected intramuscularly). 24 hours after dosing with IdeS or vehicle, animals were infused with an AAV-LK03 vector encoding for human factor VIII (AAV-LK03-FVIII), at a dose of 2×10^{12} vg/kg, through the saphenous vein. Plasma samples were collected at day 0, 1 hour, 2 hours, 6 hours, 12 hours, 24 hours, 48 hours, and 8, 15, 22,

and 29 days after IdeS infusion. Animals were euthanized following the Day 29 plasma collection.

IdeS clearance

A kinetic study was conducted in 2 Cynomolgus monkeys following a protocol similar to what previously described in humans¹⁹. Animals received 500 µg/kg of IdeS intravenously at time 0 and 24 hours later and IdeS level in blood was measured by sandwich ELISA over time. Briefly, plates were coated with a polyclonal anti-FabRICATOR antibody (A3-AF1-010, Genovis, Lund, Sweden) and blocked with PBS, 3% BSA. Plasma samples were then added to the plates and incubated for 1 hour at room temperature. Purified IdeS was used as a standard. A biotinylated anti-HIS tag antibody (AD1.1.10, R&D Systems, Minneapolis, MN) and streptavidin-conjugated HRP (Dako, Santa Clara, CA) were used for detection. TMB substrate (BD Biosciences, Franklin Lakes, NJ) were used for quantification and the reaction was stopped with a 3M H₂SO₄ solution and optical density (OD) measurements were done at 450 nm. IdeS concentration was determined against the specific standard curve using 4-parameter regression and results were expressed as µg/ml.

Anti-monkey IgG ELISA

Plates were coated with a goat anti-human IgG F(ab')₂ (Thermo Fisher, Waltham, MA, US) and blocked with PBS-6% milk. Diluted samples were incubated and a commercial purified monkey IgG (Fitzgerald Industries, Acton, MA) was used as a standard. Intact monkey IgG were then revealed using a secondary goat anti-monkey HRP-conjugated IgG (Fitzgerald Industries, Acton, MA) and the OPD substrate. The reaction was stopped with 3 M H₂SO₄ and OD was measured at 492 nm.

Human FIX ELISA

Human FIX (hFIX) protein levels in mouse plasma were measured by ELISA using a capture antibody and a HRP-conjugated detection antibody (Affinity Biological, Ancatser, Canada), following the manufacturer's instructions. In NHP samples, anti-hFIX antibody (MAI-43012, Thermo Fisher Scientific Waltham, MA), and an anti-hFIX-HRP antibody (CL20040APHP, Tebu-bio, Le Perray-en-Yvelines, France) were used for capture and detection, respectively.

Human Factor VIII ELISA

The levels of transgene-derived human FVIII in plasma were quantified using a sandwich ELISA. Briefly, 96-well microplates (Sigma Aldrich, Saint Louis, MO) were coated with FVIII capture antibody (GMA-8024, Green Mountain Antibodies, Burlington, VA) at 2 µg/ml (100 µL total) in coating buffer and incubated overnight at 4°C. After coating, plates were washed, blocked for 1 hour at room temperature, and samples were added to each well in duplicate. An 8-point standard curve was created by spiking in recombinant B-domain deleted human FVIII (Xyntha, Pfizer, New York, NY) into FVIII-depleted human plasma (George King BioMedical, Overland Park, KS) at known concentrations ranging from 300 to 1.09 ng/ml in duplicate wells. Standards and diluted samples were added to wells at a volume of 100 µl and incubated for 1 hour at 37°C. Following sample incubation, plates were washed, and incubated with a biotinylated mouse anti-human Factor VIII antibody (GMA-8023, Green Mountain Antibodies) at a dilution of 1:5000 (100 µl). Plates were washed and chromogenic signal was generated by adding 100 uL of TMB substrate to each well, followed by quenching with 100 µl 1N sulfuric acid. Absorbance was measured using an i3 SpectraMax (Molecular Devices, San Jose, CA) at 450nm and FVIII antigen levels were extrapolated from the standard curve. The lower limit of quantitation for this assay was 4.6 ng/ml.

Vector genome copy number determination

Liver VGCN was determined using a qPCR assay with primers and probes specific for the liver promoter and the mouse titin or globin genes. The promoter-specific primers and the probe²⁷ were purchased from Thermo Scientific (Waltham, MA, USA). Mouse titin and globin were used as a normalizing gene in mice and NHP studies, respectively, and were purchased from Thermo Scientific. DNA from tissues was extracted from whole-organ (mouse) or wedge biopsies followed by homogenization using FastPrep (MP Biomedicals, Irvine, CA) and Gentra Puregen Blood Kit (Qiagen, Hilden, Germany) DNA extraction. Each sample was tested in triplicates and genome copy number per diploid genome was determined against a standard curve made of linearized plasmid.

Gaussia Luciferase assay

Fifty microliters of plasma samples diluted in PBS (1:50) were loaded on white 96-well microplate (OptiPlate-96 Perkin Elmer, Waltham, USA). Gaussia Luciferase activity was measured on a Luminometer (ENSPIRETM, Perkin Elmer, Waltham, USA). The measurement was done over 1 second after automatic injection of 50 μ l of substrate solution (Coelenterazine, Sigma Aldrich, Saint Louis, USA). Signal background was subtracted from each value. The GLuc activity is expressed as RLU/sec and the values are the mean $\pm$ s.d. of duplicates from repeated assays.

Western blot for human and NHP IgG

Western blots were performed on diluted samples, (1:20) for serum from passively immunized mice or from NHPs. SDS-PAGE was performed in 4-12% gradient Bis-Tris protein gels (Invitrogen). After protein transfer with semi-dry iBlot system (Invitrogen), membranes were blocked with Odyssey buffer (Li-Cor Biosciences) overnight at 4°C. The

membranes were incubated with IgG F(ab')₂-specific antibody (Thermo Scientific, Waltham, US-MA) or IgG Fc-specific antibody (Sigma, Saint Louis, USA), or with the anti-FabRICATOR anti-IdeS antibody. The membranes were washed and incubated with the appropriate secondary antibody (Li-Cor Biosciences) and visualized by Odyssey Imaging System and the Image Studio Lite (v5.2) software (Li-Cor Biosciences).

Anti-IdeS IgG ELISA

Plates were coated overnight at 4°C with 3 µg/ml of IdeS. The next day, plates were blocked and diluted samples were incubated for 1 hr at 37°C. A secondary HRP-conjugated antibody specific for human (Southern Biotech, Birmingham, AL) or monkey IgG (Fitzgerald Industries, Acton, MA) was incubated for 1 hr at 37°C and revealed with OPD substrate. The standard curve in the assay was made of IVIg or purified monkey IgG (Fitzgerald Industries, Acton, MA) serially diluted from a concentration of 2000 ng/ml. Anti-IdeS IgG concentration was determined using 4-parameters regression and results are expressed as µg/ml of IgG.

Anti-hFIX and anti-hFVIII IgG ELISA

Anti-hFIX IgG were determined as described²⁸. Plates were coated overnight at 4°C with 5 µg/ml of recombinant hFIX (Benefix, Pfizer, Paris, France). A standard curve made of serial dilution of purified monkey IgG (Fitzgerald Industries, Acton, MA) was used as a reference to extrapolate the concentration of anti-hFIX antibodies. The next day, plates were blocked and diluted NHP sera were incubated for 1 hr at 37°C. A secondary HRP-conjugated antibody specific for monkey IgG (Fitzgerald Industries, Acton, MA) was incubated 1 hr at 37°C and revealed with OPD substrate. Anti-hFIX IgG concentration was determined using a 4-parameters regression and results were expressed as µg/ml of IgG.

The presence and amount of anti-transgene antibodies developed against hFVIII was quantified using an indirect ELISA. Briefly, 96-well microplates were coated with Xyntha (Pfizer) at 1 µg/ml (100 µl total) in coating buffer overnight at 4°C. Following coating, plates were washed, blocked for 30 hours at room temperature, and then incubated with plasma samples for 1 hour at 37°C. Sample wells were prepared in duplicate. After sample incubation, plates were washed and incubated in an HRP-conjugated polyclonal sheep anti-human IgG (NA933, VWR, Radnor, PA) at a 1:5,000 dilution for 1.5 hours at RT. Chromogenic signal was generated by adding 100 µl of TMB substrate to each well and incubating at room temperature for approximately 10 minutes, followed by quenching with 100 µl 1N sulfuric acid. An 8-point standard curve was generated by diluting human plasma with FVIII inhibitors (George King BioMedical) in naïve monkey plasma onto duplicate wells to achieve inhibitor levels ranging from 16 to 0.125 arbitrary units. Absorbance was measured on an i3 SpectraMax (Molecular Devices) at 450nm absorbance and anti-hFVIII IgG levels were extrapolated from the standard curve and anti-hFVIII IgG levels are reported in arbitrary units.

Statistical analyses

Data are represented as group mean $\pm$ s.d. Statistical significance ($\alpha = 0.05$) was tested using GraphPad Prism (v7.0) and using different statistical methods depending on the dataset to be analyzed and the experimental setup, statistical methods applied are specified in the figure legends. P values below 0.05 were considered significant. When applicable, two-sided statistical tests were used. For experiments involving a single NHP per treatment arm, samples were measured multiple times in separate independent experiments and resulting data used for statistical analysis. For mouse studies, animals were assigned randomly to treatment

718 groups. For NHP studies, animals were pre-selected based on the anti-AAV neutralizing
719 antibody titers and randomly assigned to the IdeS or control treatment arm of the experiment.
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Data availability statements

All requests for raw and analyzed data and materials will be promptly reviewed by Genethon, INSERM, or Spark Therapeutics to verify if the request is subject to any intellectual property or confidentiality obligations. Patient-related data not included in the manuscript were generated as part of a clinical trial and are not available as subjected to patient confidentiality. Any data and materials that can be shared will be released via a Material Transfer Agreement established with the relevant institution that generated the data or owns the materials.

References Methods section

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a **b** **c** **d** **e** **f** **g** **h** **i**

a **b** **c** **d** **e** **f** **g** **h** **i**

a **b** **c** **d** **e** **f** **g** **h**

a **b** **c**

a **b** **c**

□ NHP2 (control)

□ NHP6 (treated)

d **e** **f** **g**

a **b** **c** **d** **e** **f** **g**

□ NHP1 (control/treated) □ NHP4 (treated/treated)

a **b** **c** **d** **e** **f** **g**

a **b** **c**

a **b** **c** **d**