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Letter to the Editor

Delivery of genetic load during *ex situ* liver machine perfusion with potential for CRISPR-Cas9 gene editing: An innovative strategy for graft treatment

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To the Editor:

Over the years, the shortage of suitable donor organs has challenged the transplant community in performing life-saving liver transplantation (LT). Recent reports from European and American liver transplant registries show persistently high waitlist mortality rates ranging between 10% and 18% [1,2]. To cope with this, liver transplant surgeons are increasingly forced to transplant organs from extended criteria donors. However, it is well known that these organs are more susceptible to the consequences of ischemia-reperfusion injury (IRI), including primary non-function (PNF) and non-anastomotic biliary strictures (NAS) after transplantation, which, by consequence, can increase the number of retransplantations, making organ shortage an endless cycle [3]. This unfavorable scenario has created a fertile environment for the development of organ machine perfusion (MP) strategies aiming to assess and optimize organs before transplantation [4]. A large amount of research has resulted in the development of different perfusion devices and protocols, which have been tested in preclinical and clinical studies, and, more recently, in randomized clinical trials [5]. Currently, hypothermic oxygenated machine perfusion (HOPE) is mainly used to improve mitochondrial status by decreasing oxidative stress and increasing cellular adenosine triphosphate (ATP) levels [6,7], whereas normothermic MP is better suited for evaluation of graft quality during the perfusion session by measuring different biological and physiological parameters.

Furthermore, MP technology opens a door to a new era in liver graft preservation by allowing modulation of graft function through the administration of specific therapies, such as anti-inflammatory drugs, vasodilators, defatting cocktails, and infusion of stem cells [8]. In addition, our group pioneered the use of RNA interference by demonstrating the uptake of small interfering RNA against pro-apoptotic genes during both *ex situ* normothermic and hypothermic perfusion of rat liver grafts [9]. Our group started investigating the potential use of CRISPR-Cas9 (clustered regularly-interspaced short palindromic repeats and CRISPR associated protein 9) gene editing during liver MP as a promising strategy to further explore. The CRISPR-Cas9 is a natural immune bacterial mechanism against viruses, which was discovered in 2012 and it is already considered a revolution in the way we can manipulate DNA by making gene editing more efficient, faster, and cheaper. Indeed, Charpentier and Doudna, who first described the system, have just been awarded with the 2020 Nobel Prize in chemistry [10].

All in all, *ex situ* graft modulation/optimization is a high-speed research highway, which is opening up in the field of organ preservation. However, as with all roads, the journey can be long and, at some point, misleading. Hence, we have many steps left to take before reaching a safe destination. In fact, in the specific case of CRISPR-Cas9 gene editing, its efficiency will largely depend on the viral delivery performance of both Cas9 nuclease and guide RNA, which may limit the results of this highly sophisticated technology. The use of CRISPR-Cas9 gene editing for graft modulation has not been reported yet.

With this in mind, we designed a proof-of-concept experiment in which adeno-associated virus (AAV) gene therapy was used to efficiently deliver genetic load during MP before liver engraftment in preparation to be used for CRISPR-Cas9 delivery. In brief, male Lewis rats, weighting 220 to 260 g, were used as donors and recipients. Liver grafts were procured and transplanted using an arterialized rat transplant model [11]. Immediately after procurement, 6 liver grafts were subjected to 2 h of HOPE at 4 °C using University of Wisconsin MP solution, which was perfused through the portal vein as previously described [9]. At the beginning of HOPE preservation, a solution of 4×10^8 Pfu/mL of AAV (serotype 8), used as a vector for the green fluorescent protein (GFP) gene, was added to the perfusion solution in 2 of the 6 grafts. After MP preservation, controls and treated livers were immediately transplanted. All animals survived and were in good clinical conditions at 24 h after liver transplantation, when euthanasia was performed

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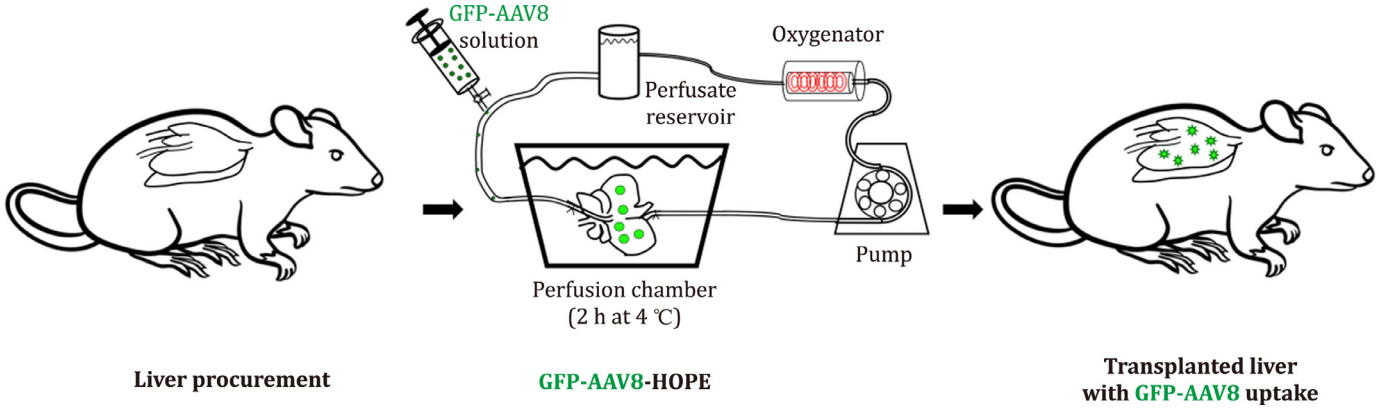

Fig. 1. Experimental design. GFP-AAV8: green fluorescent protein adeno-associated virus serotype 8. HOPE: hypothermic oxygenated machine perfusion.

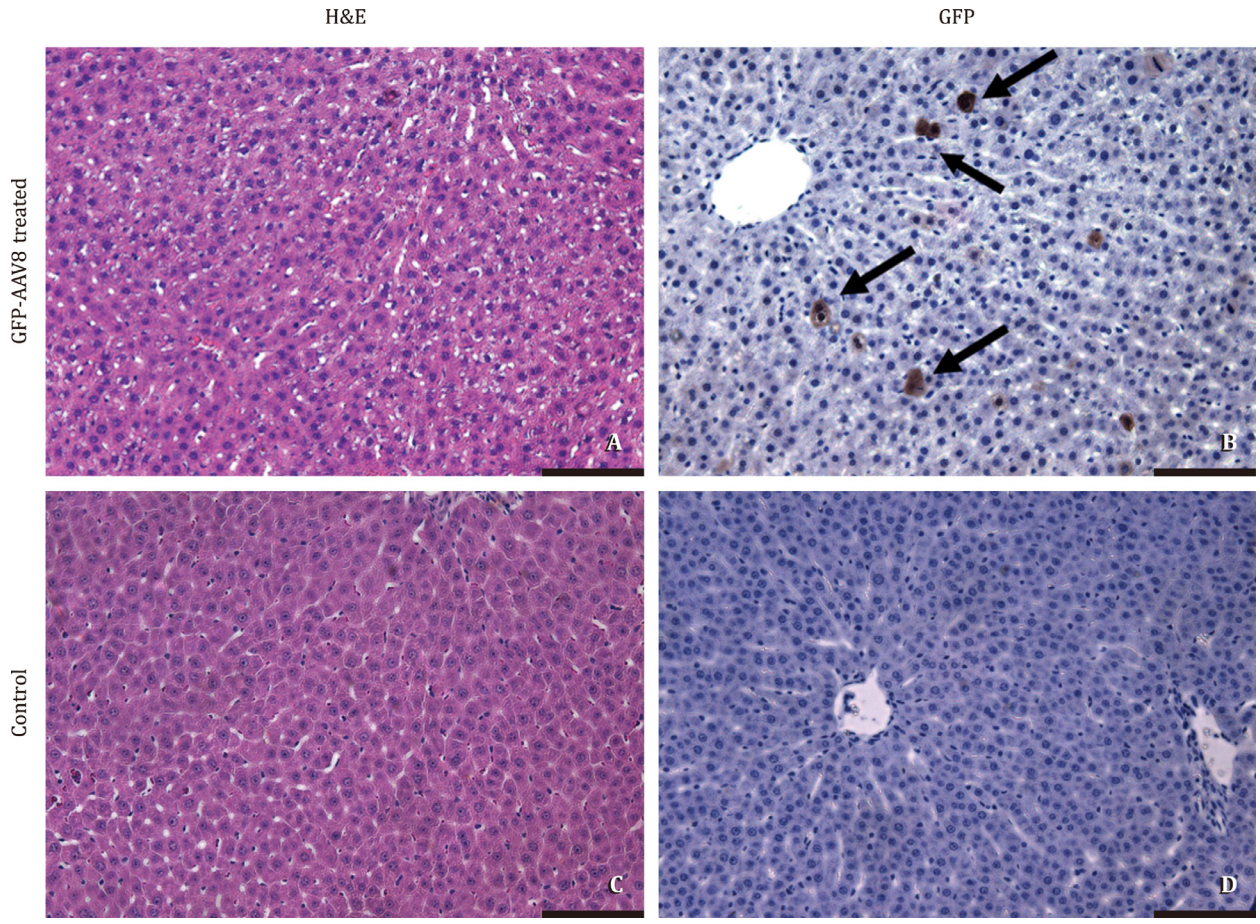

Fig. 2. Delivery of GFP-AAV8 transduces liver graft hepatocytes. Representative histology and immunohistochemistry scale bars 100 μm. Black arrows show GFP positive cells. GFP-AAV8: green fluorescent protein adeno-associated virus serotype 8.

for samples collection (Fig. 1). Immunohistochemistry analysis performed on samples of treated grafts showed 2.000 ± 1.563 GFP positive cells per × 20 field (Fig. 2), demonstrating transduction of liver graft cells 24 h after transplant. With this proof-of-principle study, we show for the first time adenoviral-mediated delivery of genetic cargo during *ex situ* HOPE liver perfusion and transplantation. Successful integration of viral gene therapy during *ex situ* machine preservation provides an opportunity to potentially introduce a therapeutic cargo to ameliorate or reduce IRI. As such, viral gene therapy could be employed to deliver and induce temporary overexpression of cytoprotective, anti-apoptotic, or immunoregulatory genes [12].

Similarly, viral gene therapy could be employed to deliver gene-editing technologies, (i.e., CRISPR-Cas9) to knockout and reduce expression of pro-ischemic, pro-apoptotic, and/or pro-inflammatory players. Indeed, CRISPR-Cas9 *ex situ* graft editing will depend heavily on the efficiency of viral delivery of both Cas9 nuclease and guide RNA, guide RNA design, and frequency of potential off-target effects, including unexpected translocations, deletions, inversions, and exon skipping [13–15]. We are currently trying to apply the CRISPR-Cas9 gene-editing platform in our *ex situ* rat liver perfusion model.

AAV vectors have been demonstrated to have robust safety profiles clinically. Further, AAV-delivered genetic material does not

integrate into the genome. AAV vectors come in a variety of serotypes with different therapeutic profiles. Here we used AAV serotype 8, which demonstrated to be a promising candidate for hepatic gene therapy clinically [13]. However, for *ex situ* perfusion and transplantation specifically, future studies will be needed to compare the efficiency of various AAV serotypes and to improve the robustness of transduction.

There are several important caveats to viral gene therapy that must be considered [15]. The first is packaging size of the vector. For most AAV vectors, the upper limit is around 5 kilobases (kb) [13]. As such, when considering future studies to use CRISPR-Cas9 to knockout genes that promote IRI, Cas9 and the guide RNA would likely need to be packaged into and delivered by separate AAV vectors. Additionally, targeted transduction of liver cell subtypes, i.e., cholangiocytes, will likely require studies evaluating multiple serotypes. Finally, while generally well tolerated, not unlike other viral vectors, AAV delivery can induce an immune response [14,15]. However, in adult mice, adenoviral delivery of CRISPR-Cas9 still generated efficient editing, despite presence of an immune response [13]. Nevertheless, the potential for AAV delivery of CRISPR-Cas9 or other genetic cargo strategy during HOPE can elicit a detrimental immune response requiring further investigation. In the same way, the number of virally transduced hepatocytes to produce a therapeutic benefit (whether overexpressing a protein of interest or editing for knock-out) and confirmation of off-target effects require more research.

In conclusion, here we show in a preliminary proof-of-concept study that AAV administration during organ machine perfusion was able to deliver genetic load to liver grafts, leading to transduction in liver grafts 24 h after transplantation. Further experimental studies using AAV as vectors to deliver CRISPR-Cas9 during organ perfusion are underway to investigate the potential use of this genetic tool to edit genes (addition/knockout) associated with post-transplant ischemia/reperfusion injury with the goal of improving post-transplantation organ function.

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CRediT authorship contribution statement

Eliano Bonaccorsi-Riani: Data acquisition, Investigation, Formal analysis, Writing – original draft. **Andrew Gillooly:** Data acquisition, Investigation, Formal analysis. **Isabel MA Brüggenwirth:** Formal analysis, Writing – original draft. **Paulo N Martins:** Conceptualization, Formal analysis, Supervision, Writing - review & editing, Funding acquisition.

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Ethical approval

This study was performed with approval from the University of Massachusetts Medical School Institutional Animal Care and Use Committee (IACUC) (A-2502-17). Rats were housed within the University of Massachusetts Medical School Animal Facilities Core. Healthy male rats were used for studies.

Competing interest

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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