Gene transfer to skeletal muscle using hydrodynamic limb vein injection: current applications, hurdles and possible optimizations

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Abstract
Hydrodynamic limb vein injection is an in vivo locoregional gene delivery method. It consists of administrating a large volume of solution containing nucleic acid constructs in a limb with both blood inflow and outflow temporarily blocked using a tourniquet. The fast, high pressure delivery allows the musculature of the whole limb to be reached. The skeletal muscle is a tissue of choice for a variety of gene transfer applications, including gene therapy for Duchenne muscular dystrophy or other myopathies, as well as for the production of antibodies or other proteins with broad therapeutic effects. Hydrodynamic limb vein delivery has been evaluated with success in a large range of animal models. It has also proven to be safe and well-tolerated in muscular dystrophy patients, thus supporting its translation to the clinic. However, some possible limitations may occur at different steps of the delivery process. Here, we have highlighted the interests, bottlenecks and potential improvements that could further optimize non-viral gene transfer following hydrodynamic limb vein injection.

KEYWORDS
gene transfer, hydrodynamic delivery, locoregional, non-viral gene delivery, optimizations, skeletal muscle

1 | INTRODUCTION

The skeletal muscle (SM) is an interesting target for delivering nucleic acids (NA).1,2 In humans, the whole body comprises as many as 650 muscles, constituting 40% of its total mass, thus making it the most abundant tissue.3 Anatomically, the SM is mainly composed of multinucleated cells, named myocytes or muscle fibers. These form syncytia covered by a layer of connective tissue called the endomysium. Muscle fibers are grouped in bundles, named fascicules. Each of them is surrounded by another connective structure called the perimysium. Finally, groups of fascicules wrapped by the epimysium form the muscle.4 SM are implicated in many functions, such as posture control, locomotion, breathing, cholesterol homeostasis, hormone secretion or thermoregulation. It can be targeted for various applications such as the induction of antibody production (antigen-based vaccination) or in situ synthesis of other proteins with diverse biomedical interests. Furthermore, SM can be affected by a wide range of diseases including myopathies. Accordingly, SM can be targeted for both direct and indirect purposes.

In this respect, the administration path is a crucial parameter, especially for the delivery of NA such as plasmid DNA (pDNA) or small interfering RNA.5 Irrespective of the delivery approach, it must be at the same time relevant, safe and efficient. Among the various hydrodynamic delivery methods, as extensively reviewed elsewhere,6-8 hydrodynamic limb vein (HLV) injection is a particular locoregional...
administration method specifically dedicated to target SM. Introduced by Professor Jon Wolff and colleagues in the 1990s, this procedure (also known as high-pressure transvenous limb perfusion or locoregional transvenous perfusion) has the potential to treat the whole musculature of a given limb at once (i.e. in a single administration)\(^9,10\). It derives from the Bier block procedure, which aims for locoregional anesthesia in orthopedic surgery or for the delivery of chemotherapies. It relies on a transient isolation of a limb, using tourniquet(s) placed at its proximal part. By analogy, HLV injection consists of a transient interruption of the blood flow of a limb followed by the rapid administration, via the venous system of that limb, of a large volume of solution containing NA constructs. For example, HLV is performed in mice according to the step-by-step procedure outlined in Figure 1 to reach the various muscles of the hind limb (Figure 2).

The potential advantages of HLV injection can be highlighted by comparison with other muscle-geared methods, including systemic intravenous (IV) or intramuscular (IM) injections, electroporation and sonoporation (Table 1).

(i) Systemic injection is meant to transfer genes into muscles throughout the whole body\(^11\). Some successes have been obtained recently using this path, notably in dogs with X linked myotubular myopathy,\(^12\) as confirmed by preliminary data in young boys with myotubular myopathy who were treated with an adeno-associated virus (AAV)-2/8 vector. Other, gene therapy clinical trials using AAVs injected via IV are currently ongoing\(^13,14\) or have been successfully completed.\(^15\) Nevertheless, widespread gene delivery remains challenging in terms of vector safety (e.g. liver toxicity of vectors, as well as anti-viral vector immune/inflammatory responses) and should even be avoided, depending on the intended application. Potential toxicity

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**FIGURE 1**  Step-by-step procedure of HLV injection as performed in the Swiss mouse. First, the animal is anesthetized (1); for example, as a result of an intraperitoneal injection of ketamine/xylazine (respectively 100 and 10 mg/kg of body weight). Hind limbs are shaved (2), a tourniquet is placed around one (3) and then is tightened at its proximal part (4). An incision of the skin (over approximately 0.5 cm) is performed (5) to identify the underlying great saphenous vein (6). A drop of saline solution is deposited over the incision (7). A 30-G needle held with tweezers is carefully introduced into the vein (8) and then held in position by hand (9). A large volume of solution containing the transgene of interest is then delivered in a few seconds (10). Of note, for a 25 g animal, this volume is \(\sim 1\) mL which corresponds to one-half of the body blood volume. Finally, the incision is sutured (11) and the second hind limb can be processed following the same procedure (12). Typically, the animal wakes up approximately 30 minutes after induction of the anesthesia and it recovers the use of his hind limbs in a few hours. For small rodents, this procedure requires the use of a binocular magnifier. In larger animals such as rats, this protocol is less invasive because no incision of the skin is required and the whole procedure can be performed with bare eyes.
of the transgene product must also be considered. For example, the normal protein, lacking in some muscular dystrophies, can be toxic when produced in non-relevant part(s) of the body (e.g. calpain 3, re-expressed to treat limb girdle muscular dystrophy (LGMD) 2A limb muscles, is toxic if expressed in the heart, which could be overcome by a more restricted tissue-specific transgene expression).

Locoregional delivery can therefore be suitable. IM injection consists of a direct administration in a given muscle, via a syringe and needle. Although HLV bears specific physical barriers (such as the passage through the vasculature and the endothelium), which are less of a problem with the IM route, subsequent steps are similar in many instances (see below). IM is relatively easy to practice and is a route of choice for vaccination. It allows high transgene expression in the injected muscle group, although this only occurs along the administration site. Thus, homogeneous gene transfer, even for a single muscle, would require multiple injections, which could potentially induce unwanted damages to the tissue. This makes such local delivery methods clinically relevant for diseases affecting large, profound and/or multiple muscles. Furthermore, issues of scaling-up from mouse to bigger animals have been reported.

Muscle electroporation generally consists of IM injections, followed by the application of a pulsatile electric field delivered by electrodes positioned on one or both sides of the muscle area to be treated. This allows a 10- to 100-fold increased transfection compared to IM injection in small and in large animals as well as in humans. Nevertheless, only mm³ to cm³ volumes of muscle can be efficiently treated with single procedures.

Sonoporation uses ultrasounds to create small pores in cell membranes, thus facilitating intracellular delivery. To date, gene-based sonoporation have rarely been applied in clinical settings. HLV gene transfer to SM is a rather quick and simple technique, which may be applied to muscle diseases using either pDNA or viral (mainly AAV) vectors. Hematoxylin and eosin staining and other histological studies (comprising Evan's blue dye) revealed only minimal and transient damages to SM fibers. The edema caused by the injected fluid resolved within 24 hours and even the minimal signs of observed muscle toxicity cleared within 2 weeks post-injection. It should be noted that arterial limb perfusion did not turn out to be safe in a canine model of Duchenne muscular dystrophy (GRMD dogs).

The highest transfection efficiencies were noticed in non-human primates; up to 40% of SM fibers expressed reporter genes following a single limb perfusion of naked pDNA. In humans, two clinical studies (NTC number on clinicaltrials.gov: 00873782) evaluated the injection of up to 20% and 35% of limb volume, respectively, in the lower and upper extremities of young adults with muscular dystrophy. These

<table>
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<th>IV</th>
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BW, bodywide; HLV, hydrodynamic limb vein; IV, systemic intravenous; IM, intramuscular; EP, electroporation; SP, sonoporation. Need for specific materials/skills; the number of stars represents the increase difficulty in performing the method. Efficiency demonstrated from small to large animal models. Clinical trials conducted with the corresponding method for skeletal muscle gene transfer applications.
studies demonstrated the clinical feasibility and safety of the procedure, with no significant side-effects or any major pain being reported. Altogether, non-viral approaches remain valid for treating dystrophic limb muscles, as an alternative to viral vectors in seropositive patients or in indications that require large transgenes with regional gene transfer.40

This review is intended to survey the current status of the HLV procedure. After listing various applications using this administration method, issues possibly occurring during this procedure are identified and further improvements are suggested.

2 | HLV GENE DELIVERY: INTERESTS AND APPLICATIONS

HLV delivery has been implemented in various gene transfer studies and applications (Table 2).

2.1 | HLV injection for in situ production of secreted proteins

HLV gene transfer can be performed with the aim to use the SM as a protein factory (Table 2). This approach is under investigation for diseases such as haemophilia. Haemophilia B is a result of mutations in the F9 gene encoding the coagulation factor IX, a deficiency of which is responsible for severe bleedings.47 HLV injection was used in haemophilia B dogs to deliver a cassette encoding the coagulation factor IX carried by a recombinant AAV2 vector. The transgene was sufficiently expressed to reach a therapeutic level of circulating factor IX protein for more than 4 years.43 Another example is anemia, which corresponds to a deficit in hemoglobin as a result of red blood cell depletion. Erythropoietin (EPO) is a key hormone for erythropoiesis that is mainly produced in the kidneys. A non-viral gene therapy protocol based on the HLV delivery of an EPO-encoding pDNA was evaluated in SM of subtotal nephrectomied Lewis rats and in rhesus monkeys.48 Long-term inducible transgene expression (for at least 5 years) of an EPO-encoding pDNA was also shown in non-human primates using an AAV vector delivered by HLV injection; no deleterious immune reaction against the transgene could be detected throughout the duration of the study.34

2.2 | HLV injection for DNA vaccination

Genetic immunization can be achieved via IM delivery of an antigen-encoding NA, with in situ antigen expression possibly triggering both cellular and humoral immune responses.49 In comparison with conventional vaccination relying on antigen protein administration, DNA vaccination exhibits several advantages, such as lower production costs, easier fabrication and avoidance of any heat instability.50 HLV DNA delivery was reported as a relevant administration method for vaccination purposes (Table 2). It was revealed to surpass other delivery methods such as IM injection of naked DNA, which failed in scale-up experiment.41,51 HLV injection for DNA vaccination was investigated in various animal models. Antitumor DNA vaccination performed by this procedure showed a protective antitumor immunity in a murine model of melanoma.41 Using a luciferase-encoding pDNA, it was shown that two administrations were sufficient to obtain a high level of anti-luciferase antibodies in mice; similar results could be obtained in rats and rabbits. Immunization could also be achieved for other proteins such as EPO.42

2.3 | HLV injection for genetic disorders

Gene therapy is an attractive therapeutic strategy to cure genetic diseases.52 It basically consists of introducing NA to correct and/or

<table>
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<th>Target</th>
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<th>Model</th>
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AON, antisense oligonucleotide; AAV, adeno-associated virus; NA, nucleic acid; DMD, Duchenne muscular dystrophy; LGMD, limb girdle muscular dystrophy. For the moment, no clinical trials using HLV have been performed to our knowledge, except one with NaCl 0.9% to evaluate the feasibility of this delivery method in dystrophic patients (limb girdle muscular dystrophy, Emery-Dreifuss muscular dystrophy and Becker muscular dystrophy).
supply a genetic defect responsible for a given pathology. To date, more than 2600 gene therapy clinical trials have been completed worldwide; successes are increasingly obtained not only for correcting the hematopoietic tissue, but also for addressing disorders affecting solid organs/tissues. For example, a recent study reported successful SM gene delivery for LGMD 2D through isolated limb infusion, a locoregional method that does not imply any hydrodynamic overpressure. The HLV procedure has been evaluated for this genetic disease, as well as others, as will be outlined below (Table 2).

LGMD comprise a broad range of neuromuscular disorders that are inherited following an autosomal pattern. More than 30 subgroups have been reported at present, displaying different genetic etiologies and phenotypes. To date, no cure exists for these diseases. HLV injection has been used in a study focusing on LGMD 2B, which results from mutation(s) in the dysferlin gene. In a murine model for this disease, the HLV delivery of a naked therapeutic pDNA allowed restoration of dysferlin in treated hind limb muscles. Following a single administration, up to 10% of myofibers were transfected in some muscles. A reduction of Evan's blue dyed positive fibers (a marker of myofiber damage) was also reported, suggesting at least partial restoration of the sarcolemma integrity.

Duchenne muscular dystrophy (DMD) is a recessive X-linked disorder affecting the whole-body musculature. It results from mutations occurring in dystrophin, the largest gene known in the human genome. Dystrophin is a 427-kDa subsarcolemmal protein that connects the actin filaments to a protein complex anchored in the membrane of muscle and heart myofibers. A major role of the dystrophin-glycoprotein complex is to maintain the integrity of the sarcolemma during muscle contractions. To date, treatments for DMD are primarily symptomatic. HLV has been evaluated in diverse DMD animal models, using different strategies implicating naked pDNA or viral vectors. (i) Regarding dystrophin delivery by HLV, Wolff and coworkers delivered a naked pDNA encoding a full-length murine DMD cassette to the hind limbs of mdx mice (a murine model of muscular dystrophy). Following a single injection, 4%-8% of dystrophin-positive myofibers were obtained. This percentage could be noticeably increased when this treatment was repeated. After six injections, it was, on average, 15% in medial thigh muscles and 25% in posterior lower leg muscles during 15 months. In another study conducted in GRMD dogs, HLV administration of a recombinant viral vector (rAAV2/8) carrying a micro-dystrophin transgene allowed long-term dystrophin expression to even much higher level. Up to 84% dystrophin-positive myofibers were indeed obtained in the extensor carpi radialis of the treated limbs. (ii) Exon skipping consists of using an expression cassette to generate antisense oligonucleotides allowing to skip a mutated exon during mRNA splicing; this restores the open reading frame of the dystrophin mRNA with subsequent translation of a shorter but functional dystrophin. Following this strategy, dystrophin was restored in up to 76% myofibers in the forelimbs of GRMD dogs after HLV injection of a rAAV8 (the follow-up time was about 3.5 months). (iii) Myostatin, a negative regulator of muscle mass, can also be targeted to alleviate the dystrophic phenotype. HLV delivery of an AAV8 vector carrying the canine myostatin propeptide gene (an inhibitor of myostatin) could enhance muscle growth of normal dogs as assessed by histology (fiber size measurement) and digital morphometric analysis.

Limb ischemia can be addressed with HLV-administered pDNA encoding short hairpin RNA targeting the prolyl-4-hydroxylase domain enzyme 2 (PHD2). This enzyme is implicated in oxygen homeostasis by hydroxylation hypoxia-inducible factor-1. The latter plays a key role in the regulation of some angiogenic factors. Thus, by inhibiting PHD2, increased recovery with enhanced vascular regeneration could be obtained in a critical limb ischemia murine model.

Mitochondrial disorders may also be targeted using HLV injection. Exogenous pDNA localization to mitochondria has been achieved using both naked or condensed pDNA (using protamine at low N/P ratios).

3 | CURRENT STATE OF HLV DELIVERY: STRENGTHS AND WEAKNESSES

Despite having been proven to be efficient in many cases, HLV gene transfer may still require improvements, especially in view of clinical applications. First, an obvious reason for improving HLV delivery relates to the need for higher gene transfer efficiency (in terms of transgene expression intensity and/or number of cells treated). Although this delivery method allows simultaneous targeting of all the SM of the treated limb, not all muscle fibers are transfected. Furthermore, notably because HLV injection generates different pressures in the various limb muscle groups, a broad range of transgene expression levels can be found across muscles, as shown, for example, upon HLV delivery of a luciferase-encoding pDNA (Figure 3). Yet, high and homogeneous transfection is crucial to assure therapeutic levels. For example, in DMD, HLV delivery of naked dystrophin-encoding pDNA is still unable to restore normal dystrophin expression in a sufficient number of myofibers in every SM, with 40% being usually considered as required to obtain a substantial clinical benefit in patients.

The efficiency of HLV gene transfer depends on the injection volume which determines the hydrodynamic pressure. From works conducted in the mdx mouse, GRMD dog and non-human primates, it was found that the optimal volume needed for efficient naked pDNA limb perfusion was 40% of the limb volume. Compared to studies performed in non-human primates, clinical studies in dystrophic patients were conducted at lower cuff pressures (450–700 versus 310–325 mm Hg, respectively). Perfusion performed at 40% limb volume in one patient was associated with short-lived physiological changes in peripheral nerves without clinical correlates. For safety reasons, the volume delivered in humans will not be as high as that in animals. Moreover, to avoid any compartment syndrome, the injected volume in man could be suboptimum, set to only 20% of the limb volume.

Also, curative treatments for congenital diseases such as DMD should take place before the onset of pathological degenerations (especially fibrosis), which will compromise phenotype reversibility with an obvious impact on therapeutic efficacy. Considering that
treatment should be initiated as soon as possible in early childhood to limit a dilution effect consecutive to muscle growth and necrosis-regeneration cycles, re-administrations may be performed throughout the patient life. The need for repeated administrations may damage and compromise the venous system and in turn the efficiency of treatments. Accordingly, the therapeutic regimen must be both convenient and highly efficient with successive administrations well-staggered over time. As for DNA vaccination, HLV injection was used to perform antitumor vaccination in mice. Following a first HLV delivery, a second one ("boost") was required a few days later to maintain antitumoral activity. Here again, increasing the yield of gene transfer could be useful to avoid such a requirement.

Furthermore, economical aspects are not to be underestimated. For example, it was reported that, compared to rodents, 20–30 higher doses of EPO-encoding pDNA were needed to reach similar efficiencies in larger animals such as dogs or monkeys. Transposed to humans, the amount of NA constructs to be delivered may reach several milligrams, with obvious consequences regarding large scale production and cost of GMP (good manufacturing practice) clinical grade material. For all of the above-mentioned reasons, the efficiency of any treatment based on HLV delivery must be increased. For this purpose, it is important to first identify the potential technical limitations to propose adequate optimizations.

In the next part of the current review, the barriers at each of the sequential steps upon HLV injection are reviewed (Figure 4), emphasizing their potential respective impact on the final transfection efficiency. For the sake of clarity, the HLV procedure is mainly considered as a non-viral method, thus focusing on its use to deliver NA either naked or combined with a synthetic non-viral system.

3.1 | Blood components

First NA crosses through the wall of the saphenous vein, thus gaining direct access to the vasculature system (Figure 4, step 1). At this stage, various interactions may occur with blood components, including nucleases. This can represent a hurdle, especially for naked NA. In vitro studies predicted a half-life of supercoiled pDNA of approximately 1 minute in rat plasma whereas a half-life of between 5–10 minutes was reported for pDNA post-IV injection in mice. Also, when using nanoparticles (NPs) for gene transfer, interactions with serum proteins (e.g. albumin, complement system) can lead to their titration, capture and elimination from the bloodstream. However, it must be noted that a typical HLV injection involves the administration of a large volume of solution flushing in the blood. This forces mechanically the NA solution to extravasate into the vasculatized tissues, this occurring very quickly and therefore limiting the interference with blood components. For this reason, the contribution of the blood barrier, with respect to the transfection yield, might not be a key determinant. This assumption is supported by the observation that no substantial benefit resulted from limb exsanguination prior to HLV injection in non-human primates.

3.2 | Blood vasculature

The endothelium barrier should not be a main barrier to HLV gene transfer, provided that the delivered volume is high enough to produce a sufficient hydrodynamic effect. The increased pressure as a result of the volume introduced into the blood vessels induces a
transient dramatic stretch of the microvasculature (Figure 4, step 2). With the capillary wall being relatively thin, stretchable and easy to break, NA can thus reach the extravascular space passing between, or even through (transcytosis), the endothelial cells. It is noteworthy that the capillary network within SM is continuous unlike in the liver where it is highly fenestrated, with gaps between adjacent endothelial cells.

3.3 Extracellular matrix

The blood vessels of the microvasculature are surrounded by an extracellular matrix (ECM), a complex biopolymer structure acting as a scaffold (Figure 4, step 3). The epimysium, the perimysium and the endomysium are mainly composed of collagen, glycosaminoglycans and proteoglycans. This composition can be modified under particular pathological conditions such as in DMD. In this disease, an aberrant accumulation of ECM is observed, with modifications of the glycosaminoglycans (with higher proportions of chondroitin sulfate and dermanan sulfate) and an increase of the collagen content. Also, a strong chronic inflammatory process is ongoing with a high load of infiltrating mononucleated cells. Thus, the activated inflammatory cells may either incorporate and degrade the vector together with the nucleases contained in this matrix and the fluids. When entrapping NA within a viral or non-viral vector, protection against degradation can be obtained but, in turn, other hurdles can be faced. Several studies suggested that the ECM could trap the positively-charged nano-objects upon IM injection. It was proposed that the ECM could indeed be considered as a selective electrostatic bandpass. According to the model reported in that study, the zeta potential of synthetic (lipid-based) NPs composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine, 1,2-dioleoyl-3-trimethylammonium-propane and 1,2-dioleoyl-sn-glycero-3-[phospho-rac-(1-glycerol)] determines the mobility of these NPs in Matrigel® (an extracellular matrix purified from mouse sarcoma) (Corning Life Sciences, Corning, NY, USA). A dramatic decrease of mobility occurred for NPs exhibiting even a slightly positive zeta potential, as low as +7.2 mV. This decrease was very sharp, with no intermediate situation between mobility and immobility being identified. When considering negatively-charged NPs, a reduction of mobility could also be observed, although it was less stringent than that for positively-charged NPs since it occurred starting from a zeta potential of −30 mV. It is worth noting here that naked pDNA typically exhibits a negative surface charge of approximately −40 mV (depending on the solvent), suggesting that its trafficking through the ECM may also be affected (and could thus be improved). All of these findings are consistent with our own experience using synthetic cationic vectors delivered by HLV injection in mice (Figures 1 and 3). For example, we evaluated 25-kDa poly-ethylenimine (bPEI), a branched polymer exhibiting a very high density of positive charges, and which is widely used for many gene transfer applications. In comparison with naked pDNA, bPEI/pDNA complexes showed much inferior (or even close to the background) transfection efficiencies in all the SM isolated from the treated hind limbs of animals (Figures 2 and 3). Thus, bPEI showed a strong deleterious effect in term of transfection upon HLV injection in every case considered, even for very low bPEI/pDNA mass ratios. Similar results have been obtained with any other (lipid or...
to the liver. Along the same line, it has been proposed that the entry mechanism may also be involved. Macropinocytosis may occur in SM cells following HLV injection. (iii) Other specific features/composition of the sarcolemma may also be involved. Macropinocytosis may occur in SM cells such as described in hepatocytes during hydrodynamic gene delivery to the liver. Along the same line, it has been proposed that the entry of naked DNA into muscle fibers could happen following a specific recognition via a receptor-mediated endocytosis process. In support of this hypothesis, IM injection studies reported that the transfection efficiency of naked pDNA was impaired when a polyanionic compound such as heparin was co-administrated, suggesting a competitive inhibition of pDNA endocytosis. We have observed a similar phenomenon following HLV injection in mice of a pDNA mixed with dextran sulfate (Y. T. Le Guen, T. Le Gall and T. Montier, unpublished data). Regardless of the exact mechanism of entry of pDNA into muscle fibers, the sarcolemma constitutes a noticeable barrier to HLV gene transfer.

### 3.4 | Plasma membrane

The cell membrane (Figure 4, step 4) is a major obstacle for gene transfer in general. For example, in vitro studies using flow cytometry showed that only 0.1%–20% of pDNA copies actually cross this barrier and reach the cytoplasm. Following HLV injection of naked pDNA, the mechanism of pDNA entry through the sarcolemma is not fully understood yet. Several (nonexclusive) hypotheses have been proposed.

(i) First, the hydrodynamic pressure likely promotes a local transient mechanical destabilization of the plasma membrane, allowing NA translocation from the outside to the inside of the cells. This assumption is supported by the finding that, upon IM injection, the transfection efficiency increases proportionally with the injection speed. (ii) In the context of hydrodynamic gene delivery to the liver, it was proposed that the hydrodynamic pressure can generate pores through which NA constructs may enter into the cell (via so called "hydroporation"). Such a mechanism could also take place during HLV delivery. (iii) Other specific features/composition of the sarcolemma may also be involved. Macropinocytosis may occur in SM cells such as described in hepatocytes during hydrodynamic gene delivery to the liver. Along the same line, it has been proposed that the entry of naked DNA into muscle fibers could happen following a specific recognition via a receptor-mediated endocytosis process. In support of this hypothesis, IM injection studies reported that the transfection efficiency of naked pDNA was impaired when a polyanionic compound such as heparin was co-administrated, suggesting a competitive inhibition of pDNA endocytosis. We have observed a similar phenomenon following HLV injection in mice of a pDNA mixed with dextran sulfate (Y. T. Le Guen, T. Le Gall and T. Montier, unpublished data). Regardless of the exact mechanism of entry of pDNA into muscle fibers, the sarcolemma constitutes a noticeable barrier to HLV gene transfer.

### 3.5 | Cytoplasm

Once inside the cell, the cytosol is also an obstacle for HLV gene transfer since the NA can remain trapped inside (Figure 4, step 5). The diffusion of molecules through this natural hydrogel indeed largely depends on their molecular weight. For large macromolecules such as pDNA > 2 kb, the mobility is strongly decreased. Moreover, the cytosol contains nucleases. The half-life of naked pDNA was estimated to be approximately 4 hours in the cytoplasm of a murine myoblast (C2C12) cell line. Hydrodynamic delivery by itself can allow to overcome this step to a certain extent because the transient increase of the intracellular volume can enlarge the mesh size of the cytoplasm hydrogel. As a consequence, the exclusion diameter is increased and the diffusion of exogenous NA can be enhanced, allowing it to get closer to the fiber nuclei. It is noteworthy that, in healthy conditions, nuclei are located at the periphery of muscle cells; thus, the path from the sarcolemma to nuclei might be shorter than in centronucleated cells. However, following HLV delivery, a relationship between the size of NA constructs and their transfection efficiency is generally still observed. This indicates that the hydrodynamic positive effect on NA mobility in the cytoplasm is probably limited, especially for large NA.

### 3.6 | Nuclear membrane

Myofibers correspond to terminal non-dividing cells. Under normal (non-pathological) conditions, they exhibit a very low renewing rate, with an average lifespan of 10–15 years in humans (this duration being most certainly shorter in dystrophic muscles). This has an obvious impact on gene transfer into these cells because the entry of NA constructs into their nuclei (Figure 4, step 6) cannot rely on mitosis, during which the nuclear envelope transiently disappears. Furthermore, in some pathological contexts, the nuclei can be located far from the fiber periphery (centronucleated fibers). Once inside the nuclei, other parameters, notably related to the design of the NA delivered, can impact on transgene expression (see below).

### 4 | POTENTIAL OPTIMIZATIONS: STEP-BY-STEP IMPROVEMENTS

Subsequent to the first description of the HLV injection procedure by Wolff and colleagues, optimizations have been proposed to enable improved benefits from this technique at the same time as preserving its simplicity, safety and clinical translatability. With respect to the successive barriers opposing to HLV gene transfer as listed before, the next part of this review discusses the interest of potential improvements that were already underscored and/or would deserve to be tested in further studies (Table 3).

#### 4.1 | Extravasation from vessels

This step mainly relies on a controlled hydrodynamic pressure applied to enhance endothelial and parenchymal cell permeability (Figure 4, steps 1 and 2). By contrast to arterial delivery, a pre-injection of papaverine (a phosphodiesterase inhibitor) did not provide any benefit following HLV delivery in non-human primates. However, because the injection volume may be reduced when practicing this procedure in humans (for safety reasons as stated before), it could help to counterbalance a lower hydrodynamic effect. In this respect, preconditioning treatments would deserve further attention. It should also be highlighted that compounds such as neutral amphiphilic tri-block copolymers (TBCP) (see below) could open new muscle vascular territories, thereby expanding the vessel ‘surface for extravasation.
Investigations using various chemical compounds can provide clues to better understand this process, which, in turn, could help for improving gene transfer at this step. As for positively-charged (polymer or lipid-based) vectors, they can be efficient for *in vitro* transfection of muscle cells, whereas naked pDNA is inefficient.\textsuperscript{114} By contrast, the same chemical compounds delivered *in vivo* via IM or HLV routes usefully show detrimental effects compared to naked pDNA (as discussed before).\textsuperscript{115} Opposing this, some neutral synthetic compounds such as some block copolymers (described hereafter and in Figure 5) that are inefficient under *in vitro* conditions can provide some benefit for gene transfer in the context of IM delivery. This again illustrates a sharp contrast between *in vitro* and *in vivo* settings, making preclinical (animal) experimentation both an indispensable and unavoidable step.

Neutral amphiphilic TBCP are structurally composed of three subunits. “A-B-A” block copolymers correspond to a sequence of hydrophilic/hydrophobic/hydrophilic moieties, whereas “B-A-B” block copolymers correspond to a sequence of hydrophobic/hydrophilic/hydrophilic moieties. Among the “A-B-A”-type neutral amphiphilic TBCP, Pluronics (BASF, Ludwigshafen, Germany) (also called poloxamers) have been the most widely used for gene transfer to SM. They consist of two poly (ethylene oxide) (PEO) chains surrounding a central poly (propylene oxide) (PPO) chain.\textsuperscript{107} For example, Pluronic F-68© (also named Poloxamer 188 or Lutrol© (BASF, Ludwigshafen, Germany) allowed to increase the gene transfer upon IM injection in the tibialis anterior muscle of mice.\textsuperscript{116} This compound has been shown to promote the interaction of pDNA with membranes and also its cell internalization.\textsuperscript{111} As regards the “B-A-B”-type neutral amphiphilic TBCP, the reverse Pluronics (corresponding to PPO-PEO-PPO) also enhanced gene transfer into SM.\textsuperscript{100} Aside from Pluronics, other original “A-B-A”-type neutral amphiphilic TBCP were designed with alternate chemical moieties, such as poly(tetrahydrofuran) (pTHF) and poly(2-methyl-2-oxazoline) (pMeOxz). Such PEO-pTHF-PPO\textsuperscript{106} and pMeOxz-PPO-pMeOxz\textsuperscript{101} showed some efficiency under similar experimental conditions. Tetronics (BASF, Ludwigshafen, Germany) (star-like polymers) comprise the same chemical groups as Pluronics but with a specific organization (i.e. with four branches grafted on an ethylenediamine core). For example, Tetronic 304 increased the gene transfer efficiency upon IM injection.\textsuperscript{115,117} It is noteworthy that all the above-mentioned copolymers could also be useful for DNA vaccination.\textsuperscript{118} Furthermore, increased transfection could also be obtained by coupling some of the previous compounds with physical gene delivery methods such as electroporation and sonoporation.\textsuperscript{102,105}

In addition to a possible correlation between surface charge of a given compound and its role, as helper or antagonist, for SM gene transfer (notably during ECM crossing), a strong electrostatic interaction and condensation of NA may prevent their recognition with a receptor participating in cell entry, an assumption in support of the “cell internalization via NA receptors” hypothesis. By contrast, compounds such as amphiphilic copolymers that do not (or only slightly) interact with NA would not interfere with such a process. The benefit conferred by the latter class of compounds may also rely on other cell entry mechanisms. This includes a local permeation of the cell membrane.
4.4 | Traffic through the cytoplasm

In the cytoplasm, NA assisted-movements along the microtubules network may be used to get closer to the nuclei, thus escaping the trap constituted by this hydrogel and avoiding degradation by nucleases present in that compartment (Figure 4, step 5). For this purpose, some peptides were designed to bind to NA and to interact with dynein; this led to a notable increase (by a factor 5) of luminescence in the liver of mice upon HTV injection of a luciferase-encoding pDNA. To our knowledge, the potential of such an approach remains to be evaluated in the context of HLV gene transfer to SM. However, again, the relevance of such a strategy may be driven by the intended application and eventual pathological context. For example, in DMD, the lack of dystrophin also results in a strong disorganization of the microtubule network.

4.5 | Translocation into nuclei

Under healthy conditions, SM is a long-lasting stable tissue. With mitosis of muscle fibers being a very rare event, the entry of exogenous NA into the nuclei of the myofibers occurs mainly through the nuclear pores (Figure 4, step 6). Because this translocation is generally poorly efficient, optimizations of this step should be beneficial. Improvements can be obtained by taking advantage of specific (experimentally, pathologically or even therapeutically-induced) conditions, as detailed below. DNA nuclear targeting sequences (DTS) are short DNA sequences capable of binding proteins that shuttle from the cytoplasm into the nucleus. The incorporation of such motifs has been reported to enhance the translocation of pDNA into the nucleus of non-dividing cells in vitro. For example, cyclic AMP response element-binding protein DTS-carrying pDNA displayed a rapid movement through the cytoplasm and a rapid translocation in the nucleus. Nuclear factor-kappa B (NF-kB) was also used for assisted nuclear import of pDNA. An optimized DTS called 3NF (corresponding to three NF-kB binding sites with optimized spacers) was inserted in the backbone of a pDNA; compared to a control pDNA devoid of such DTS, 3NF-equipped pDNA showed significantly increased transfection not only in vitro, but also in vivo (following HTV delivery in mice). It is noteworthy that NF-kB is upregulated under inflammatory conditions and in some muscular diseases (e.g. at an early stage in DMD). It can also be experimentally induced; for example, as a consequence of an hydrodynamic injection or with some amphiphilic block copolymers such as Pluronic p85. Therefore, the HLV delivery of combination of such chemicals with NF-kB DTS-equipped pDNA might be useful. Furthermore, Pluronic p85 can elicit some pro-inflammatory pathways and immune responses, which may be relevant for DNA vaccination purposes. Along the same line, glucocorticoid responsive elements (GRE) DTS might also be useful because glucocorticoids are often included in the therapeutic regimen of muscular diseases such as DMD; GRE DTS-carrying pDNA delivered via nebulization to the lungs in mice has indeed led to a 4.7-fold increased gene expression upon glucocorticoid treatment. Moreover, some studies have highlighted an interest in DTS-equipped pDNA when delivered to SM via a physical gene delivery method (e.g. SV40 DTS-pDNA together with electroporation and NF-kB related transgene expression in mouse tibial cranial muscle after pDNA injection + electrottransfer). Taken together, these findings suggest that the "DTS strategy", optionally combined with some specific chemicals, might be relevant in the context of HLV gene transfer, especially for gene therapy of inflammatory muscle diseases such as DMD.

5 | OTHER MISCELLANEOUS CONSIDERATIONS

Other parameters should be considered because they can also impact on the yield of HLV gene transfer. This includes first the final product formulation to be delivered, with physical/physicochemical properties
that may impact on each step of the delivery procedure. Considering the NA construct, its size impairs transfection efficiency,\textsuperscript{29} emphasizing the need for short and functional NA backbones and transgenes, such as mini- or micro-dystrophin pDNA for DMD.\textsuperscript{109} The purity of pDNA batches is also a crucial parameter, especially regarding residual bacterial genomic DNA that can cause muscle damages.\textsuperscript{133} The viscosity of the solution to deliver can affect the injection rate and biodistribution.\textsuperscript{75} The composition of the solution may disturb normal blood clotting and isotonicity, with direct and/or indirect influence on the bioavailability of the NA product. This was substantiated by experiments using hydrodynamic intra-arterial limb delivery with hypotonic or hypertonic solutions, which reduced gene transfer.\textsuperscript{75} Consequently, formulations must be optimized with regard to purity, viscosity or osmotic strength, aiming to ensure optimal flow and efficient/safe transit across the various physiological barriers. Other parameters such as tourniquet inflation, pressure/duration and rate of injection are also modulators. These can tolerate a range of variations, as seen in rats and non-human primates, demonstrating the robustness of the method.\textsuperscript{38} Accordingly, HLV injection parameters could be tuned to some extents, depending on the intended application.

Besides optimizations dedicated to obtain high and widespread pDNA delivery into the nuclei of muscle cells, improvements could also concern the transgene expression cassette. In addition to the potential benefit taken from an active nuclear import, some DTS might also further enhance the transgene transcription.\textsuperscript{131} This may also be achieved thanks to peculiar properties of some amphiphilic block copolymers.\textsuperscript{134} Pharmacological treatment may also help; for example, it was shown that corticoids, which are used to treat DMD patients, could also increase transgene expression in SM.\textsuperscript{135} Moreover, re-expression of a silenced transgene might possibly be reactivated thanks to subsequent HLV administration(s) of saline, as shown previously for liver transfection using HTV.\textsuperscript{136}

Adequate transgene expression requirements may differ between HLV gene therapy intended for protein production or for DNA vaccination. For the latter application, the strong CMV promoter could be used because it confers a high expression level of the transgene in mammalian cells, whereas the duration of expression is probably not determinant.\textsuperscript{137,138} For long-term expression applications, the use of a muscle specific promoter could be more adequate and ensure to restrict transgene expression to SM.\textsuperscript{16,109} However, it is important to note that, upon HLV, sustained expression could be obtained using various promoters (including the CMV promoter).\textsuperscript{27,29,109} The duration of transgene expression does probably also depend on the CpG dinucleotides present on pDNA because of their immunogenicity-inducing recognition by TLR9.\textsuperscript{139} Accordingly, CpG-free pDNA is less prone to inducing inflammation in lung transfection studies\textsuperscript{140} (as well as following IV injection).\textsuperscript{112} Of note, however, long transgene expression has been achieved following HLV administration of non-CpG free pDNA.\textsuperscript{27} Along the same lines, CpG-enriched pDNA should be more relevant for DNA vaccination.\textsuperscript{111} By contrast, GpG-enriched pDNA has been shown to protect against anti-transgene immune response.\textsuperscript{141} To date, gene therapy pre-clinical studies (in animal models) have shown no induction of any deleterious immune reaction against the transgene following HLV injection.\textsuperscript{34} However, this remains to be confirmed in humans.

6 | CONCLUSIONS

HLV injection is a locoregional delivery method with a broad biodistribution to any SM of a given limb. It was shown to be very efficient in both small and large animals, with a good safety profile and the potential for a variety of clinical applications. However, the efficacy of today HLV non-viral gene transfer might not be optimal and sufficient to reach therapeutic levels because of limitations occurring at the various stages of this multistep process. Some possible improvements of this delivery method have already been discussed above. An example consists of using neutral amphiphilic TBCP, which could have beneficial effects on some steps of the HLV delivery process. It is noteworthy that such compounds could be advantageously combined with DTS-equipped plasmids. It should also be stressed that additional transfection benefits might be obtained by combining HLV delivery with other physical gene transfer methods (e.g. sonoporation). In a broader perspective, some additional improvements might be foreseen. For example, regarding the NA constructs to be transferred, the use of novel oligonucleotide chemistries might be of great potential. Gene editing might also be performed using HLV injection. For example, CRISPR/Cas9 has already been efficiently delivered to the liver via the hydrodynamic HTV method. Finally, taken altogether, the HLV approach might become an efficient delivery method for clinical SM gene transfection.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest.

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REFERENCES


110. Bello-Roufaï M, Lambert O, Pitard B. Relationships between the physicochemical properties of an amphiphilic triblock copolymer...


