D2.5
Strategy paper on viral/non-viral vector development for *in vivo*/*ex vivo* gene therapy

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1. Deliverable's description

The purpose of this document is to outline the RESTORE strategy to improve the accessibility of gene therapies by addressing the technical challenges faced in the development and wider implementation of these potentially life changing therapies. The focus of RESTORE is on genetic modification for therapy or prevention of disease (no genetic enhancement) and limited to modification of somatic cells (no germline) for functional repair or for sustained and regulated expression of therapeutic gene products. Genetic modification can be achieved via *in vivo* (e.g. Luxturna) or *ex vivo* (e.g. Kymriah) gene transfer which have both overlapping and specific challenges. Overcoming these challenges will unlock the full potential of these therapies to address a broad spectrum of inherited and acquired diseases. Here we will focus on mode of delivery, the success of which is crucial to clinical translation, covering both viral and non-viral approaches; the manufacturing challenges of *ex vivo* cell handling, which are common to both genetically modified and unmodified therapies, are covered in Deliverable 2.4.

2. State of the art

There are currently 10 EMA approved, marketed ATMPs; 5 others have been approved and then withdrawn for various reasons, some of which due to challenges outlined in this document. Of the 10 marketed therapies, 3 are *in vivo* gene therapies (Luxturna, Imglygic and most recently Zolgensma) and 4 are comprised of *ex vivo* gene modified autologous cells (Zynteglo, Kymriah, Yescarta and Strimvelis).

Gene transfer strategies can result in either transient modification, for varying periods of time depending on the vector/format used, or stable integration with long term impact of the genetic modification. Both of these have their advantages and disadvantages which need to be considered in the context of the disease being targeted. Both Luxturna and Zolgensma use non-integrating AAV whereas the *ex vivo* modified therapies employ integrating viral vectors to achieve stable integration.

Transient expression resulting in replacement function can be very effective in treating disease states but often requires repeat dosing to manage chronic conditions. Some non-integrating vectors (e.g. rAAV) can persist as extrachromosomal elements for extended periods. The benefit of this approach is to avoid the potential safety risks associated with integration. To date, most integration-based strategies have been relatively straightforward gene insertions which integrate randomly into the host cell genome and are expressed under an exogenous promoter. Viral vectors such as γ-retrovirus or lentivirus have evolved to efficiently deliver and integrate nucleic acid payloads into target cells. However, viral vectors also have several limitations not least the substantially high costs of clinical-grade production, which hinders more widespread clinical applications. As a result, there has been growing interest in the application of non-viral vector delivery systems that have the potential to reduce the costs and complexity associated with, for example, current-gen CAR-T therapies.

Recent analysis of the UK clinical trials database (Cell and Gene Therapy Catapult 2019) exemplifies the range of genetically modified therapies currently in clinical trial globally. 74% of the trials involved genetically modified therapies, 59% of which were *ex vivo* modified and the remaining 41% *in vivo* delivery. *Ex vivo* delivery was solely achieved using viral vectors, the majority of these are virally transduced T cell products (TCR and CAR). For this reason, amongst others, we have chosen CAR-T cells as an exemplar to discuss some of the challenges of *ex vivo* gene modification and propose some solutions in terms of non-viral approaches and the inclusion of gene editing technologies. Of the *in vivo* delivered gene therapy products in the database 89% of these are AAV based, 8% other viral vectors and 3% non-viral.

Across both *in vivo* and *ex vivo* delivered gene therapies it is clear that availability of high quality viral vectors is a challenge given the huge increase in demand over recent years which shows no sign of abating. In this paper we have focused on AAV manufacturing to demonstrate the challenges and potential solutions however many of these can be extrapolated to other viral types, e.g. lentivirus which is currently required in large volumes for *ex vivo* gene delivery.

2.1 *Ex vivo* gene modification

For Chimeric antigen receptor (CAR) T-cell therapy, successful therapeutic outcome depends on long-term expression of CAR transgene in T cells which has resulted in viral vectors being the most widely used technology in advanced clinical trials and regulatory approved CAR-T therapies. Successful adoption of alternatives to viruses to express a CAR transgene in T cells has been underpinned by advances in non-viral delivery techniques that have significanty enhanced nucleic acid delivery into therapeutically relevant cell types. This includes liposomal formulations, nanoparticles, cell penetrating peptides and advanced electroporation methods such as nucleofection. Non-viral strategies used in the clinic to generate CAR-T cells include the delivery of a plasmid-based transposon/transposase system via electroporation or mRNA transfection (Hudecek et al.; R. Zhao et al.). Transposon systems, such as *piggyBac* or *Sleeping Beauty* (SB), are appealing because they unite the favorable characteristics of integrating viral vectors with those of non-viral delivery systems (i.e. lower immunogenicity, reduced costs of GMP manufacture).

Regardless of CAR loading strategy (i.e. viral or non-viral), the immunosuppressive tumor microenvironment has implications for T cell function in terms of differentiation and exhaustion.
One negative regulator of T-cell activity is the inhibitory checkpoint ligand PD-L1, which is expressed on some tumor cells and functions through binding of programmed death-1 (PD-1) receptor on activated T cells. This downregulation of T cell function can be reversed using PD-1 or PD-L1 monoclonal antibodies (mAbs) and studies have shown CAR-T cell therapy and PD-1 blockade to be highly synergistic (McGowan et al.; J. Xu et al.). Ideally, PD-1 blockade will be limited to the specific CAR-transduced T-cells and not T cells more broadly as this can cause widespread immunogenic side effects. This means a combination strategy reliant on cell-intrinsic disruption of the PD-1 gene loci rather than the systemtic administration of of PD-1 mAbs. With the advent of site-specific nucleases, precision modification at specific sites in the human genome is now possible. In particular, CRISPR (clustered regularly interspaced short palindromic repeats) coupled with CRISPR-associated protein 9 (Cas9) endonuclease allows the ability to target multiple genes in T cells to improve cancer immunotherapy. Recently, the antitumor activities of CAR-T and TCR-T cells have shown great improvement with the utilization of CRISPR/Cas9 gene editing to disrupt the endogenous PD-1 gene (Stadtmauer et al.; Dai et al.; Ren et al.; J. Zhao et al.). The scope of this foundational work has since been expanded to

simultaneous targeting of multiple endogenous targets (i.e. TCR, β-2 microglobulin (B2M) and PD1) as efforts continue towards development of optimal 'off-the-shelf' allogeneic CAR-T cells (Stadtmauer et al.; Dai et al.; Ren et al.; J. Zhao et al.). To be successful therapeutically, the functionality of multiplex gene editing strategies depends on safe and efficient delivery of the gene editing machinery into the cell nucleus. Non-viral delivery is unveiling a new era for CAR-T cell therapy by enabling targeted genome engineering. Moreover, electroporation-alternatives and improvements in DNA and RNA synthesis are poised to further enhance CAR-T engineering efforts. Looking beyond *ex vivo* routes, therapy developers will look to leverage advances being made with virally and non-virally delivered gene therapies. Progress here will be essential for truly unlocking the clinical potential of genome editing.

2.2 In vivo gene delivery

In vivo gene delivery has wide therapeutic potential and areas of intervention: genetic diseases, by adding functional genes or replacing dysfunctional genes, correcting or disrupting mutated disease-causing genes through pre-natal, post-natal or adult intervention; endogenous regeneration by delivering factors for *in vivo* tissue protection/engineering; cancer, by direct/indirect tumour cell elimination. Worldwide there are >300 clinical trials testing *in vivo* genetic engineering, the vast majority rely on gene addition, only two trials are based on gene editing strategies so far. Viral vectors are the most used delivery vehicles currently (e.g. AAV, LV, oncolytic vectors) while non-viral vectors (e.g. nanoparticles), or vector-free approaches (RNA, proteins) are currently at an earlier stage of development. Disruptive technologies such as genome editing platforms and non-viral gene delivery systems are expected to play a critical role for future gene-modified therapies (covered in the *ex vivo* sections of this document), but the momentum that has driven viral vectors into the clinical pipeline will ensure the manufacturing requirement for this technology for the foreseeable future.

The extensive use of AAV for *in vivo* gene delivery reflects its relative safety and ability to achieve persistent transgene expression; natural AAV serotypes have been re-purposed as effective recombinant gene delivery vehicles (Sun et al). However, the need for enhanced transduction efficiency and target cell specificity have led to engineered 'evolution' of natural AAV serotypes including point mutations to alter binding and antibody neutralisation activity (Lochrie et al), tyrosine mutants with increased transgene expression (Zhong et al) and alternate receptor footprints for re-directing viral tropism (Asokan et al). Clearly there is still scope for further optimisation of vector design for specific applications however this will not be discussed further in this paper.

Historically, AAV and LV manufacturing workflows mainly relied on the use of 2D planar technologies and fixed bed bioreactors however rapid progress has been made towards suspension culture in stirred tank bioreactors (STRs). Most early clinical trials have used vectors produced using multi-layer cell culture systems (e.g. Cell Factory or HYPERstack) and it is expected that these will continue to be used for these existing products despite their obvious limitations. Comprehensive development and greater adoption of suspension based manufacturing processes would be advantageous, especially for high dose and/or high demand indications.

3. Challenges and Limitations

3.1 *Ex vivo* gene modification

Early generation T cell therapies have relied on integrating viral vector systems to deliver the CAR component. This presents inherent challenges including concerns around patient safety, limited genetic cargo capacity, cost of goods and a more mature phenotype of the final product. Recent estimates calculate an overall cost of 150,000-300,000 $ for CAR T cell therapy products generated using current manufacturing approaches (Vormittag et al.). The generation of viral vectors is expensive and availability of vector at clinical grade is a major barrier to widespread implementation of CAR T cell therapy.

Nonviral integrative vectors, such as *piggyBac* or SB transposons, provide an alternative to modify primary T cells. Compared with retroviruses and lentiviruses, this approach can significantly reduce the complexity of the manufacturing process and are comparably inexpensive (Vormittag et al.). The transposon system is conventionally used as two plasmids, once encoding the transposase and the other encoding a gene of interest (i.e. CAR) within the transposon. However, cell viability can be significantly impacted by this approach as both electroporation and exogenous plasmids are known to be cytotoxic. Moreover, as with any vector that integrates semi-randomly into chromosomes comes the potential risk of insertional mutagenesis leading to transcriptional activation or inactivation of cellular genes.

Site-specific transgene integration mediated by CRISPR-cas9 is a developing strategy to circumvent oncogenic mutations potentially caused by stochastic integration. The approach involves the integration of a functional gene cassette by homology-directed recombination (HDR). Studies have shown that HDR can take place at a very high rate with efficient nuclear delivery and expression and final assembly of CRISPR/Cas9 protein complexes (X. Xu et al.; Puig-Saus and Ribas; Liu et al.). Although viral vectors are an obvious candidate for highly effective delivery, this can result in long-term expression of Cas9 and sgRNA proteins, increasing the risk of off-target cleavage, which is a major concern in establishing the safety of CRISPR/Cas9-based therapeutics. In addition, viral vector cargo size limitations limit efficacy, particularly in adeno-associated virus (AAV) systems, which can necessitate packaging of editing enzymes and donor templates in separate viral particles. Non-viral delivery methods have emerged as a viable alternative because they lack the viral machinery to integrate exogenous DNA material into the host genome. Payload flexibility has also been key in migrating CRISPR/cas9 delivery away from plasmid-based systems, where the protein expression process is delayed, to more transient formats like mRNA and protein. Transient *ex vivo* delivery of CRISPR guide RNA (gRNA) along with the Cas9 mRNA or Cas9 protein has been achieved using several non-viral methods including but not limited to electroporation (Kim et al.), lipids (Zuris et al.), peptides (Suresh et al.), gold nanoparticles and other nanostructures (Lee et al.). Electroporation is the most widely used method currently, however disadvantages include toxicity and proliferation stalling. Nonetheless, commercial availability for GMP-aligned electroporation-alternatives that can support clinical scalability remains limited.

Recently, it was reported that the CRISPR/cas9 system could be used for site-specific *CAR* gene transfer into the T-cell receptor α constant (*TRAC*) (Eyquem et al.). In these cells, referred to as *TRAC*-CAR T cells, the CAR is placed under control of endogenous regulatory elements, reducing exhaustion and tonic signalling in comparison to conventional CAR-T cells (Eyquem et al.). A cGMP-compliant, clinically validated TRAC-CAR T cell manufacturing platform has now

been developed. This platform uses a combination of Cas9 ribonucleoprotein (RNP) electroporation and AAV6 for delivery of the CAR-encoding HDR template. AAV6 donor delivery is not only scalable, but it also performs better than the conventional approach of using dsDNA as a donor template. The use of AAV6 as an HDR donor template is attractive as its genome is composed of single-strand DNA (Albers et al.). Previous studies have demonstrated that single-strand DNA donor templates integrate more specifically at the target site in comparison with double-strand DNA donor templates, which also integrate in an HDR-independent manner at other sites of double-strand breaks (Roth et al.). In summary, we can now exploit HDR for clinical applications including CAR-T generation, but reliance on viral vectors (AAV6) risks impacting the cost-effectiveness of the approach.

A major challenge for CAR-T generation regardless of platform (viral, transposon, gene editing) is the use of a patient-derived (autologous) cells. From an immunological perspective, autologous CAR-T therapy does not elicit an allogeneic reaction. However, autologous CAR T cell therapies require a bespoke manufacturing process for every patient after leukapheresis. This places significant emphasis on the need for high-quality starting material to prevent manufacturing failure, which can be challenging as patients are typically lymphopenic from their disease or previous chemotherapy. The ability to use cells from healthy donors, referred to as 'off- the-shelf' allogeneic CAR T cells, can circumvent this constraint and potentially be the mainstream direction in the future. To be successful, 'off- the-shelf' CAR T products will need to overcome graft-versus-host disease (GVHD) and rejection of the infused allogeneic T cells.

3.2 *In vivo* gene delivery

The major challenges of *in vivo* gene therapies can be grouped into 4 categories: Efficacy, Safety, Immunogenicity and Manufacturing.

Efficacy – delivery efficiency; cargo capacity (AAV ~4.5 kb, LV ~ 10kb); suboptimal control of expression of gene product expression; limited efficiency of gene editing methods; limitations of current animal models; limited efficiency in crossing the blood-brain-barrier

Safety - acute and long-term toxicity related to the delivery system (e.g. genomic integration), to the transgene (expression/overexpression), to other components of the gene therapy product; effects on the biology of the target cells; off-target effects related to both vector load and limited specificity and off–target expression of the transgene or gene editing machinery; limited detection of gene editing-mediated off-target effects or integration of persistent episomal DNA

Immunogenicity - innate or adaptive immune responses to any of the components of the gene therapy product may impact in different ways on the efficacy and safety of the approach (e.g. potential for re-administration)

Manufacturing - scale-up and standardisation of pre-GMP/GMP manufacturing (low yield vs. high costs). Current capacities several logs of magnitude below what is required; extended release time and cost related to post-production quality control; control of all steps of production up to fill and finish, supply chain/stability; suboptimal unit operations; highly variable analytical assays

For the purposes of this paper we will focus on putative manufacturing solutions however these will not be able to resolve all the challenges e.g. gene product expression will require better promoter design and capsid engineering may enable crossing of the blood-brain barrier.Novel solutions to the issue of neutralising antibodies are also being investigated (Leborgne et al).

4. Putative solutions

4.1 *Ex vivo* gene modification

Non-viral transposon systems provide a simple and inexpensive alternative for CAR-T cell production. Until now these systems have been plasmid based, facilitating economical vector amplification in bacteria. Amplified plasmids have known toxicity issues and have several undesirable features for clinical translation including bacterial genetic elements and antibiotic-resistance genes. Recently, studies have demonstrated that CAR-T cells can be engineered through SB transposition of CAR genes from mimilistic DNA vectors called minicircles (MCs) (Holstein et al.). These vectors are attractive because they lack antibiotic-resistance genes, thereby significantly enhancing the safety profile of non-viral gene delivery in clinical settings. Another approach recently described is to use linear, covalently closed, minimal DNA vectors referred to as Doggybones (dbDNA). dbDNA incorporating *piggyBac* has been used to generate CAR-T cells; promoting stable CAR expression comparable to that of conventional plamids (Bishop et al.).

There are numerous chemical and physical and viral-based methods for gene transfer into cells in culture. In the *ex vivo* space, non-viral delivery is appealing as it removes the cost and complexity associated with viral vector manufacturing. Various techniques for non-viral cell engineering are described in research literature, and may become more clinically or commercially applied over the coming years. Meanwhile, electroporation is the most widely used method non-viral technique currently for preclinical and clinical development of cell therapies. While electroporation can be efficient for delivery of nucleic acids to some cell types, toxicity can be high, particularly in primary cells. One strategy to overcome this is use mRNA, which has been shown to cause significantly reduced cellular toxicity as compared to nucleofection with plasmid DNA. For example, mRNA has been described as a source for transient delivery of an SB transposase (Hudecek et al.). Another strategy is to use an electroporation-alternative delivery method. Lipid nanoparticles (LNP) are the most clinically advanced, but conventional methods have faced several challenges including low encapsulation efficiency, batch-to-batch variability, limited control over particle size and limited scalability. End-to-end, commercially available platforms are emerging to address these issues, but custom manufacturing will be required for scale-up and GMP-grade compliance. Finally, Avectas are developing a chemically-mediated platform that temporarily permeabilises the cell membrane. The technology is expected to translate into the clinic in the near future.

Efficient delivery of a nuclease or base editor has opened the door to more sophisticated engineering of CAR-T cells. For example, CRISPR/Cas9 can be used for targeted disruption of PD-1 to enhance CAR-T anti-tumour activity. Mutiplex geneome engineering, whereby several targets are disrupted simultaneously, has expanded the scope of this work and is now looking towards the generation 'off- the-shelf' allogeneic CAR-T cells. For this strategy to work, the αβ T-cell receptor (TCR) on T cells needs to be eliminated to avoid graft-versus-host-disease (GVHD), and human leukocyte antigens class I (HLA-Is) need to be removed to minimize their

immunogenicity. One ongoing clinical trial has been designed to test whether it is safe and feasible to do multiplex CRISPR gene editing, which has never been done before (clinicaltrials.gov; trial NCT03399448). Chromosomal translocations were observed in vitro during cell manufacturing, but these decreased over time after infusion of the engineered cells into the patients. In future, safety risks associated with double-stranded breaks may be circumvented using base editor technology, which can mediate highly efficient multiplex gene disruption with minimal double-strand break induction. A key feature of base editing technology is the fusion of the DNA-binding domain of a catalytically dead Cas9 (dCas9) to an enzymatic activity that changes a single target DNA base. For instance, base editors can knock out genes by changing single nucleotides to create early stop codons. Longer-term, the source cell material for allogeneic is likely to be determined by ability to enrich/select for specific editing events. Even a low presence of residual TCRαβ T cells less than 1% of total cell inoculum constitutes a risk for GVHD (Georgiadis et al.). CAR-T cell derivation from induced pluripotent stem cells (iPSC) is promising owing to the self-renewing ability of iPSCs and their permissiveness to gene editing. A bank of iPSCs with common HLA haplotypes can be generated to minimize the risk of allorejection of CAR iPSC T cells (Iriguchi and Kaneko). Of note, safety and the efficacy of this approach have not yet been clinically assessed.

4.2 Viral vector manufacturing and analytics

As stated above, early clinical trials and products (both *in vivo* and *ex vivo* gene delivery modalities) are very much dependent on scaled out manufacturing workflows using 2D planar technology with obvious limits on output. Improvements in both manufacturing workflows and analytical panels are required to meet the ever-growing demand.

Manufacturing

Depending on the indication and treatment approach the scale of vector manufacture required (at current yields) can vary from $\leq$10L up to >10, 000L so it is clear that there is not one single solution. It is also the case that differing quantities and grade of material will be required at different stages of the product life-cycle therefore extensive and robust characterisation is required to ensure comparability can be demonstrated as manufacturing processes evolve to match requirements. Long lead times and high batch costs for GMP material, even small batches required for pre-clinical work, can stifle the development of these novel therapies. Despite the variable requirements of different sections of the gene therapy community, any advances that improve yield and consistency and reduce time and costs will obviously benefit all therapy developers.

First generation viral vector manufacturing platforms were based on the use of adherent tissue culture plastic; CaCl2 plasmid transfection protocols; ultra-centrifugation and depth filtration for up and down stream processing (USP/DSP). This 1st gen platform is still used in many academic labs to generate small amounts of material for development work. Contract manufacture organisations are largely moving towards 2nd generation platforms using advanced adherent

systems (both 2D planar and 3D); PEI/Lipofectamine plasmid transfection; multi-step chromatographic purification and single use technologies all the way from USP to Drug Product.

In order to fulfil the demands, and therefore realise the therapeutic potential of gene therapies, considerable improvements need to be made and translated into a manufacturing environment. A ‘3rd and beyond generation’ process could include:

- Suspension based technology for scalable expansion/production – use same technology at different scales to support product development cycle
- USP intensification – scaling up is not the only way to increase output
- Continuous elements throughout the bioprocess – improving step yield, throughput and decreasing risk of contamination and subsequent batch failure
- Alternative transfection methods e.g. HTP electroporation – increase transfection efficiency, reduce loss of producer cell viability
- Continuous chromatographic purification – improved utilisation of costly single-use consumables for the processing
- Process analytical technologies (PAT) implementation to identify and manage sources of variability and ultimately support adaptive manufacturing – reduce batch failure, increase consistency
- Continuous biomanufacturing – learning from biologics manufacturing where this has been demonstrated to be cost-effective

There are multiple options for each unit operation and they cannot be developed as stand-alone steps, the entire bioprocess and therefore impact on subsequent steps in the process needs to be considered e.g. changing the lysis step could result in requirement of an additional buffer exchange prior to commencing chromatographic purification. In addition to the bioprocess engineering and technological approaches, generation of higher yield producer cell lines.

As demonstrated by this overview, there is considerable need and considerable opportunity to improve viral vector manufacture to address the access to material challenges faced by gene therapy developers.

Analytics

A typical analytical assay panel measures safety (sterility, endotoxin, mycoplasma etc.) content (titre), purity (host cell protein/nucleic acid) and potency and utilises a number of different methodologies. As the field has progressed and expanded as has the demand for increased sensitivity (e.g. reduction of material use) and throughput (e.g. to support process development activities). As mentioned above, extensive and robust product characterisation supports progression through the development cycle and therefore requires a well-developed analytical plan.

Progress has been made with the use of newer methodologies, examples below and Table 1, however broader validation (e.g. across multiple serotypes) and implementation of these technologies would greatly increase product understanding. This greater understanding would underpin improvements in vector manufacture, *ex vivo* delivery workflows, dosing strategies and even interpretation of pre-clinical and clinical data.

- qRT-PCR for detection of residual HEK-293 host cell DNA (Norman)
- Digital Droplet PCR (ddPCR) for Genome Copy Determination (Li, et al). better accuracy and precision compared to qPCR

- Microfluidic electrophoresis-SDS to characterise AAV8 particles as an alternative to standard silver stained SDS-PAGE (White & Rodionova)
- *In vitro* relative infectivity assay to compare test preparation to standard material

	1st Generation	2nd Generation
Physical titre	ELISA	MADLS
Packaged genomes	qRT-PCR	ddPCR
Packaging ratio	ELISA/PCR	HPCE
Viral capsid proteins	Western blot	Automated WES
Aggregation	DLS	MADLS
Infectious titre	FACS	ddPCR
Functional titre	*In-vitro*	FACS/Impedance
Total protein	Coomassie	LC-MS
Sterility	Growth based	ddPCR
Purity		ddPCR / Seq

Table 1. 2nd generation approaches to viral vector analytics. Taken from Delahaye et al.

Development of standardised and robust suites of assays for each of the commonly used virus/serotypes would contribute to the optimal progress of these promising therapies towards broader clinical availability. Availability of assay standards would also enhance understanding and comparability and demonstrates the type of advance that can better be achieved by consortia approaches for the benefit of the whole field rather than individuals.

5. Challenges for RESTORE

There continues to be steady progress in the advancement of gene therapies, and these are expected to continue to emerge as commercial ATMP products over the next ten years. As these therapies progress toward clinical application, establishing robust commercial scale manufacturing solutions (both for viral vectors and non-viral approaches) will be essential. The greatest challenges facing the translation of *ex vivo* gene therapy at present are the unknowns and emerging technologies such as the fast-developing field of gene-editing. Added to this is the bottleneck of efficient delivery and the lagging auxiliary fields governing manufacturing and analytics on the one hand, and safety and regulatory issues on the other. As described in this paper one of the main challenges for *in vivo* therapies is viral vector manufacturing and analytics. Broken down into their key elements, these combined challenges can be addressed under four

strategic priority areas concerning standardization, Smart QC, Cargo and Combinatorial (Figure 1).

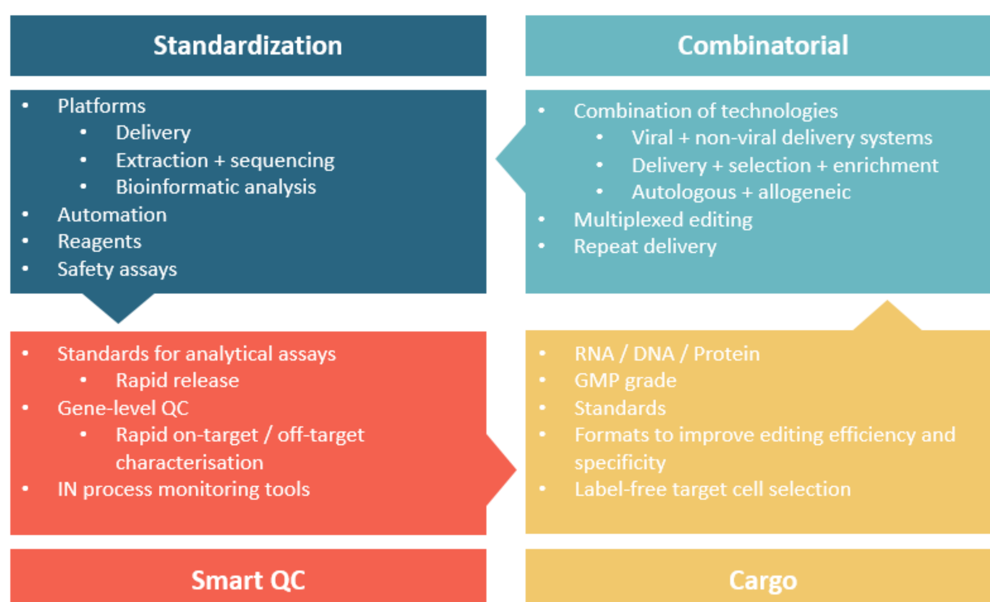

Figure 1: *Four strategic priority areas identified as areas where growth is needed to support the scientific and commercial maturation of gene-modified therapies.*

6. Summary

The majority of the *ex vivo* gene therapy studies are still based on a single gene transfer. However, novel modalities are emerging that rely on genome editing technologies as well as multiplexed approaches combining gene transfer and gene inhibition. In addition, research efforts exist to genetically engineer cell types other than CD34+ cells or T cells, such as gene-modified NK cells, or B cells, as well as other stem cells, such as MSC, for novel *ex vivo* gene therapies. More specifically, in this document, we have explored how the use of gene editing has already resulted in strategies to improve CAR-T cell cytotoxic activity and unlock a new area of exploration in the form of universal CAR-T cells. Whilst the therapeutic promise of gene editing is clear, efficacy of these complex editing systems largely depends on efficiency of intracellular delivery and suitability of the delivery system. Non-viral delivery is unveiling a new era for *ex vivo* modified cell therapy by enabling targeted genome engineering. Moreover, electroporation-alternatives and improvements in DNA and RNA synthesis are poised to further enhance cell engineering efforts.

For both *in vivo* gene modification and current/future generation *ex vivo* gene modified cell therapies availability of appropriate viral vectors (scale, purity, titre, cost) remains a significant bottle neck. This is an area where concerted collaborative effort could help to unlock the full

potential of these therapies to address a broad spectrum of inherited and acquired diseases in parallel with the work on next generation gene delivery and editing approaches.

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