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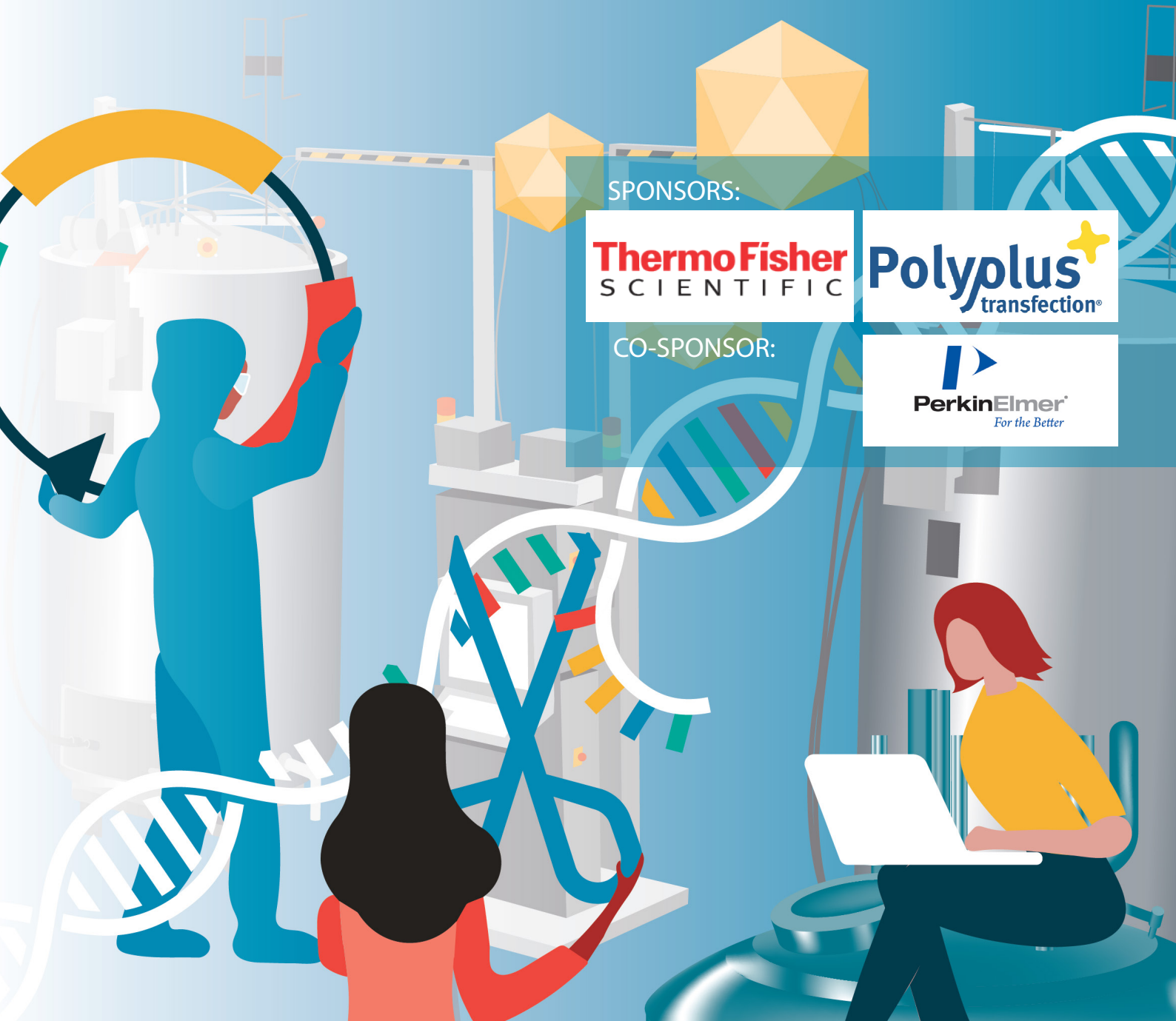
CELL & GENE THERAPY INSIGHTS

SPOTLIGHT ON:

Viral vector bioprocessing & analytics: today's key tools and innovation requirements to meet future demand



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EXPERT INSIGHT

Lentiviral vector manufacturing process enhancement utilizing TFDF™ technology

Thomas Williams, Oliver Goodyear, Lee Davies, Carol Knevelman, Michael Bransby, Kyriacos Mitrophanous & James Miskin

Oxford Biomedica (OXB) is a leading gene and cell therapy company that focusses on Lentiviral Vector (LV) technology innovation, with over 22 years of experience in process development and manufacturing. To address increased LV supply demand, OXB transitioned a GMP LV cell culture manufacturing platform from a cell factory-based adherent cell process to a more scalable, serum-free suspension process performed in single use bioreactors, scaled-up to 200 L in volume at the current time. The relative sensitivity of lentiviral vectors to environmental pH, salt concentration and shear stress during vector harvest and downstream processing continues to present a challenge for the development of efficient manufacturing processes [1]. This work evaluates the TFDF™ (Tangential Flow Depth Filtration) technology developed by Repligen Corporation (Repligen) for the harvest of lentiviral vectors from cell culture supernatant from the bioreactor. The TFDF™ technology effectively separated cells and cell debris from vector particles. Harvest yields typically exceeded 90% with flux rates between 700–750 liters/m^2/hour (LMH) at both 5 and 50 L scales. The tangential mode of TFDF™ was found to be sufficiently gentle on the cells to support multiple harvests, opening the possibility of greatly increasing LV vector production in a perfusion mode.

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THE CHALLENGES OF SCALING UP LENTIVIRAL VECTOR MANUFACTURING

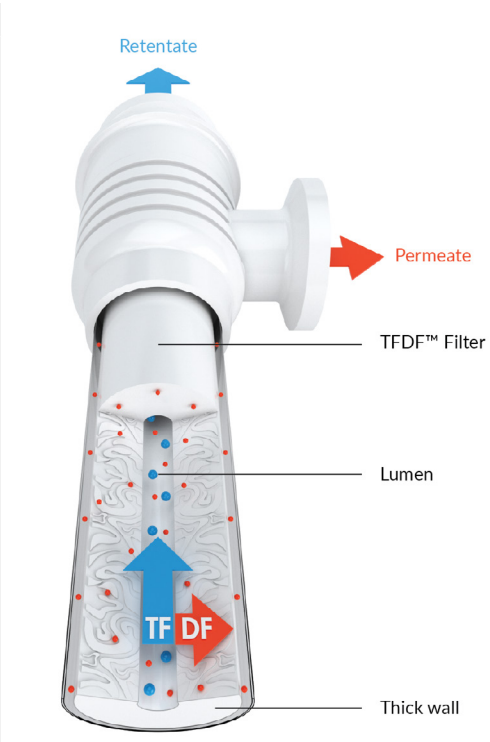
Gene therapy utilizing lentiviral vectors to introduce therapeutic transgenes into the cells of patients represents a unique treatment option for an ever increasing range of genetic and acquired diseases. Key clinical successes, an increase in clinical trial activity in the space, and the approval of lentiviral vector based Advanced Therapy Medicinal Products collectively result in a dramatic increase in global demand for manufacturing capability [2]. However, development of a manufacturing process capable of generating lentiviral products with suitable quality attributes and at the capacity and cost of goods required to provide security of GMP supply remains a challenge across the biotechnology industry. OXB has been a pioneer in the development of products based on lentiviral vectors and has developed a commercial lentiviral manufacturing platform using transient transfection of mammalian cells grown in a serum-free suspension culture. During production, mature vector particles are secreted into the supernatant requiring physical separation of the vector from production cells and cell debris for vector harvest. This initial clarification step is typically performed via normal flow filtration methodologies utilizing filters appropriately sized to enable efficient transmission of vector whilst retaining cells and cell debris. Depth filtration based processes have shown variable recovery and can compromise product quality due to exposure of either the cells or the vector to excessive hydrodynamic stresses [3,4].

TANGENTIAL FLOW DEPTH FILTRATION: A NEW TECHNOLOGY FOR THE ISOLATION OF LENTIVIRAL VECTORS

Repligen recently developed a novel filtration technology, tangential flow depth filtration (TFDF™) based upon passing a cell culture feed stream through a tubular depth filter *in tangential mode* (Figure 1). Cell culture feed travels through the lumen of the tube where retentate returns to the bioreactor and permeate passes through the depth filter wall. The setup of the system resembles that of a hollow fiber but the TFDF™ filter is highly distinct from a hollow fiber. Whilst a hollow fiber measures 0.075 mm to 0.2 mm wall thickness, 0.5 mm to 1 mm lumen diameter, and is typically constructed of mPES, with an anisotropic structure, the TFDF™ filter has 5.0 mm wall thickness, 4.6 mm lumen diameter, and is constructed of polypropylene/polyethylene terephthalate with an isotropic structure (Figure 2). Importantly, the 2–5 µm effective average pore rating of a TFDF™ filter indicates a potential to pass vial vector particles (typically 20–200 nm in diameter) into the permeate whilst retaining the larger

FIGURE 1

TFDF™ tubular depth filter.



Cell culture feed travels through the lumen with tangential flow against the depth filter wall. Retentate (blue) returns to the cell culture feed stock while permeate travels to a permeate reservoir.

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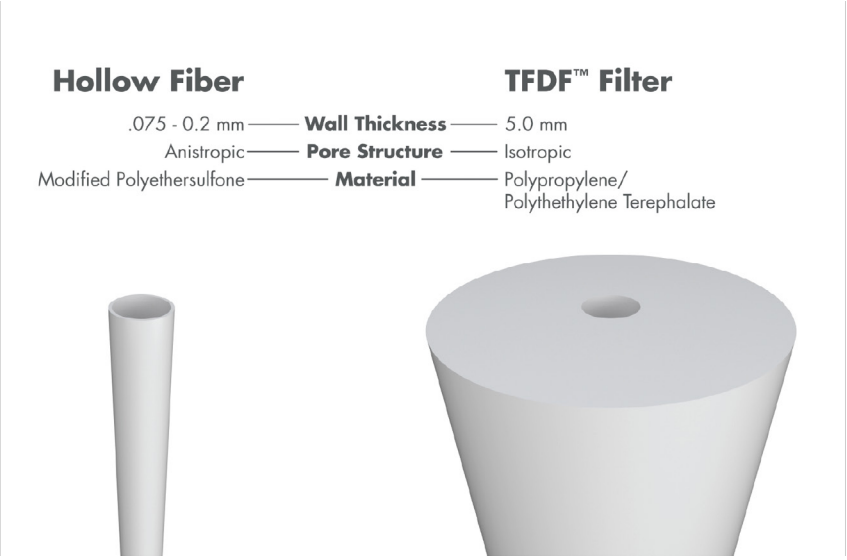
FIGURE 2

Comparison of a hollow fibre filter and TFDF™ tubular depth filter.



cells and cell debris. This study assessed the potential of TFDF™ to separate lentiviral vectors from cells and cell debris during vector production in batch and perfusion modes in bioreactor volumes ranging from 5 to 50 L.

ATTRIBUTES OF THE TFDF™ TECHNOLOGY

The TFDF™ technology leverages aspects of both tangential flow (TF) and depth filtration (DF). Tangential flow through tubular depth filter supports high densities while the structure and capacity of the depth filter enable high product transmission (Figure 3). The combined technology enables cell separation operations of high cell density samples with high flux and high recovery. The filter units scale by increasing both the length of the tube and the number of filter tubes per module (Figure 4).

TANGENTIAL FLOW DEPTH FILTRATION (TFDF™) SYSTEM

The TFDF™ technology comprises hardware, software and ProConnex® TFDF™ flow path components. Both the hardware/software systems and the ProConnex® flow paths have been designed to support bioreactor volumes ranging from 1 to 2000 L (Figure 4). The ProConnex® TFDF™ flow paths are closed, single use, gamma-irradiated and supplied with integrated TFDF™ filter and pressure sensors (feed, retentate and permeate). Genderless AseptiQuik® connectors provide connectivity from the ProConnex® TFDF™ flow path to the bioreactor, additional flow paths and reservoirs. A non-invasive, clamp-on ultrasonic retentate flow meter measures the flow delivered by a magnetic levitating pump from the bioreactor to the filter.

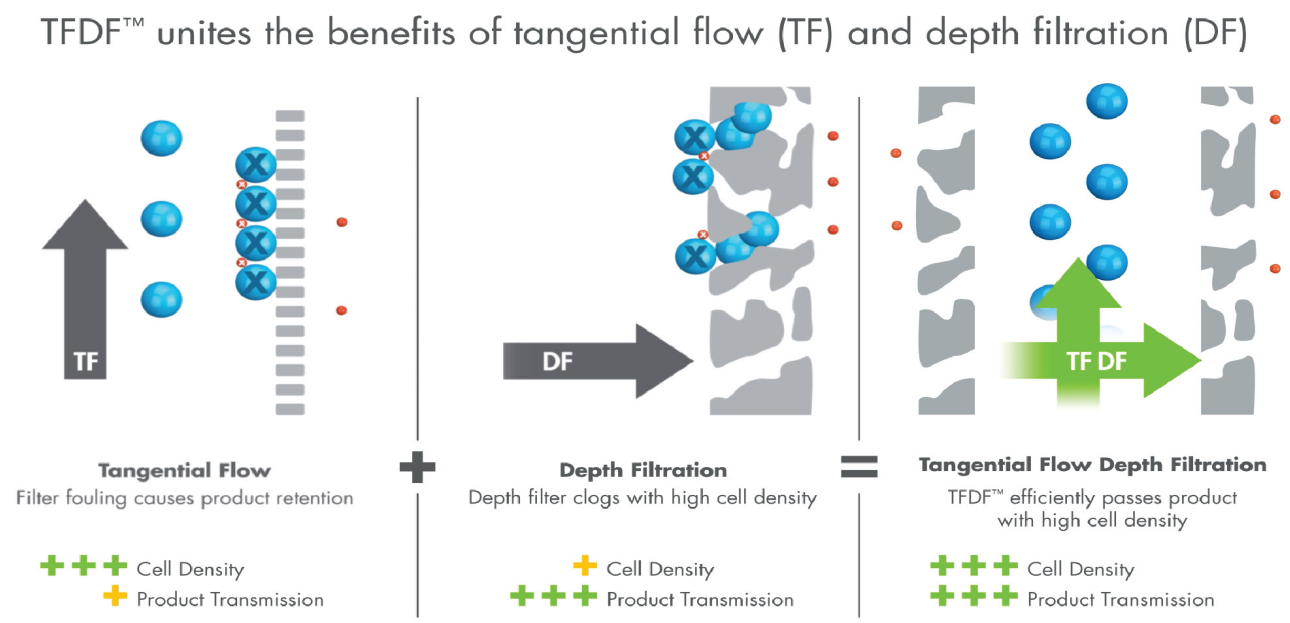
KROSFLO® TFDF™ EVALUATION METHODOLOGY

Experiment #1: harvest of HIV-GFP in batch & perfusion modes

In order to assess the potential use of TFDF™ technology for the isolation of lentiviral vectors during manufacture in serum-free suspension culture, initial studies were performed using a Human Immunodeficiency Virus (HIV-1) vector expressing the Green

FIGURE 3

Tangential flow directs the majority of cells and cell debris over rather than through the filter.



The unique structure of the depth filter enables high product transmission. Operation of a depth filter in tangential mode synergistically enables high yield and flux with high cell density samples.

Fluorescent Protein (GFP) reporter transgene and pseudotyped with the vesicular stomatitis virus G protein (VSV-G). Vector was produced at small-scale in a glass 5 L stirred tank bioreactor (STR) via transient transfection of mammalian cells using lentiviral expression plasmids complexed to a lipid based transfection reagent. To enable harvest of vector via the KrosFlo® TFDF™ System, a ProConnex® TFDF™ flow path with a filter surface area of 55 cm^2 was connected to two *in situ* bioreactor dip-tubes using AseptiQuik® G connectors such that the bioreactor feed and retentate dip-tubes were coupled to their respective lines on the ProConnex® TFDF™ flow path. In a fashion distinct from a hollow fiber tangential flow filtration (TFF) process, the KrosFlo® TFDF™ System applies a pump to the permeate line and during vector harvest the flux for the permeate line was set at 700–750 LMH, a recommended starting point for initial process studies. Following harvest of vector containing supernatant, production cells retained within the bioreactor were re-suspended in fresh media and cultured for an additional period in order to assess the potential for performing multiple vector harvests from a single transient production process. A second vector harvest was performed using the same TFDF™ filter used for initial harvest. In order to assess the efficiency of the TFDF™ harvest process and the impact of TFDF™ harvest on lentiviral functionality, samples of vector containing supernatant were removed from the bioreactor immediately prior to each harvest and also from harvest material collected via the TFDF™ membrane. Functional vector titer in all samples was determined following transduction of adherently grown Human Embryonic Kidney (HEK) 293T cells with transduced (GFP positive) cells quantified using flow cytometry. Briefly, 72 h after viral transduction, treated cells were detached from the assay plate and analyzed for GFP

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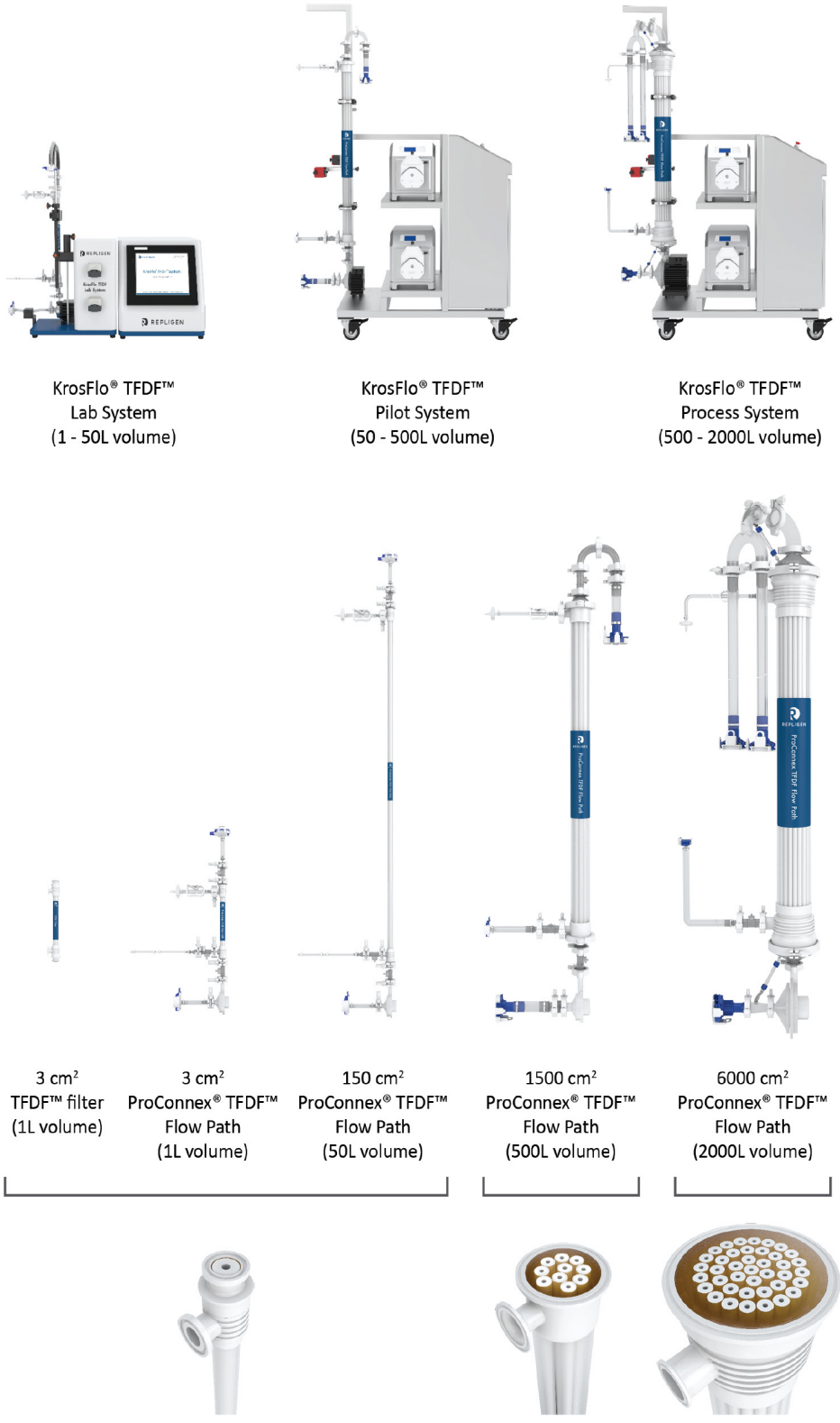
FIGURE 4

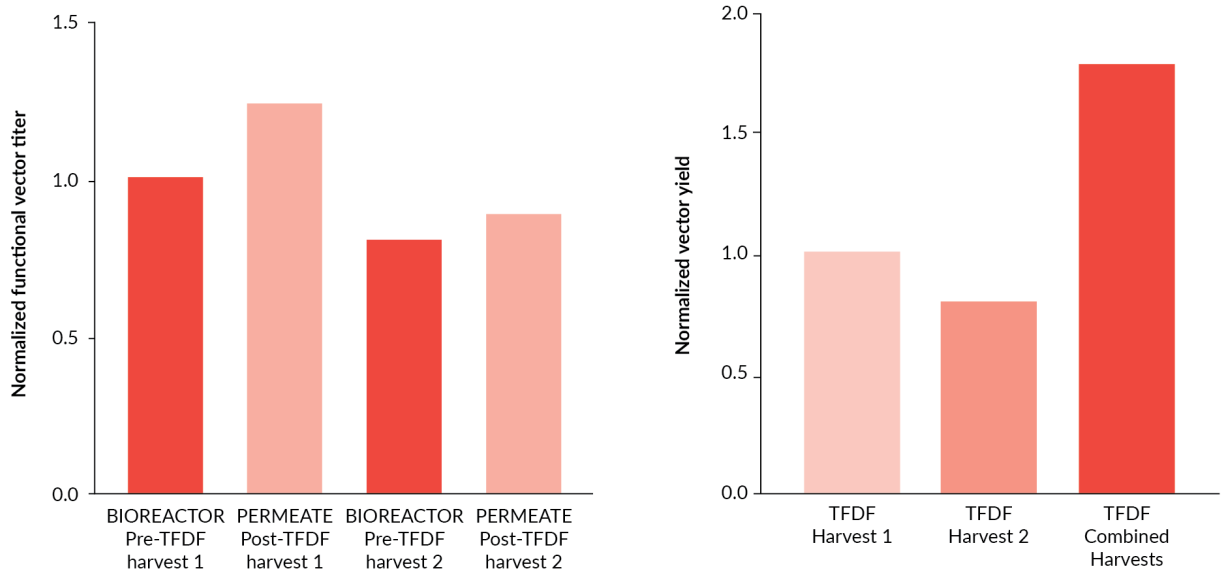
KrosFlo® TFDF™ Systems and ProConnex® TFDF™ flow paths scale from 1 to 2000 L.



The additional flow paths with 55 cm² and 450 cm² surface area are not shown here.

FIGURE 5

(A) Efficient recovery of HIV-GFP lentiviral vector through the TFDF™ membrane. (B) Improved process yields are achieved following multiple TFDF™ harvests.



(A) HIV-GFP vector was produced in a single 5 L STR and harvested from the bioreactor using the TFDF™ system. Following an initial harvest (Harvest 1), cells within the bioreactor were re suspended in fresh media and vector production continued for several hours until a second vector harvest was performed (Harvest 2) using the same TFDF™ system. HIV-GFP vector titers were determined in the bioreactor immediately prior to each harvest and in the TFDF™ permeate following each harvest. All data has been normalized relative to the initial titer in the bioreactor.
(B) Combination of HIV-GFP yields from Harvest 1 and Harvest 2 material resulted in an 80% increase in overall process yield compared to a standard single harvest approach. All values are normalized relative to TFDF™ harvest 1.

fluorescence using a BD FACSVerse™ flow cytometer in conjunction with BD FACSuite™ software. Size and fluorescence data was collected for 10,000 individual events per sample and all samples were analyzed in duplicate. Appropriate analytical gating was utilized to identify the percentage of HEK293T cells that exceeded a fluorescence threshold determined by the background fluorescence measured in non-transduced cells. Functional vector titer was calculated assuming a single transducing unit per GFP positive cell.

Experiment #2: harvest of therapeutic lentiviral vectors

Whilst studies performed utilizing vectors expressing reporter genes such as GFP can be hugely informative for process development, the gene of interest encoded within the lentiviral vector construct can significantly impact both upstream and downstream process performance. Consequently, it is essential that any process development activities are assessed in the context of a directly relevant vector construct. In order to assess the performance of the KrosFlo® TFDF™ System for the harvest of clinically relevant lentiviral vectors, two 5 L STRs were utilized for the production of an Equine Immune Anaemia Virus (EIAV) based lentiviral vector expressing a therapeutic transgene pseudotyped with VSV-G. In one bioreactor, a single vector harvest was performed utilizing the KrosFlo® TFDF™ System operating with an applied permeate flux of 750 LMH. In the second bioreactor, vector was harvested utilizing a commercially available depth filter commonly used for clarification of material derived from mammalian cells grown in suspension culture. Samples were removed from both bioreactors prior to harvest and also from the respective clarified harvest material.

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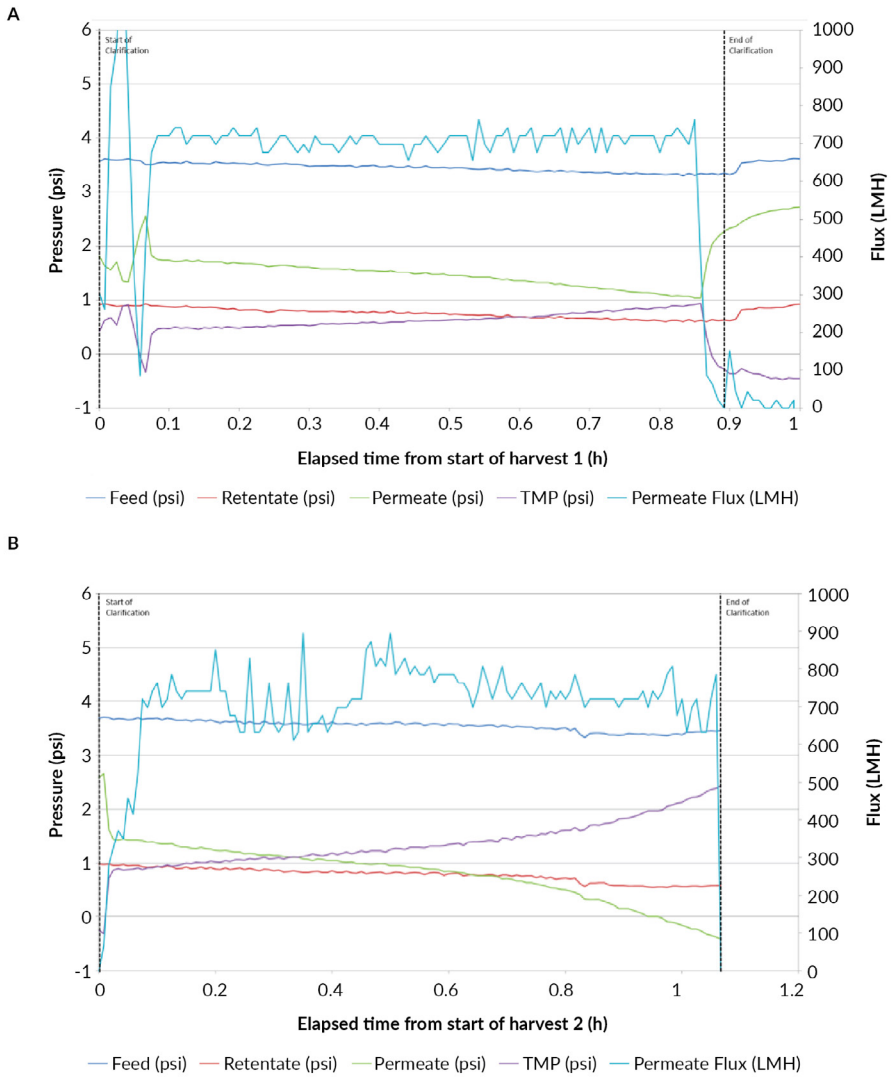
FIGURE 6

(A) TFDF™ Flux and pressure profiles during harvest 1. (B) TFDF™ Flux and pressure profiles during harvest 2.



(A) Flux, feed pressure and retentate pressure remained relatively constant. TMP increased with a slight positive slope and permeate pressure decreased as the inverse of TMP.
(B) Flux, feed pressure and retentate pressure remained relatively constant. TMP increased by 1.5 psi with no impact on flux and permeate pressure decreased as the inverse of TMP.

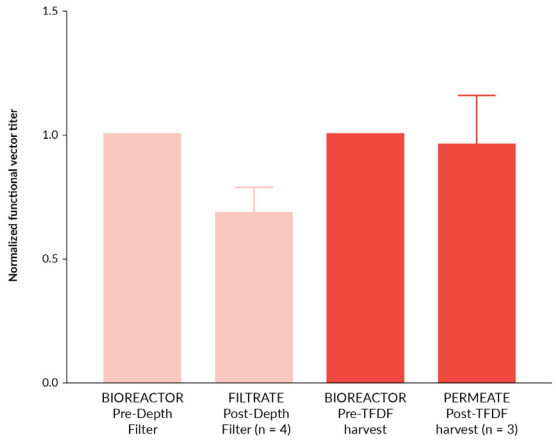
Functional vector titer in all samples was determined following transduction of adherently grown HEK 293T cells with transduced cells identified by immunostaining and subsequent quantification using flow cytometry. Titer determination was performed as described above but transduced cells were identified by flow cytometry following treatment with a fluorescently labelled antibody conjugate targeting the therapeutic protein.

Experiment #3: increased process scale from 5 to 50 L

To evaluate the performance of the KrosFlo® TFDF™ System at a scale more applicable for the clinical manufacture of lentiviral vectors, production and clarification of an EIAV vector incorporating a therapeutic transgene was performed following transient transfection of mammalian cells in a 50 L Single

FIGURE 7

Efficient recovery of a therapeutic EIAV lentiviral vector through the TFDF™ membrane.



EIAV vector expressing a therapeutic transgene was produced in 5 L STRs and vector was harvested from the bioreactor using a standard depth filter approach (n = 4 STRs) or using TFDF™ technology (n = 3 STRs). EIAV vector titer was measured in all bioreactors immediately prior to vector harvest and in the harvested material. Results were used to determine vector recovery during the harvest process. All data is normalized to the titer in the associated production bioreactor and error bars, where present, represent mean ± one standard deviation.

Use Bioreactor (SUB). All vector production process operations were scaled in accordance with accepted engineering principles and in order to accommodate the larger process volume a ProConnex® TFDF™ flow path with a filter surface area of 450 cm^2 was utilized at a permeate flux of 750 LMH for vector harvest. Samples were removed from the bioreactor prior to clarification and also from harvest material collected immediately following clarification through the TFDF™ membrane. Functional vector titer in all samples was determined as described above.

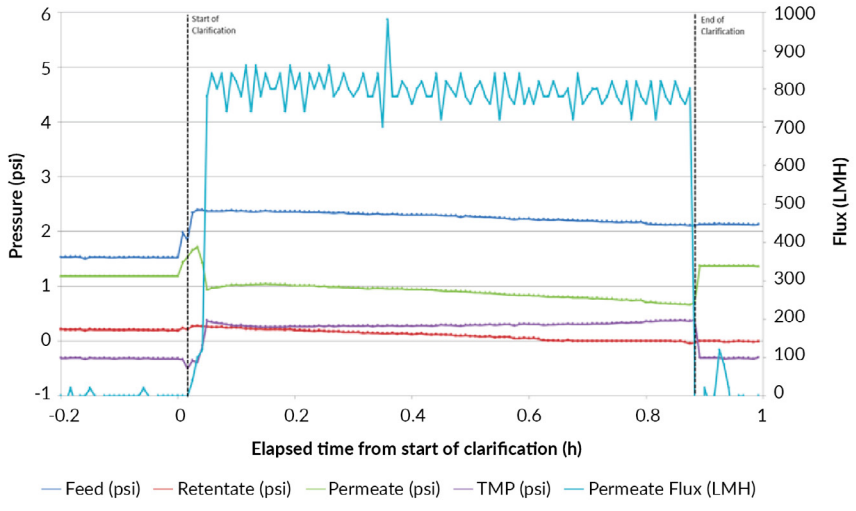
TANGENTIAL FLOW DEPTH FILTRATION: A NEW MODALITY FOR MANUFACTURING LENTIVIRAL VECTORS

This study assessed the feasibility of the TFDF™ technology to harvest lentiviral vector particles in both batch and perfusion modes from serum-free, suspension cell culture. Performance of the TFDF™ technology was compared to a depth filtration method commonly used for clarification of viral supernatant. Experiments were scaled from 5 to 50 L.

Initial evaluation of the TFDF™ technology demonstrated clear separation of HIV-GFP

FIGURE 8

Flux and pressure profiles from TFDF™ system during harvest of a therapeutic lentiviral vector.



During the run, all pressure values were maintained within ±1 psi and a steady permeate flux was achieved.

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lentiviral particles from production cells and cell debris during the harvest process. Analysis of the functional titer in Experiment 1 indicated successful recovery of vector through the TFDF™ membrane at the first harvest and also following a second harvest (Figure 5A). To calculate vector recovery, samples were removed from the bioreactor immediately prior to TFDF™ mediated harvest and from the collected permeate at each harvest time point. For both harvests, the functional titer determined in the bioreactor and associated permeate were similar indicating that there was no measurable loss of vector during TFDF™ mediated harvest. In this initial proof of concept study, the measured functional titers in the TFDF™ permeate samples were marginally higher than those measured in the bioreactor prior to each harvest (Figure 5A). Vector harvest utilizing TFDF™ technology is not associated with concentration of the harvest material and this observation may be attributed to the inherent assay variability associated with titer determination for lentiviral vectors [5]. Importantly, the data demonstrates that the TFDF™ technology is appropriate for use with shear sensitive lentiviral vectors as no loss of vector functionality was observed across the harvest process.

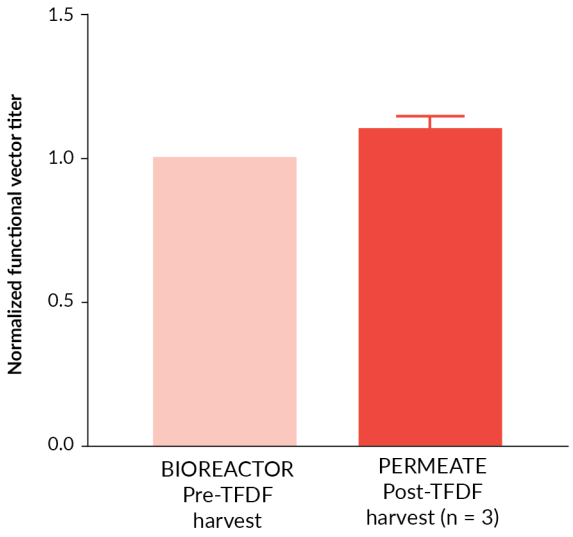
The potential benefits of TFDF™ technology for increasing overall lentiviral vector process yields was demonstrated by the ability to perform multiple vector harvests from a single transient transfection process. Although the observed vector titer in harvest 2 samples was lower than that observed in harvest 1 samples (Figure 5A), pooling of TFDF™ permeate material from both harvests resulted in an increase in overall process yield of approximately 80% compared to the single harvest process (Figure 5B). Multiple harvest steps are not feasible when primary vector clarification is performed using standard depth filtration approaches since the filters result in trapping of the cells outside of the production environment.

Harvest via the TFDF™ system was executed at a relatively high flux of 750 LMH with little evidence of membrane fouling. The flux profile as a function of time indicates relatively minor pressure changes in the feed, retentate, permeate and transmembrane pressures (TMP) over the course of the one hour unit operation (Figure 6A). The only pressure increase of note was a rise of TMP from 0.5 psi at the start of harvest to approximately 1 psi after 45 minutes. Operation at a flux of 750 LMH with a TMP of less than 1 psi differentiates TFDF™ from traditional TFF and depth filtration technologies that operate at significantly higher TMP. The design of the TFDF™ filter itself in combination with a tangential mode that directs the majority of cells and cell debris to be retained within the fiber lumen rather than through the filter is most likely responsible for achieving the observed high flux with low TMP values.

A similar flux profile was generated during harvest 2 using the same TFDF™ filter as harvest 1 (Figure 6B). Flux was again maintained at 750 LMH for approximately 45 minutes. Feed and retentate pressures remained stable

FIGURE 9

Efficient recovery of a therapeutic EIAV lentiviral vector through TFDF™ membrane at 50 L scale.



EIAV vector expressing a therapeutic transgene was produced in 50 L STRs (n = 3) and vector was harvested from the bioreactors using appropriately scaled TFDF™ membranes. EIAV vector titre was measured in all bioreactors immediately prior to vector harvest and in the TFDF™ permeate. Results were used to determine vector recovery during the harvest process. All data is normalised to the titre in the associated production bioreactor and error bars, where present, represent mean ± one standard deviation.

with less than ±1 psi variation. TMP started at approximately 1 psi and increased by only 1.5 psi to a final value of 2.5 psi, indicating that while some cells and cell debris restricted flow through the membrane, the amount did not impact flux.

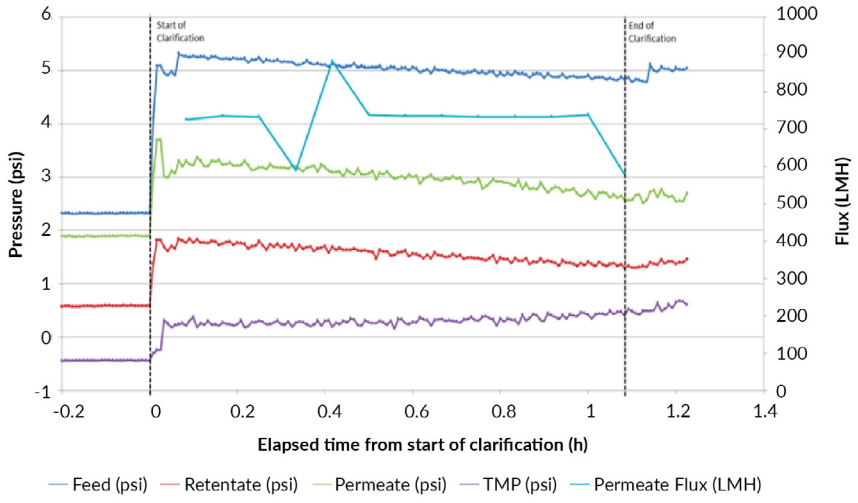
In order to extend the TFDF™ evaluation to include a clinically relevant vector, lentiviral vector harvest utilizing the TFDF™ system was repeated utilizing an EIAV based lentiviral system incorporating a therapeutic transgene. In three independent studies, vector was produced in 5 L STRs and harvested utilizing TFDF™ technology (Figure 7). Similar to results utilizing the HIV GFP vector, efficient transmission of the EIAV vector across the TFDF™ membrane was observed with an average process recovery of approximately 95%. The flux and pressure profiles for the LV vector harvest were similar to those of HIV-GFP (Figure 8). Flux was held constant at 750 LMH and all pressure varied by less than ±1 psi. Similar to runs with HIV-GFP, TMP started at approximately 0.5 psi and progressed with a slight positive slope. The pressure curves illustrate robust filter performance without filter fouling, enabling completion of the harvest unit operation in less than one hour. In contrast to vector harvest utilizing TFDF™ membranes, comparable studies utilizing a standard depth filtration approach for vector harvest in four independent STRs resulted in average vector recovery of only 70% (Figure 7).

Overall, these data demonstrate the efficient recovery of lentiviral vectors utilizing TFDF™ technology and application of a multiple harvest approach could potentially be utilized for further increases in process yield

Process scalability represents a critical requirement of manufacturing technologies. Harvest clarification of an EIAV lentiviral vector expressing a therapeutic transgene was therefore scaled ten-fold from 5 to 50 L in a single-use bioreactor. Vector recovery was assessed in three independent bioreactor studies (Figure 9). In all studies, the ProConnex® TFDF™ flow path was scaled appropriately. While operating at a similar flux of 750 LMH (Figure 10), the TFDF™ technology once again effectively separated cells and cell debris from LV vector at a constant flux with completion of the unit operation in approximately one hour. Comparison of vector

FIGURE 10

TFDF™ Flux and pressure profiles during harvest of a LV vector at a 50 L scale.



Flux, feed pressure and retentate pressure remained stable ± 1 psi. TMP increased with a slight positive slope by 0.5 psi and permeate pressure decreased as the inverse of TMP.

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titer in the permeate to that of the bioreactor again indicated efficient transmission of lentiviral vector across the TFDF™ membrane and complete recovery of the EIAV lentiviral vector was observed (Figure 9).

TOWARDS A PERFUSION MODE?

This study evaluated the TFDF™ filtration technology developed by Repligen for separation of cells and cell debris from lentiviral vectors as a means of harvest clarification in both batch and perfusion mode at scales between 5 and 50 L. This study demonstrates the first practical demonstration and application of this approach for the clarification of lentiviral vector material prior to further downstream purification using the effective 2–5 µm pore rating of the TFDF™ filter. The clarification unit operation was completed in less than an hour, enabled by flux rates between 700–750 LMH, with yields typically greater than 90%. Comparable results were found for both HIV and EIAV based lentiviral vectors and for vectors expressing a reporter gene as well as a therapeutically relevant transgene.

Whilst the high process flux and vector yields observed using TFDF™ mediated harvest clarification offer significant improvements over standard clarification approaches, extension of the TFDF™ to support a perfusion mode for lentiviral vector production holds the potential to significantly increase productivity. Using the HIV-GFP vector, use of TFDF™ as a cell retention device for perfusion of vector particles was shown to be successful with inclusion of a second harvest resulting in an overall increase in process yield of approximately 80%.

Setup and operation of the TFDF™ technology was technically simple, facilitated by the integration of pressure sensors, TFDF™ filter, tubing and clamps into a single ProConnex® flow path, requiring less than 30 minutes for consumable installation and filter priming prior to the run. Users with knowledge of hollow fiber or flat sheet methods will find the overall user experience to be familiar.

To conclude, the KrosFlo® TFDF™ system was found to be an effective tool for lentiviral vector harvest clarification. The process is efficient, easy to use, scalable and offers the potential to improve overall vector yields via repeated harvesting from the production vessel.

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AUTHORSHIP & CONFLICT OF INTEREST

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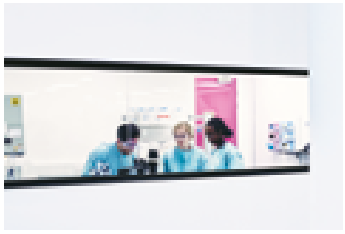
Building the future

Personalised gene-based medicine is entering the mainstream. It is an area of healthcare bursting with new ideas around many unmet needs.

We have always seen its potential and are playing a crucial role in making new curative treatments.

Oxford Biomedica offer tailor-made vector production solutions for companies around the world seeking to develop and commercialise innovative gene therapy products. Discover how our LentiVector® platform is delivering life-changing gene therapies to patients by supporting the successful development and commercialisation of gene and cell-based medicines for our own products and those of our partners.



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CELL & GENE THERAPY INSIGHTS

VIRAL VECTOR BIOPROCESSING & ANALYTICS: TODAY'S KEY TOOLS AND INNOVATION REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

INTERVIEW with (from left to right):
Pim Velthof, Bioprocess Scientist, Upstream Bioprocessing;
Pranav Puri, Bioprocess Scientist, Upstream Bioprocessing specializing in cost modelling; and
Jolanda Van Vliet, Director Quality Assurance & Qualified Person, Batavia Biosciences.

How are fixed-bed bioreactors set to change viral vector processing?

Cell & Gene Therapy Insights 2020; 6(3), 215–220

DOI: 10.18609/cgti.2020.028

Q Batavia possesses capabilities and expertise across a wide range of vector types and culture systems. How has your work evolved as novel technologies have emerged?

PV: I've been with Batavia for 8 years and when I started, there was a lot of effort going into cell line and media development – trying to increase or optimize the production yield. In the last 4 years, I see a shift towards process intensification. We are now involved in many projects with a technology development aspect, specifically for fixed-bed bioreactors such as the iCELLis® (Pall Biotech) and the scale-X™ (Univercells) platforms. So, we've moved from trying to optimize the yields to also optimizing the technologies we use, which is a very interesting development area.

Many process development trajectories for viral vectors are comparable, we work with a transfection system in combination with gene of interest A or gene of interest B. Alongside that, we have gained an extensive amount of knowledge on the fixed-bed bioreactors that we can utilize from project to project.

Q Fixed-bed bioreactors have caught the imagination of the viral vector bioprocessing space. What are the chief pros and cons of these novel systems, versus the more traditional Cell Factory™/cell stack adherent systems?

PP: Cell stacks are uncontrolled systems. It is difficult to track the progression of your process, and as a result of that when using these systems there's an inherent chance you have variability in the batches that you produce. They are also very labor intensive in terms of the manual intervention they require, and are therefore prone to more contamination.

On the other hand, if we talk about the yields of a particular viral vector or any viral vaccine candidate, it depends on the process. As we have a lot of experience, we find that after proper process development fixed-bed processes have similar or higher yields than the traditional systems. If you also take other cost determining factors into account, such as the amount of manual labor and capital investment needed, the fixed-bed system results in significantly lower Cost of Goods (CoGs).

PV: What I observe with most of our projects is that clients come in with a really promising gene therapy product candidate and are typically producing it in small-scale T-flasks. Then the initial thought is "let's do that in a Cell Factory™, so you get the same process in a slightly bigger format".

> "I believe fixed-bed systems to be a great improvement ... It's just one handling of a single machine, and you can do the transfection in a well-controlled high cell density environment."

That may well provide sufficient material for clinical Phase 1 but beyond that to late stage or commercial scale, you have to scale out creating more batch-to-batch variations that later have to be pooled into one clinical batch. The manual handlings required with Cell Factories just creates a multitude of opportunities for human error and contamination. And that's one of the reasons I believe fixed-bed systems to be a great improvement compared to traditional systems. It's just one handling of a single machine, and you can do the transfection in a well-controlled high cell density environment. Moreover, in the fixed-bed systems all cells are already centered in the bed in a high cell density to low volume ratio providing a very clear target for your transfection, minimizing the use of costly transfection reagents and DNA.

PP: It does require more investment in process development at an early stage, which could create a struggle for clients who want to rush to clinical phase. On the other hand, you need to have your end goal in mind. Particularly with gene therapies, where production yields are typically quite low, you will need larger scales quite early in the process. That's one area where fixed-bed systems provide better support.

Additionally, one of the chief roles of fixed-bed bioreactors will be, since the process is totally controlled, to make it easier to implement the principles of Quality by Design; and ultimately to have better product characterization. These things are difficult to implement in cell stack-based production where the process is not under your control.

> "...one of the chief roles of fixed-bed bioreactors will be ... to make it easier to implement the principles of Quality by Design; and ultimately to have better product characterization."

JVV: On the other hand, about 50% of new products will not enter or fail during Phase 1 clinical trials. Many academic centers, for example, won't have the money to invest in early stage process development. Therefore, in that setting, you see the need to move as quickly and as efficiently as possible into Phase 1 clinical trials. Once they have met that criteria and pass that phase, you will then typically see the move to a fixed-bed production method and more robust processes. So, with new drug products there's always a balance between benefit, costs and perceived risk.

For us, from a quality and development point of view, fixed-bed bioreactors are promising for the scale up to commercial manufacturing. It's much easier to perform comparability studies with this production method.

Q How do the currently available fixed-bed bioreactors compare to each other?

PV: I've personally worked a lot with both iCELLis Nano and the scale-X hydro and carbo bioreactors. The iCELLis systems have been around longer, and are therefore further evolved towards a good manufacturing practice (GMP) environment.

Both systems are truly ground-breaking innovations. However, their introduction into GMP settings is not straightforward. There are a number of technical aspects to optimize; but we're seeing progress in this regard.

In terms of scalability, I personally feel the scale-X has a strong advantage at the moment. The scale-X systems are available at three different scales, supporting a larger range than the iCELLis. Univercells offers the research-suited, scale-X hydro, which has 2.4 m^2 surface area, similar to the iCELLis Nano with up to 4 m^2. Then there's the mid-sized reactor, the scale-X carbo, which is in the 10–30 m^2 range, which is excellent for process development projects, because it makes enough product in our hands for clinical Phase 1 and 2 trials. The process can then linearly be translated to the largest scale, with 200–600 m^2. In contrast, for the iCELLis there is a gap between the 4 m^2 and the 500 m^2 system, which can be too large for some projects.

> "...fixed-bed bioreactors are promising for the scale up to commercial manufacturing. It's much easier to perform comparability studies with this production method."

PP: What we can safely say is that both of these fixed-bed bioreactor systems are actually very similar in terms of supporting high cell density growth of adherent cultures, and obtaining the desired yields. This is supported by numerous studies that have been presented at conferences and in scientific publications, showing a lot of similarity between these two systems when it comes to production yields.

Q Considering the cost aspects of the fixed-bed adherent bioreactors, what are the key cost drivers for each of these platforms?

PP: Currently, most viral vectors are still being produced using the traditional systems, which have a high CAPEX demand with respect to the size of the facility and infrastructure they require. On top of that, they have high labor costs, and since these are totally manual and uncontrolled systems, it results also in high OPEX costs. In addition, in the CoGs calculation you should also take into account the inherently high batch-to-batch variability and high contamination rate. These considerations add up to high manufacturing costs.

On the other hand, if we look at the fixed-bed bioreactors, these are single-use systems that offer full control over the process. However, they do require individual development for a particular vector candidate with some process-specific development and implementation in the facility, as well as personnel training, which must be calculated in your cost modelling.

In a very recent study where we compared cell stack systems with fixed-bed systems for the production of several vaccine candidates, we see that the CoGs for fixed-bed systems is between 20 to 80% lower than the traditional systems (depending on the cell line used and the characteristic of the viral vector candidate).

Taking into account all the cost drivers, fixed-bed bioreactor systems do result in significantly lower manufacturing costs per dose.

Q What about the cost comparison with suspension cultures?

PP: In principle, suspension cultures always have a lower CoGs compared to adherent cultures. That's simply because with suspension cultures it's easier to scale up and well established for protein and antibody manufacturing. When it comes to microbial or even mammalian cultures with microcarriers, suspensions always tend to have a lower CoGs, and that's simply based on the scale at which production can be carried out. However, in general you don't often have a choice. Not every product can be produced with suspension cells and not all adherent cells can handle the sheer-stress needed in microcarrier systems to keep the beads in suspension.

In the gene therapy field, having to perform transfections, we don't often see comparable titers from suspension cultures versus adherent cultures. It is much more difficult to obtain those yields ratios to the same level, which of course play a really important role in cost modeling.

Q Are there any particular regulatory and quality considerations related to use of novel fixed-bed bioreactors for viral vector production to consider?

JVV: When introducing new technologies, you always have challenges with the regulatory authorities. You have to show that your product is at least as safe, high quality and effective when using the new technology compared to the old/previous technologies and for that, you need data. That's why at Batavia we are so focused on high quality data in early development. Your process development is the key to the data delivered later on when you go into the GMP facility for clinical production.

Another aspect is that you also should look into compatibility of the product with these new disposable systems, for example, USP class 6 materials. But also the controllers are important to qualify as they deliver the data that dictates the process, and should therefore be CFR part 11 compliant.

Your whole validation strategy and qualification strategy is important, and you should think about that from the beginning and evolve it over time. Defining your operating ranges is therefore very important, because if you

> "...a lot of focus is now shifting towards measuring online oxygen consumption and metabolites, trying to integrate online sampling systems to get more consistent data."

perform your process within the validated operating ranges, you can deliver a high-quality product.

PV: At a recent user day for iCELLis, I asked whether anyone was already talking to regulatory agencies about how they deal with the cell count and state of the cells at the moment of infection? It's an obvious critical process parameter, but at the same time everyone in the field knows that it's very hard to do a representative count and make a representative measure of the cells at the moment of infection, or the moment of harvest for that matter. I found it very interesting that no one has been talking to regulatory agencies on this particular topic yet. Or if they are, they didn't want to share it.

JVV: Cell count is a great example of where we are looking for a replacement for the traditional cell count method as it's just not possible to take representative samples from the fixed-bed bioreactors. It requires a new way of thinking for us, but also for the regulators. For decades that they've looked at cell density at infection, or transfection, and now there is no representative value for this critical process parameter. You must show that you have a stable process, and that the product you deliver complies with the critical quality attributes that you have defined upfront. I think is set to be a tough discussion with the regulators. They are not as involved in the process development as we are. You have to educate them in the new technologies and process control which can be challenging.

Q What is the state-of-the-art in terms of process analytical technologies that are applicable with these platforms?

PV: One of the key developments at the moment is the implementation of online cell density probes, and analyzing their performance as cell count replacements. At the same time, I think a lot of focus is now shifting towards measuring online oxygen consumption and metabolites, trying to integrate online sampling systems to get more consistent data. Continuous measuring of critical process parameters instead of getting just one data point and validating that for go/no-go, provides a more valuable profile and enables more in-depth process understanding.

Q Where can improvements be made in this regard?

PV: I think a lot of the previously mentioned technologies are more targeted at the cell culture phase, but one approach that could help optimize the infection step is integration with online Raman technology. I think this is a very promising approach and I look forward to see how it will provide valuable insights in the vector production process to determine the best moment to harvest.

PP: In addition, there's also some work being done in electron microscopy, where you can obtain information on how much of the viral vector you have produced, much faster than with a classical cell-based assay (e.g., TCID50, PFA or FFA). I see electron microscopy being used more and more, to help provide insights into how the virus is being produced in your system within a couple of hours. I can imagine it would be difficult to integrate such a system in a GMP setting, but at least it gives you controlled information during your process development when you're using fixed-bed bioreactor systems. Also, innovation is being done in the qPCR technology field, potentially becoming a faster and more accurate alternative for cell-based assays.

> "At Batavia, the same team that develops the production process, executes the production in GMP. This way we avoid a time consuming and risky tech transfer."

Q What does Batavia's approach to a viral vector process development project utilizing a fixed-bed adherent culture system look like? What would be the key considerations for each of you?

PP: We normally start with a small-scale development, which involves work in T-flasks or in cell stacks. We would typically do a Design of Experiments (DoE) study to determine the critical parameters which could influence the yield. Then those results are used to come up with the initial settings to be used in the fixed-bed bioreactor. These could include parameters such as seeding density, cell density at infection, multiplicity of infection, as well as the day of harvest. DoE studies are powerful tools to predict your yields as well as some of these critical parameters.

Next, we do the initial process development in fixed-bed bioreactors using the smaller scale fixed-bed systems, like the scale-X hydro or iCELLis Nano. Depending on the viral vector product, and how much process development it would require, it could be somewhere between 4 to 10 runs depending on how well the process is established. The process development also includes upscaling the bioreactor process to 10–30 m^2 fixed-bed bioreactors. Once, the scaled-up process is established, it is used for the manufacturing of product for toxicology studies and then later for Phase 1 clinical studies in a GMP environment.

At Batavia, the same team that develops the production process, executes the production in GMP. This way we avoid a time consuming and risky tech transfer.

PV: The benefit of going over this process multiple times with different clients, is that we know the most efficient route and can make proper risk assessments of potential 'short cuts'. I think that's the added benefit of being a contractor: you do this more often than a biopharmaceutical company.

Pranav has painted a picture of the complete process, but when a customer comes in we can tailor our approach to their needs. It all depends on how well established their current process is and what is still needed to make it fully GMP compliant.

JVV: For me as a qualified person (QP), it's very important to be involved in the early development, because it's essential to keep in mind that you want to implement it in GMP conditions. You have to consider various aspects, such as your raw materials: Are they compliant with GMP requirements? Do we see risks for the process? From a quality or safety point of view, can we introduce the choice of equipment in the GMP facility?

I'm involved in the whole process starting at the beginning, and define what we are going to measure together with the USP and DSP team members, what the critical process parameters are, and what samples we need to take. The analytical part is also extremely important, because you can have a nice process but if the data coming from your assays is not reliable, the process development will fail. Therefore, we are closely involved in the development and validation of the assays, to ensure the data is reliable and useful.

Q Looking to future, where next for viral vector culture system innovation? In what directions do you expect this field to move?

PV: I think the whole industry is moving towards further understanding these systems, and the next critical step will be standardizing methods. There is a lot to standardize! Understanding the growth of different cells in these systems and establishing the optimal transfection environment are central to optimize and standardize these production methods.

JVV: Ultimately standardization will also help reduce variability from batch to batch and increase process knowledge, which in turn will have a positive impact on lowering costs for the currently very expensive gene therapies.

PV: There are so many gene therapy candidates, it's incredible and the pace of developments is rapid. In order to support this growth, standardizing the fix-bed bioreactor methods towards robust production of high quantity and quality material is vital.

In my view that's what Batavia can offer – we can help to put down that standard track.

PP: Another direction Batavia is moving in is working with its partners to develop what we call modular production platforms. These are production units where the USP, DSP, as well as the final inactivation/aliquoting/fill are combined in three modular units. This gives the manufacturer an opportunity to have a cellular growth area of 600 m^2 in a GMP room that's roughly 60 m^2. Having these steps linked together in a modular fashion increases the productivity, lowers CoGs drastically, and is capable of generating commercial amounts.

With the design of such a facility in place, we look forward to the first facility with these modular production platforms being put into use. We are currently busy with CoG calculations to see how such a facility would perform in the long term.

Q What will be the key priorities for each of you, and for Batavia Biosciences, moving forward?

PP: I'm currently working with several viral vector and viral vaccine candidates. All these projects involve performing DoE studies and then setting up the starting point for fixed-bed bioreactor development processes. I will also be doing CoG analysis simultaneously for some of the clients.

JVV: We have recently finalized a pre-clinical batch on a fixed-bed bioreactor. Now, the aim is to move that into GMP to produce a clinical batch, and that will start a discussion with the regulators. Because we provide support for the Investigational New Drug or Investigational Medical Product Dossier, this technology will need to be described as part of the CMC section. This will be the first time, for us at least, that we describe this process in a submission for a clinical trial, which is exciting.

You see a lot of movement in the regulatory guidelines, such as the new ATMP guidelines in Europe. Therefore, GMP becomes more and more important for gene therapy products too. Our aim is always to follow the field and requirements next to identifying opportunities to improve our processes and deliver the quality we want for our customers.

PV: Too many to mention! We have a multitude of small-scale projects going on but what was just mentioned regarding the isolators is one that I am heavily invested in. It's a project investigating a highly intensified polio vaccine (sIPV) production process completely taking place in isolators. These isolators would have the benefit that they can be placed anywhere without high demands on the production environment and already provide the necessary containment and safety. With this, we expect the supply of polio vaccines could be connected close to the location of demand. We want to show the potential of these new production methods from a CAPEX and OPEX perspective. So really interesting times ahead.

BIOGRAPHIES

Jolanda van Vliet is Director Quality Assurance (QA) and Qualified Person (QP) at Batavia Biosciences since 2013. Prior to Batavia, she worked for 3 years at the Dutch Institute for safety, health and environment (RIVM) before joining Janssen Biologics. During her 15 years at Janssen, she mastered all aspects of manufacturing and quality assurance of diverse biological products including antibodies and recombinant proteins. At Janssen, Jolanda was responsible for change control, validation and batch review and release. At Batavia Biosciences, Jolanda is responsible for maintaining the GMP status and to assure proper quality in executing customer programs. As QP, Jolanda is responsible for the technical disposition of Investigational Medicinal products as well as material intended for preclinical safety studies.

Pranav Puri is Bioprocess Scientist at Batavia Biosciences, where he is responsible for a number of upstream process development projects and in silico cost modeling. For the past 10 years, he has held positions at the Rijksuniversiteit Groningen and University of Amsterdam, where he worked within a public–private partnership project. During this time, he has been involved in the development of several microbial platforms and the discovery and production of therapeutic proteins. He has a profound background in Microbial Process Development for all major classes of biopharmaceuticals. Pranav has a PhD in Molecular Biology and Biotechnology and is co-author of 8 scientific publications and book chapters.

Pim Velthof is a Bioprocess Scientist at Batavia Biosciences responsible for a number of upstream process development projects, including the performance of Design of Experiments (DoE) studies. He has 13 years of USP industry experience. Prior to Batavia, Pim has build a profound background in bioprocess development at companies like UniQure, Abbott Biologicals and Centocor, working with various cell lines and viruses such as AAV and influenza virus. At Batavia, Pim holds an MSc degree in Life Science & Technology.

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BATAVIA biosciences

CELL & GENE THERAPY INSIGHTS

SPOTLIGHT

INTERVIEW

Drawing parallels and cross-pollinating ideas between viral vector and vaccine fields

AMINE KAMEN is Professor of Bioengineering at McGill University, and Canada Research Chair in Bioprocessing of Viral Vaccines. He is Researcher Emeritus of the National Research Council of Canada (NRC) where he was employed until the end of 2013, as Head of the Process Development section of the Human Health Therapeutics Portfolio. At NRC, he established one of North America's largest and most advanced governmental centers for animal cell culture addressing process development and scale up of biologics. Along with his team he also licensed multiple technology platforms for efficient manufacturing of recombinant proteins, viral vectors, and vaccines, and led technology transfer to manufacturing sites for clinical evaluation and commercialization.

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Q What are you working on at the moment?

AK: I tend to work between gene and cell therapies and vaccines. The link between the two fields of application is the tool you are using: viruses.

In the case of vaccines, the dominant application I'm working on was triggered by the influenza pandemic 2009, and I think it is highly relevant to what's going on with the coronavirus right now.

"...in the application of both vaccines and of gene and cell therapies ... Regulatory considerations can make it difficult to implement changes, and this has a significant impact on progress."

My approach proposes using cell culture technology to accelerate the delivery of an influenza vaccine during pandemics. We revisited the manufacturing process and using recombinant technologies, we developed the process to start from the sequence variant, HA-NA, and move very rapidly to the vaccine. The preferred platform is our human cell line, HEK-293.

The second vaccine application is focused on taking advantage of the success of the vesicular stomatitis virus (VSV) platform. Originally developed by members of the Public Health Agency of Canada, this platform was used to develop a vaccine against Ebola. The VSV glycoprotein was replaced with the glycoprotein of the Zaire strain of Ebola; this was approved by the FDA just last year. The next step is to use this as a platform with other glycoproteins, such as HIV. This is a large project in Canada involving a number of collaborators, and our contribution is in developing the process and increasing the yield by taking a streamlined approach to the downstream processing of the vaccine.

I'm also working with the adenovirus vector for vaccination. In this case we're again using the HEK-293 cell line to produce a recombinant vaccine. My main activity is looking at process development and downstream processing, and on developing analytical tools with a focus on acceleration and intensification of processes.

For gene therapy, we're again working with the adeno-associated (AAV). As a small virus that is developed as a vector for delivering a transgene, it has started to see significant success in the clinic, with a few products now approved. But the problem with AAV is that currently, it is produced by transient transfection. Our key contribution over the years was to do this in suspension culture, which allowed production on a larger scale – up to 500 liters. Another challenge I'm working to address is that for tissue-specific cell types you have to adapt the process to the variation in serotypes, which translates to challenges in purification and quantification.

Finally, there's CAR-T cell therapy, where the main tool for transforming the T-cell is the HIV-derived lentivirus. The problem is the same: it is produced using transient transfection, and as an additional challenge, the virus is enveloped. That adds additional pressure and on top of that, it is extremely unstable and can lose infectivity. Therefore, the process continues to be adapted.

This may sound like a lot of different angles, but I am lucky to work with a large team who can focus on these different aspects. There is also a kind of cross-pollination between the different activities. When you gain understanding of one system, you are able to translate that knowledge to other issues you are facing.

Q What are your thoughts on the ongoing evolution and emergence of cell culture systems across both vaccine and viral vector areas? Where do you see promise, but also potential roadblocks?

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AK: There is no doubt that this space is predominantly driven by commercial applications and incentives, and I think the reference for that are the Chinese hamster ovary (CHO) cells used in monoclonal antibody production. There, we started at 0.1 gram per liter and people were happy. Now we are at 5–6 grams per liter and people are saying: "you can do better!"

The trend is there and there is fabulous potential. But the reality is that in the application of both vaccines and of gene and cell therapies, the field is behind. Regulatory considerations can make it difficult to implement changes, and this has a significant impact on progress.

However, we are seeing forward movement – the Ebola and influenza emergencies are two examples. In these cases, the drive was less commercial and more society- and health protection-oriented, which is traditionally what you find in the vaccine space: it has been supported predominantly by governments because it is a public health issue. In parallel, there is an acceleration in the space of cell and gene therapy, in the form of the approvals of new products.

The critical limitation I foresee in this space is the people – specifically, the expertise that is required to operate in the spaces of vaccine and cell and gene therapy product manufacturing. Investing in equipment and facilities is not sufficient; there is a pressing need to significantly invest in training, which is something I've been saying for the last 20 years. Unfortunately, we only realize this when we face critical situations such as the current events with Covid-19. We should have taken this lesson seriously with SARS and with MERS, which are both similar viruses. But we didn't, and now we're facing this situation again. The lesson is to prepare in times of peace. We need to operate in a continuous way, and take advantage of the accelerated development we're seeing, as happened with monoclonal antibodies.

However, I am optimistic, because as someone who practices in both of these fields, I believe we are all in the same space. There is specific expertise that is required when dealing with viruses, but the transition for an expert in cell culture is quite straightforward.

Q Focusing specifically on gene therapy viral vector upstream bioprocessing, what would you consider to be the main priorities moving forward? Where is technological innovation most needed?

AK: Transferring genes in vivo is now dominantly achieved using AAV. As I mentioned, this is a very small virus and you have to adapt your process to the serotype. The current limitations for producing large quantities are considerable, because your starting point is transient expression using either the baculovirus-insect cell system or the plasmid transient transfection of mammalian cells.

One recently approved product for inherited disease requires a dose of 10^{14} Viral Genome (VGs) per kilogram. That's a huge quantity that very few manufacturers will have the capacity to produce. The treatment is currently estimated to cost US$2.5 million per patient. By significantly increasing the yield and our capacity to produce these products efficiently, we can reduce these costs.

Improving product quality is also a major concern, specifically improving the purification aspect in order to get the bioactive vector instead and prevent the product from being highly contaminated by empty capsids. This is characteristic of AAV: you might produce a lot of material but if it is empty capsids, it is not delivering the appropriate gene.

In the case of lentivirus, the ongoing challenge is how unstable the product is – the half-life is between 8 and 12 hours, depending on the construct. The particle is still there, but it is no longer functional in terms of transducing the cells. There are so-called 'stable' producing cell lines, but even these will produce over 1 or 2 weeks maximum. Ideally, we need something that will produce over a longer period of time. I see this area evolving alongside the concept of continuous integrated manufacturing.

Although we have seen a lot of forward movement in gene therapy from where the field was two decades ago, we haven't invested significantly in the manufacturing aspect in those intervening years. There has obviously been a lot of research done at the fundamental level, and at the medical level, but not sufficiently at the processing level.

Q Looking at downstream bioprocessing, can you tell us more about the focus and progress of your work?

AK: Moving to more scalable techniques would be extremely beneficial, both across the board for virus applications, and especially for any virus that is enveloped, such as VSV, Influenza, or lentivirus.

In the case of AAV, the only efficient way to absolutely ensure you separate successfully that is in use today is ultra-centrifugation. Now, I have nothing against ultra-centrifugation – I think it's a great method. But for many applications, the implementation is not straightforward. And for industrial ultra-centrifugation, the critical investment is significant. I anticipate it will still be used when needed for large quantities, but I think there is a potential for the downstream process to utilize other methods – such as membrane, ion exchange or affinity chromatography – in order to respond to the specific needs of the vaccine and the cell and gene therapy field.

Q What process analytical tools or methods show the greatest promise in both upstream and downstream applications?

AK: It is important to note that this is one of the most limiting areas of process development. So often at the end of a project, people think "if I had better tools for quantification, I would have done a better job." It is something that often does not get enough attention in the early stages of development, but it is critical and we should invest in it better and earlier.

"My approach proposes using cell culture technology to accelerate the delivery of an influenza vaccine during pandemics."

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"In the case of lentivirus, the ongoing challenge is how unstable the product is ... There are so-called 'stable' producing cell lines, but even these will produce over 1 or 2 weeks maximum. Ideally, we need something that will produce over a longer period of time. I see this area evolving alongside the concept of continuous integrated manufacturing."

An important concept in this space is considering the function of your virus versus the particle itself. Most current work essentially relies on TCID50, which is the dominant infectivity assay, but completely neglects what we might call the load (to the patient) or the total particle. This has been recognized in early clinical trials with gene therapy using AVV. The concept of total particle versus functional particle should always be taken into consideration, especially in the vaccine space, because people define their dose based on the infectious units but behind the infectious units, there is maybe two or three orders of magnitude of total viral particles present that might contribute to the immune response.

This notion is difficult to pass on to the community and it is creating a lot of noise in the field. But we really need as many methods as we can find to describe viral products as completely as possible.

However, for the purpose of process development, we also need something that is inline and that is rapid response. So we use both: we tend to rely heavily on HPLC for analysis of the total particle, and to validate that the material is truly functional, we use either TCID50 or gene transfer units. We also rely on PCR, and are in the process of moving to digital drop PCR (ddPCR).

As it becomes more accessible, I predict we will see more and more use of imaging. In the same vein, advanced microscopy in high or super resolution is becoming more available and accessible. I don't see it as a new process method, but it will help us better understand the problems related to the functionality and stability of these products.

Q What would be your advice to someone planning a viral vector manufacturing facility today?

AK: Times are better than they were some years ago. There used to be a lot of fluctuation – manufacturers building capacity and then stopping. I have visited many exciting GMP facilities that were totally empty.

As I said earlier, the element that's lacking is the human resource – this is the big mistake being made. Governments and organizations are happy to give one-shot deals with a capital

investment, but these operations are not really a luxury. In Canada alone, I know of 6 or 7 fully equipped GMP facilities, but none of them are operational.

My advice to anyone going into this is to think about the operational aspect, not only the physical or equipment aspects. It is not enough to invest in concrete and equipment – you have to truly invest in the people who are going to run your facility, and ensure they are properly trained.

I think the momentum is building now and more people are recognizing this, although a lot of mistakes have been made in the past. The US may be the exception, as there are some very strong gene vector labs there. They do have small capacities, but they are operational and there has been constant investment.

Q What are your chief goals and priorities in your work over the foreseeable future?

AK: Something I mentioned at the beginning was using a VSV platform to develop an Ebola vaccine. It turned out that vaccine needs to be delivered at minus 65°C so my next step is to look at practical issues such as if this can be freeze dried to make it easier to deliver. In some cases, it's the simpler things like this that I work on – to tackle the problems that can arise when you look not only at manufacturing vaccines, but at delivering them to the right place at the right time.

I am also contributing to building cell culture capacity. I have a project in Ethiopia which is dealing with animal vaccines, using advanced recombinant adenovirus technology. Using the technology in this manner is a way to contribute to increasing and building production capacity where it is most needed. In a further example, I worked quite actively with the Institute Pasteur de Dakar in Senegal. I tried to convince them to move to a viral cell manufacturing platform. Unfortunately, though, they were still investing in egg-based manufacturing for yellow fever in order to commercialize more rapidly.

So I have multiple aims, but they're focused on the practicalities. I want to translate cell-based technologies and make them available where they are needed. That's my driving motivation.

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CELL & GENE THERAPY INSIGHTS

VIRAL VECTOR BIOPROCESSING & ANALYTICS: TODAY'S KEY TOOLS AND INNOVATION REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

INNOVATOR INSIGHT

A reproducible, high-throughput platform to quantitatively study AAVs

James White & Natalia Rodionova

The adeno-associated virus (AAV) has the potential for major therapeutic advances in the future due to its low immunogenic response in humans. Studying AAVs through sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis presents multiple challenges in user variability and time consumption. In order to resolve these challenges, a microfluidic platform, the LabChip® GXII Touch™ system, was used to characterize AAV serotype 8 (AAV8) particles. This reproducible technology can be used for high-throughput, quantitative characterization of AAV particles.

Cell & Gene Therapy Insights 2020; 6(3), 449–453

DOI: 10.18609/cgti.2020.055

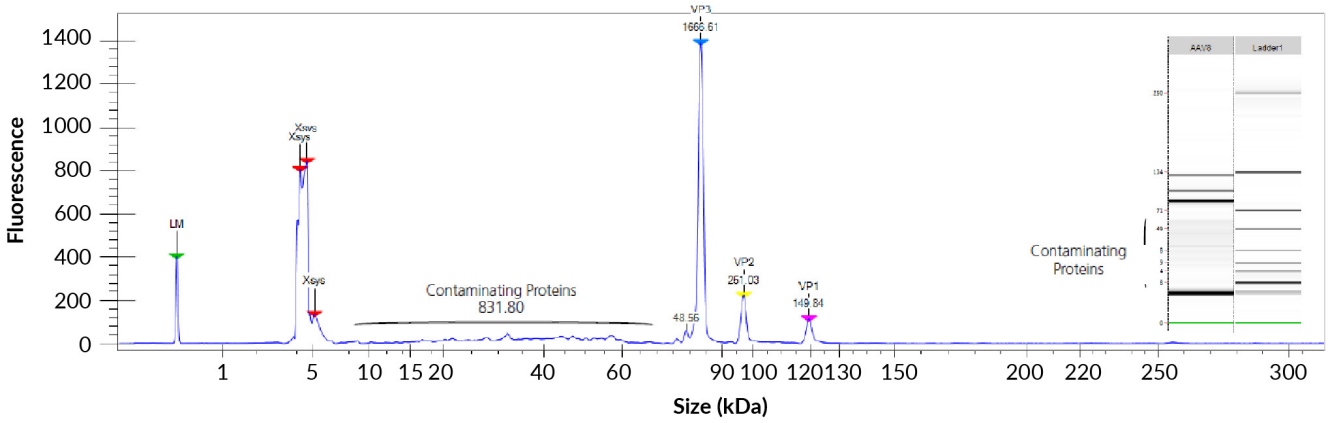
Adeno-associated virus may play a major role for gene therapy applications in the near future. This is due to their ability to infect dividing and quiescent cells while only causing a mild immune response in hu-mans. These small viruses, when engineered to carry specific genes, can be a powerful tool in fighting many diseases. In order to achieve this, AAVs must be well characterized and studied.

Typically, AAVs are studied using SDS-PAGE gels with silver stain. This highly sensitive method yields con-vincing qualitative results but is time consuming and user dependent. Studying AAVs through SDS-PAGE with silver stain in a high-throughput manner is a difficult task [1,2]. This article will specifically look at a microfluidic electrophoresis-SDS platform as a potential solution to simplify the quantitative study of AAVs.

µCE-SDS uses electric fields to create a migration difference based on the charge of the analytes. The ana-lytes are then exposed

FIGURE 1

The output of the LabChip® GXII Touch system is an electropherogram or a virtual in-silico staining gel (insert).



LM is the lower marker and Xsys are system peaks. VP3 (blue), VP2 (yellow) and VP1 (pink) represent the denatured VPs and their concentration (ng/µl). Each peak is annotated with the expected VP type and concentration (ng/µl). Identifying the presence of contaminating proteins is very useful to ensure the overall quality control process for AAV characterization and further analysis.

to fluorescent light to measure the amount of each in the sample. This process is highly reproducible and has comparable sensitivity to SDS-PAGE with silver stain [2,3]. µCE-SDS platforms, such as the LabChip® GXII Touch™ system, have the potential to quantitatively study AAVs in a consistent manner.

The LabChip® GXII Touch™ system is a reproducible quantitative instrument to characterize analytes. This system has a highly sensitive standardized analysis for the size, concentration and purity of proteins. Run-ning 150 nl samples in under 65 s, this automated system is 21 CFR 11 GMP compliant. In this article, we show how AAV8 was characterized using the LabChip® GXII Touch™ system.

AAV8 CHARACTERIZATION

AAV8 particles (catalog#A81000, Welgen Inc., Worcester MA) were generated from HEK-293 cells and contained at least 5×10^{12} genome copies (GC)/ml. This material was placed in a hardshell 96 well V-bottom SBS plate (catalog #6008870, PerkinElmer, Waltham MA) along with the ProteinEXact™ assay's nonreducing sample buffer. The plate was sealed and heated at 70°C for 10 minutes. Once cooled to room temperature, Milli-Q® water (Millipore, Bedford MA) was mixed into each well. The plate was then analyzed on the LabChip® GXII Touch™ system once the plate was spun at 1200 RCF to remove any bubbles.

The AAV capsid forms an outer shell with three viral proteins (VP) known as VP1, VP2, and VP3. These proteins not only protect the AAV genome, but also perform host cell binding. The denatured AAV8 cap-sid proteins were measured using the ProteinEXact™ Assay on the LabChip® GXII Touch™ system. The resulting electropherogram is shown in Figure 1. This assay classifies the three viral proteins as well as determining the presence of contaminating proteins. This is highly useful in the overall quality control process for AAVs manufacturing and study. LabChip® assays are designed to minimize cross contamination between sips.

The AAV8 VP ratios were determined through automatic calculation of the corrected area under the curve (AOC) by the LabChip® Reviewer software. To demonstrate the reproducibility in this determination, 12 samples were superimposed and compared (Figure 2).

This AAV capsid ratio profile can be further used to distinguish AAV serotypes. Purified AAV serotypes were run through the LabChip® GXII Touch™ system and the

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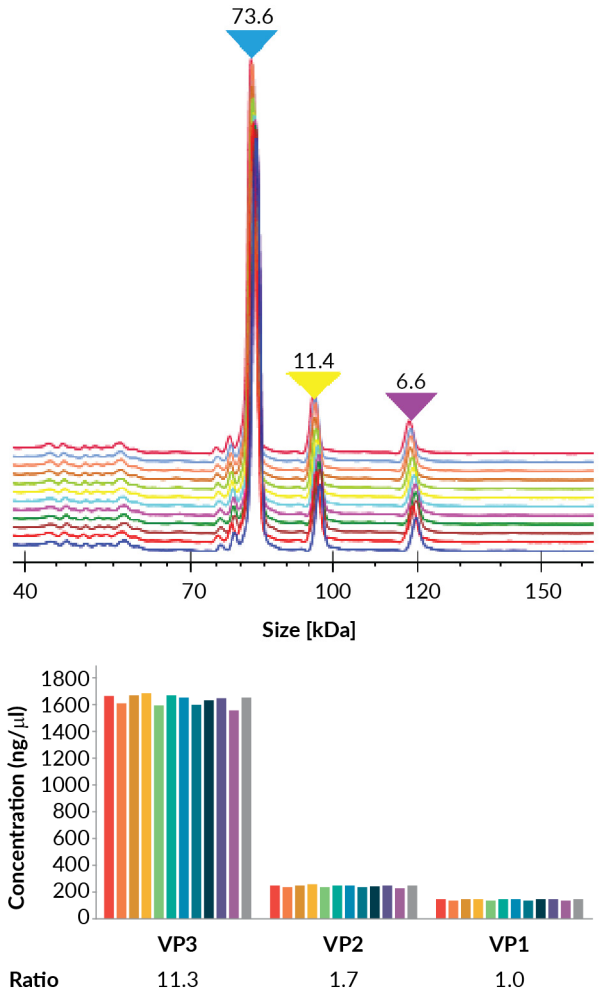
FIGURE 2

An electropherogram overlay (A) and calculated ratio (B).

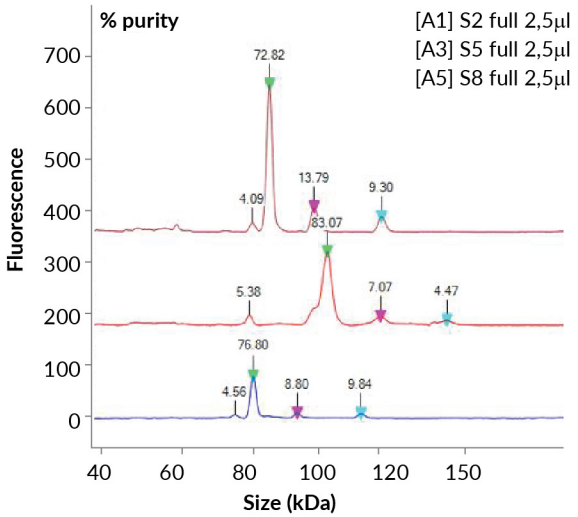


(A) Electropherogram overlay showing 12 runs of each denatured VP peak: VP3 (blue), VP2 (yellow) and VP1 (pink). (B) The calculated ratio was based on the average concentration of each peak.

peaks were determined (Figure 3). In order to properly validate serotype ratios in your

FIGURE 3

AAV capsid profiles of serotype 2 (blue line), serotype 5 (red line), and serotype 8 (brown line).



The peaks for VP3, VP2 and VP1 are shown in green, pink and turquois respectively. Serotypes were highly purified before they were characterized with the LabChip® GXII Touch™ system.

experiments, it is essential to confirm complete capsid breakage.

The LabChip® GXII Touch™ system is an efficient, accurate and reproducible µCE-SDS alternative to SDS-PAGE with silver stain for the quantitation of proteins. This system yields high-quality quantitative results with a faster analysis time and increased throughput than SDS-PAGE with silver stain4. The LabChip® GXII Touch™ system supports high-throughput AAV research by standardizing the analysis of the size, concentration and purity of proteins.

For research use only. Not for use in diagnostic procedures.

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APPLIED GENOMICS

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CELL & GENE THERAPY INSIGHTS

VIRAL VECTOR BIOPROCESSING & ANALYTICS:
TODAY'S KEY TOOLS AND INNOVATION REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

INTERVIEW with:
Nicole Faust, CEO, CEVEC, now part of Cytiva

"The major challenge I anticipate will be to bring down production costs. The current price of gene therapies makes it inaccessible to so many patients worldwide."

Scaling AAV vector manufacture: overcoming roadblocks to the translation of gene therapies

Cell & Gene Therapy Insights 2020; 6(3), 617–623

DOI: 10.18609/cgti.2020.074

Q What impact are the current limitations on AAV vector manufacturing having on the translation of gene therapies, and what do you see as the key challenges when using current AAV production methods?

NF: One of the biggest challenges for manufacturing AAV gene therapies stems from the fact that the methods for producing AAV vectors were developed at the universities where the gene therapies were invented. In this setting, researchers only needed small amounts of the AAV material, and needed it quickly, so they used the methods that were at hand. As these initial therapies were all directed against ultra-rare diseases, there was no need for a huge amount of material for patients either. There simply was not a lot of pressure to develop scalable production methods.

The result was that the R&D methods from the universities were modified and adapted to be performed under GMP, and to be acceptable for clinical studies and commercial material. But they were never developed and evolved to fit production needs at an industrial scale. What we see now are more therapeutic approaches for common diseases, such as Alzheimer's and Parkinson's, moving into the clinic with much larger patient numbers and with a need for high doses of AAV particles per patient. So those adapted lab methods have really hit their limit due to the fact that they are based on adherently growing cells.

The standard cell line for AAV vectors in marketed products is adherently growing HEK-293 cells. These cells are substrate-dependent to be able to proliferate, and they can be grown either using cell culture plates, e.g., cell stacks with 10 plates on top of each other, or more sophisticated devices, like the iCELLis, where cells are grown on fibers. However, these devices have their limitations with respect to capacity and the handling of the cells. Very often serum is involved in adherent production, which is acceptable but does not really meet modern biotherapeutic production standards.

> "The predominant production method right now is still triple transfection of plasmids."

There are suspension methods being developed, and for HEK-293 cells these efforts are evolving, but what they all have in common is that the production step is always based on transient transfection using plasmid DNA, preferably of GMP grade quality. This is needed in huge quantities, so you have a significant cost factor – the plasmid cost can be up to one third of the actual batch production cost. Sourcing the plasmid can additionally become a time constraint as it can take up to half a year. You have to apply a relatively complex process involving mixing the plasmid and transfection reagent and adding that to the process. This might not seem too complex, but when you imagine doing this on a scale of several hundred liters, it can become complicated and also makes the process less robust than a standard protein production

process, for example. All of these features mean that even suspension cell production is not truly scalable.

There are some other methods based on helper virus infection of your production cells – but again, you have to produce the helper virus first, and you have to prove the absence of the helper virus at the end of the process. Methods using adeno helper virus or baculovirus are being used despite these challenges, but they are not broadly applied. The predominant production method right now is still triple transfection of plasmids.

> "Our dream was to make AAV production as easy as antibody production."

Q Could you tell us more about CEVEC, now part of Cytiva's work on viral vectors, particularly your stable AAV production platform ELEVECTA®?

NF: When we started our viral vector work, we initially asked ourselves, which viral vector types are the most important ones in gene therapy. As it turned out, the most common viral vector types are lentiviral and AAV-based vectors. We have production systems for lentiviral vectors, all based on transient transfection, with very competitive titers and yields. But we quickly realized that the real production gap is in the AAV field. Lentiviral vectors are mostly used in *ex vivo* therapy, which means you are adding them to cells that have been isolated from the patient. While this brings its own challenges, these are not so much centered on the vector itself, which is usually required in relatively low doses, making vector production less of an issue here. On the other hand, with AAV there is an increasing demand for big production batches. For this reason, we turned our focus to simplifying AAV production.

Consider monoclonal antibodies: making a monoclonal antibody was a huge challenge 25 to 30 years ago, and now antibody production services have become a commodity; something more or less every CMO can do using standard methods. Our dream was to make AAV production as easy as antibody production, and we decided that the only way to truly achieve this was to move away from transient transfection towards *bona fide* stable producer cells.

To achieve this, we had to integrate all necessary components stably into our cell line, thereby generating a true producer cell that carries everything that is required for AAV production, including the therapeutic gene. Then, all you have to do is expand your cells for the production volume, which can be whatever bioreactor format you are working with, up to several thousand liters. After expanding the cells to that volume, induce induction of the AAV, and essentially the AAV production in this culture works like for a monoclonal antibody.

This makes it sound easy – but of course in reality it was not so simple! Our platform provides the only genuine stable AAV producer cell lines. Components required to make the AAV vector, like the AAV rep genes and some of the adenovirus helper genes, are quite toxic to cells. You thus have to make the system inducible, and due to the molecular setup of AAV, this is not trivial. But we have succeeded – and the result is our ELEVECTA® platform, which allows us to

> "Our platform provides the only genuine stable AAV producer cell lines."

generate producer cells for a desired AAV vector irrespective of the serotype-specific capsid or gene of interest and then help our clients produce their specific therapeutic AAV vector using these producer cells.

We are not only making the process truly scalable because you can run the upstream production process in the big bioreactor format, but this production is now also much more reproducible and robust than what you get from a transient transfection. For example, from a quality perspective, it is important to have a certain amount, ideally a high amount, of full particles. You always have empty particles that do not carry the therapeutic gene in the preparation, but you want that number to be relatively small, and you want it to be constant. That's something you can achieve with a clonal stable cell line, but is very hard to do with transient transfection.

Q What challenges can viral vector production pose at the different stages of gene therapy manufacture, and how are you working to meet those challenges?

NF: The biggest challenge is at the commercial stage, because you have to secure supply for a sufficient number of doses. When you submit your market application to the regulatory authorities, you have to outline how you can guarantee supply of your product.

In earlier project phases, time appears to be a key factor – ideally you want to be the first in the clinic with your product, and you want to be the first to get to market. We see this with clients who have their AAV vector developed: they know what it has to look like, they have convincing animal data, and now they need cGMP material quickly to go into the first clinical phase. The times it takes to make a stable cell line – about a year – can be considered too long in such cases. We are addressing such constraints by offering a fast route to GMP material by moving back to transient production for the first clinical phases. This uses our so-called Alpha cell line, the precursor of the final producer cell. In this way we can deliver clinical GMP material relatively fast while the cell line development is ongoing. For the next clinical phase, our client will have material from the stable cell lines which is very closely related to the cell line that was used for the transient production, thereby avoiding a major platform switch.

At the other end of the development pathway, we also hear from larger Pharma companies who run into obstacles at a later stage – they have just licensed a project which looks very promising after the initial clinical trial, but they now find the production platform being used will not work at larger scales. When they look at production for Phase 3 and beyond, they realize using transfection of adherently grown HEK-293 cells is not going to be a viable option. This is a totally different category of clients we are working with, and who we are helping to make the transition to our ELEVECTA® platform.

Q Looking at the area of viral vector manufacturing as a whole, what are your predictions for the field over the next 5–10 years?

NF: I predict in the AAV field, that for *in vivo* gene therapy we will see many new serotypes, with specific tissue tropisms in order to help direct the therapy to the target tissue. There are a number of companies out there screening for and developing new AAV serotypes. The good thing is that production technologies are adaptable to all such serotypes; if you use transient transfection for serotype 1, it will also work with serotype 2. The same is true for ELEVECTA® – it has worked consistently well with all the different serotypes we have so far tested.

The major challenge I anticipate will be to bring down production costs. The current price of gene therapies makes it inaccessible to so many patients worldwide. Increasing the number of patients does mean you can lower product prices, but if the production costs are too high, there is no way of lowering prices sufficiently. Therefore, we need better, more affordable production methods.

Q You also provide production cell lines for adenoviral vectors. Could you tell us about the interest you've had in utilizing these for the potential production of a COVID-19 vaccine?

NF: When COVID-19 started to become a major health threat, our first thought was to find a way to contribute, but initially we drew a blank. Then, we learned that a number of companies are developing COVID-19 vaccines based on non-replicating adenoviral vectors presenting Sars-CoV2 antigens on their surface. The advantage is that the adenoviral vector itself is harmless, but it boosts the immune response. As of this morning, eight COVID-19 vaccines are in clinical trials, and two of them are based on adenoviral vectors.

Our cell line was originally made to produce adenoviral vectors, and it is designed in such a way that it cannot accidentally generate replication-competent adenovirus. With the standard producer cell line, which again is HEK-293, the genetic setup is such that accidental homologous recombination can happen leading to up to 100 replication competent adenoviruses in 1010 particles. That does not sound like a big number but it is a significant risk as such replication-competent adenoviruses may replicate within the patient's body. Since safety is crucial for a vaccine, which is after all given to healthy people, we receive requests from companies working on such COVID-19 vaccines to use our platform which avoids the risk of replication-competent adenovirus formation.

AFFILIATION

Nicole Faust
CEO, CEVEC, now part of Cytiva

AUTHORSHIP & CONFLICT OF INTEREST

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VIRAL VECTOR BIOPROCESSING & ANALYTICS: TODAY'S KEY TOOLS AND INNOVATION REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

INTERVIEW with:
Patrick Erbacher, CSO, Polyplus-transfection® SA

"What we are seeing in the gene and cell therapy field is that more and more virus manufacturers are coming to us with their suspension cell systems that can support very high cell density per culture volume."

Are suspension-based systems for cell and gene therapy key to commercial-scale manufacture?

Cell & Gene Therapy Insights 2020; 6(3), 221–226

DOI:10.18609/cgti.2020.029

Q What is your take on the current status of viral vector manufacturing for gene and cell therapy?

PE: The first successes of cell and gene therapy prompted us to anticipate future demands for the production of viral vectors at the commercial level, especially for AAV viral vectors that require more production capacity to be able to treat patients. Many clinical trials of rare diseases are in very advanced clinical stages that will lead to rapid commercial approvals. At this stage, demand for viral vectors will require industrial production from manufacturers to meet the needs of patients. Despite the current efforts in building new factories to manufacture these viral vectors, current productivity does not seem to be able to meet emerging demands. In addition to addressing the capacity shortage, there are specific leverage points that can improve production efficiency, primarily the optimization of plasmid DNA, cell line, transfection reagent and cell culture medium. These leverage points play a role during the development phase of the upstream process.

Q There is the recurrent question of adherent or cell culture systems. Do you see a trend in the gene and cell therapy field?

PE: Adherent cell culture systems are historically more present in laboratories especially during early development processes. With adherent cell culture systems, cells are usually grown in presence of serum and on a standard 2D flatware which leads respectively to reproducibility and space limitation concerns. There are several ways to increase productivity by using specialized culture vessels instead of standard flatware, by increasing manual unit operations and by increasing cell density to overcome time and space limitations. What we are seeing in the gene and cell therapy field is that more and more virus manufacturers are coming to us with their suspension cell systems that can support very high cell density per culture volume. In the future, suspension systems will most likely be widely adopted for larger scale industrial processes, inspired by protein and antibody manufacturing trials. With these systems based on cell culture in suspension, the objective is to be able to adapt the production scale to the demand, reduce variability from one batch to another by eliminating various parameters of cell culture (for example, the seeding of cells, serum, etc.) and by simplifying the collection and purification of downstream viral vectors. In addition to the flexibility of these systems using cells in suspension, this approach using high cell densities can be more easily optimized and allow for much higher production yields. Manufacturers of viruses from suspension cells mainly produce AAVs for which a scaling approach is more

“In the future, suspension systems will most likely be widely adopted for larger scale industrial processes, inspired by protein and antibody manufacturing trials.”

appropriate since the objective is to increase the number of viral vector particles produced in order to obtain more doses to treat patients. For the production of lentiviruses used for CAR-T cell therapy, opting for an evolutionary approach in a more sophisticated adherent cell culture system, such as fixed bed bioreactors, is clearly an option. The advantage here is that you can quickly harvest the lentivirus from the supernatant.

Q As a provider of transfection solutions for Gene and Cell therapy, can you share your insight on the current needs for viral vector manufacturing?

"We are now on to the next challenge: increasing viral vector yields to meet the growing demands especially for AAV manufacturing, which is currently the most promising viral vector for gene therapy."

PE: There are several critical parameters that should be considered when striving to improve productivity, and it is ideally during process development that theses parameters should be optimized to ensure later on process industrialization. Process development is divided into two parts: upstream development during which the virus is produced from the cell culture and downstream development during which the virus is purified. For AAV viral vector manufacturing, a 40-50% of recovery rate of viral vectors can be reached with the current set of tools. Hence, a 2-fold increase in recovery is the maximum that can be expected even with further improvements in downstream purification strategies. There is however still considerable room to increase viral vector yields by optimizing key parameters in the upstream development, including cell lines, cell culture media, plasmid DNA and transfection solutions. The idea in the field is to develop each of these components specifically for recombinant AAV production.

Regarding cell lines, the current aim is to develop suspension HEK-293 derived cell lines that grow at very high densities, are easy to culture at different scales of production and last but not least can produce high viral vector yields. For these cell lines to grow at high density and to produce higher yields, it is important to use a chemically-defined cell culture medium that can improve health of cells, notably by reducing cell clumping tendency which in turn negatively impacts transfection efficiency and cell viability. A related question is of course, stable or transient production of viruses? Transient transfection is the gold standard for viral vector production, because it leads to highest production yields and is a quick process. Generation of stable cell lines has not proven to be a reliable alternative to transient viral vector production. Stable cell line generation is time-consuming for in the end a lower productivity. Inducible expression systems have also been developed as an alternative to control in time production of viruses which are highly toxic to the cells, but these systems require further tuning for optimal expression. In the end, most commercially available stable cell lines are developed as packaging cell lines that still need to be transfected with a plasmid containing

the gene of interest. Transient or stable, the question that remains is which transfection solution to efficiently deliver plasmids in order to achieve high viral vector yields? From a transfection solution developer's point of view, we cannot control the number nor the size of plasmid DNA used, we also cannot control the synthetic medium and certainly not the cell line chosen by manufacturers. Hence from the get-go, we focused on developing a transfection reagent that would be used across-the-board, especially because we were seeing a rise in the use of different types of recombinant viral vectors, such as adenovirus, retrovirus, lentivirus and AAVs. This reagent PEIpro® is now the preferred transfection reagent for gene and cell therapy, especially because this is a robust reagent available at all quality grades. GMP compliant PEIpro®-GMP is highly sought after for commercial grade manufacturing.

Q Transfection being a key step for the upstream process, what do you see as innovation requirements for this step?

PE: With our expertise in developing transfection reagents dedicated to viral vector production, we developed the transfection reagent PEIpro® that allows production of viral vectors in adherent and suspension cell systems from process development up to larger scale manufacturing. Since its commercialization in 2012, we've been continuously working hand in hand with viral manufacturers to optimize large-scale transfection. For this, our initial focus was to improve process performance by maintaining comparable infectious virus titer yields during scale-up process, and to meet quality requirements for commercialization by launching PEIpro®-GMP. We are now on to the next challenge: increasing viral vector yields to meet the growing demands especially for AAV manufacturing, which is currently the most promising viral vector for gene therapy. More precisely, we are developing a new transfection reagent dedicated to AAV production in suspension cells. By narrowing the window of use of this new reagent, we are convinced that we can maximize AAV viral yields, while maintaining reproducibility at different production scale.

BIO

Patrick Erbacher, PhD is the CSO of Polyplus-transfection® SA. With more than 20 years of experience in developing nucleic-acid based delivery reagents for research, biologics, Patrick has driven the development of innovative transfection reagents for several key applications, including mRNA-based gene expression, RNA interference, CRISPR-based genome editing and biologics production at R&D and industrial scale.

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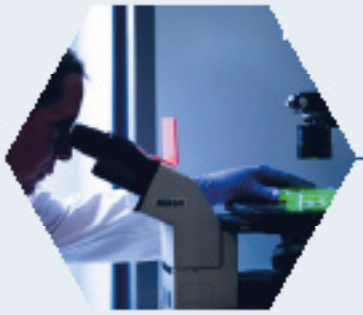

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SPOTLIGHT

INTERVIEW

What is 'quality' in viral vector manufacture? Strengths and limitations of current analytical tools

MARKUS HÖRER co-founded Freeline in March 2015 and serves as Chief Technology Officer and GmbH Managing Director since May 2015. Markus has 30 years' experience working in AAV biology, as well as over 23 years' experience in industrial vaccine and biologics development. Markus brings experience in research, process and analytical development, quality control, preclinical and business development. He joined Freeline from Rentschler, where he was responsible for setting up a Virus-based Biologics business unit, focusing on AAV vector development. Before that, from July 1996 to March 2010, he held various positions at MediGene, where he was responsible for developing AAV vector technology, its successful transfer into a GMP environment, and its use for clinical development of autologous Melanoma cell vaccines. Markus was also in charge of setting up a quality control and assay development division for an oncolytic HSV technology platform. Finally, Markus developed and was head of a novel AAVLP B-vaccine platform that was acquired by 2A Pharma, Sweden. Markus received a Ph.D. in the Department of Tumor Virology at the German Centre for Cancer Research in Heidelberg, Germany and has filed more than ten patents and claims 12 further inventions in the AAV field.

Cell & Gene Therapy Insights 2020; 6(3), 347–358

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Q What are you working on right now?

MH: I'm wearing two hats. One is my Managing Director/site head hat, where I have the responsibility to manage the site according to LLC law as a representation organ of the

GmbH and to further grow the Munich site in line with Freeline business needs. Then in my role as Chief Technology Officer of Freeline, I'm responsible for driving the company's platform technology development strategy and keeping us at the cutting edge of AAV biology.

Compared to the recombinant protein/antibody space, the AAV field is still comparatively new and the manufacturing technology is in a rather immature state, with the need to develop platforms enabling us to make high quality vectors at scale.

When talking about CMC, platform development starts with a continuous improvement of starting materials used for AAV manufacturing, such as optimization of the molecular design of our unique and proprietary two-plasmid platform, or engineering of the AAV manufacturing cells aiming at best in class vector yields, potency and quality. CMC platform development further comprises upstream and downstream drug substance process development, as well as drug product and formulation development. A very important piece of platform technology work comprises the implementation of a steadily increasing number of analytical assays, which allow us to further improve our manufacturing platform and vector design and to generate an in-depth understanding of our product. A panel of assays is applied for routine testing of process intermediates and characterization of drug substance/product in Munich as well as for CMC feasibility studies helping us to select the best development candidates for new programs. QC assays are transferred to our Quality Control department in UK and to CROs, where assays are validated with technical support provided by the Munich team. Likewise, manufacturing platforms are developed in-house and then transferred to CMOs, and into our own Freeline manufacturing facility.

Coming back to platform development, CMC is just one important piece. Others include the development of next generation capsids, with further improved transduction profiles for the human liver compared to our current S3 capsid, which we believe is the best-in-class AAV capsid for human liver gene transfer currently in clinical development. We also closely monitor and plan to implement new, enabling technologies that will allow Freeline to extend our portfolio into disease areas that are currently inaccessible for gene therapy.

Q Can you go into more depth on your and Freeline efforts to answer the challenging question of what actually constitutes or defines AAV vector 'quality'?

MH: Let's start with the question of what does vector quality mean for safety and efficacy in humans? There are in fact quality attributes whose impact on vector potency, immunogenicity and overall safety is currently unknown or at least not fully understood. As an example, when you're packaging AAV vectors with your gene of interest, the full-length vector is unfortunately not the only piece of genetic information that you pack into a capsid. The design of starting materials, vector cassette and upstream manufacturing platform are the major determinants for how much nucleic acid junk ends up in the capsid, including truncated vector genomes, plasmid- or virus-derived DNA, and host cell DNA. Packaging of truncated vector genomes means packaging of non-functional junk expression cassettes at first glance. However, co-transduction of cells with N- and C-terminally deleted

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genomes still overlapping in the middle part could still contribute to overall vector potency via reconstitution of a full-length genome within the cell.

> "...the AAV field is still comparatively new and the manufacturing technology is in a rather immature state..."

Packaging of plasmid-derived sequences could result in de-novo expression of peptides or genes in a target cell and increase the immunogenic payload of a vector. Host cell DNA packaging means you could pick up not only the known oncogenes that have been used for generation of a respective manufacturing cell (e.g., E1A in HEK293 cells, SV40T in HEK293T cells or HPV18 E6/E7 in HeLa cells, which is why you have to develop release assays to quantify known oncogenes – typically qPCR or ddPCR assays), but any kind of potential proto-oncogene, which could turn into an oncogene when taken out of the natural context. These include but are not limited to genes regulating the cell cycle. If such genes are picked up in full-length, dependent on the precise nature of the packaged HCD, these DNA impurities could transform a human target cell and bear the risk of tumorigenicity. However, as such processes are very slow, HCD-related safety issues might be observed many years if not decades after patient treatment.

Therefore, one obvious and big effort for Freeline is to reduce the payload of DNA mis-packaging, and the other big focus is on developing sophisticated analytical methods to allow us to characterize the nature of all the DNA packaged – the key thing here is not so much 'how much', but rather the potential of each piece of DNA to produce a functional protein. This is driven by the size and nature of the pieces of DNA that are packaged. Getting this information is very challenging but something we believe is very important. I'll come back to this point later.

Another of our primary development goals is to design and produce vectors with the highest possible potency. We believe we have at least three important assets in the company that ensure highest possible vector potency. One is our proprietary AAV capsid for highly efficient human liver gene transfer. Secondly, we optimize and continuously improve every single piece of our vector expression cassette for any program we are developing: from careful selection and evolution of potent liver-specific promoters, codon optimization of transgenes, comparison of a large panel of signal peptide sequences, cloning of intron sequences and other elements increasing transcription rates, mRNA export and stability, to the choice of polyA signals. Importantly, we also review the various attributes of the protein to be expressed such as protein stability, size, kinetics, affinity to partners and immunogenicity and when relevant we engineer a protein with improved properties. This means that we generate hundreds of candidates for a given program from which our lead development candidate is selected based on a battery of *in vitro* and *in vivo* preclinical experiments and CMC feasibility studies. And thirdly, we think that the selection of a mammalian cell culture platform combined with our unique plasmid system gives us the highest possible potency from a manufacturing perspective.

Having a potent vector isn't important solely to enable low doses and allow you to achieve the lower possible cost of goods for any given level of targeted protein expression. It also

enables you to achieve a higher level of expressed proteins for any given dose, which is important for opening up the widest possible range of therapeutic targets.

Q Tell us more about the limitations of current AAV vector analytical tools as you see them

MH: I'll start by saying that the gene therapy space can benefit from a wide range of analytical tools, which have been developed for classical biologics characterization and release testing. Of course, a viral vector consists of nucleic acids as well as proteins, so that's a major difference between classical biologics like recombinant proteins or antibodies and viral vectors. With respect to molecular characterization, we have a really limited number of tools that are industry standards. PCR methods are designed and optimized for measuring low levels of DNA at high sensitivity, both for determining the vector genome dose, and for quantifying DNA impurities. However, a major limitation of qPCR and also, more recently, of droplet digital PCR (ddPCR), is that you cover only a very small piece of a sequence of interest – maybe 100 or 200 base pairs. You quantify something, but whatever it is that you quantify, you will still have no data about the functionality of that particular sequence. So, if you talk about a vector dose, for example, is a qPCR or ddPCR result really a reliable indicator for full length vector packaging? Couldn't you also be picking up truncated vector genomes, provided those truncations are outside of your PCR amplicon? Could you quantify a sequence that has single nucleotide mutations, which could render a sequence non-functional, and yet still say it's your dose, it's your vector? And for impurities, one of the critical questions is what's the size profile of a given impurity? If it has a length which is below something that could comprise a functional gene, obviously the safety – or the potential harmfulness – of such an impurity is much lower than if you can cover a complete oncogene, for example. The key limitation of qPCR and ddPCR for impurity analysis is that, simply put, you don't look into functionality, which is much more important than just copy numbers.

The molecular characterization tools that are industry standard for releasing AAV vector batches have significant limitations. And it doesn't end there. If you quantify a vector dose by qPCR, you get a first impression of how many vectors you have packaged, but you are then faced with another important quality attribute – the full-empty ratio: how many capsids are functional, and how many are empty capsids? Is the PCR result a good indicator of 'full' particles? What do we actually mean when we talk about an empty or full particle and how relevant is this for product potency and safety? The term 'full-empty ratio' is a huge oversimplification for a quality attribute which is much more complex than simply having real empty and full particles carrying a functional genome. We currently do not even know whether AAV manufacturing results in completely empty capsids as a by-product, or

"The molecular characterization tools that are industry standard for releasing AAV vector batches have significant limitations."

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"If you quantify a vector dose by qPCR, you get a first impression of how many vectors you have packaged, but you are then faced with another important quality attribute – the full-empty ratio: how many capsids are functional, and how many are empty capsids? Is the PCR result a good indicator of 'full' particles?"

whether those capsids actually carry a payload that is simply too small to identify. A 'full' capsid is not only a capsid that carries a full-length, functional vector genome – it could be a capsid carrying any other piece of DNA of a given size. It could be DNA impurities, for example, or truncated vector genomes. It could even be a proteinaceous payload. So what does a reported full-empty ratio really mean? Not much. To make the situation even worse, there is a panel of assays that is accepted as 'full/empty method' and different companies use different assays to report full/empty ratios. We have compared different industry standard methods like qPCR/capsid ELISA, cryoEM and AUC to determine 'full-empty ratios' and could show that results can vary from as low as about 10% to as high as 80% full particles for the same vector batches, even though each individual method provides very consistent results. Therefore, you also see a lot of discrepancies when different groups present full-empty ratios and discuss their downstream process capabilities to specifically enrich full particles. If you want to, you can 'cheat' a lot by simply choosing the methods that you think give you the best and most appropriate ratio to report.

In vitro potency assays have to be developed to compare potency of every new vector batch to that of a reference standard. So-called TCID50 assays are run to determine the infectious titer of an AAV vector batch. Both assays rely on the infection of cells with a range of vector doses, followed by quantification of protein production and functional protein activity in the case of potency, and quantification of replicating vector genomes in the case of the TCID50 assay as a function of input virus. Therefore, these cell-based assays are directly linked to the vector dosing assay. Depending on the vector genome dosing assay chosen, you will report different vector potency and infectious titer for a given vector batch, meaning data must be interpreted with maximum care.

We have talked already about the weakness of qPCR and ddPCR regarding lack of sizing information. Another weakness is that these assays do not provide any information regarding the state of packaged DNA. By 'state', I mean is it a single strand or a double strand of DNA sitting in the capsid? The latter may be unlikely, as the known mode of action of DNA packaging is active pumping of single-stranded DNA molecules into preformed capsids through their pores in a Rep protein and energy dependent process. The capsid pores are

"The limitation of the current standard industry assay is the comparably low assay sensitivity with a detection limit of about 1–10 rcAAV in 1e8 vector genomes."

too small to accommodate double-stranded DNA. Nevertheless, capsid assembly around double-stranded DNA fragments might be one way to explain how dsDNA packaging could occur. Furthermore, if a packaged DNA comprises a single strand, could it ever be converted into a transcriptionally active double strand in the cells of a patient, or will it remain single stranded and therefore degrade very quickly? You can imagine that these different states of mis-packaged DNA impurities have a different impact on the safety profile of an AAV vector batch.

The last example regarding weakness of current industry standard assays that I would raise is rcAAV (replication competent AAV) testing. If you produce recombinant vectors such as AAV, you have to dissect the wild type genome into pieces, which allows you to put a foreign cassette into your AAV vector and to provide the viral genes on separate starting material entities such as plasmids (for transient transfection-based manufacturing platforms) or recombinant viruses (e.g., for the insect cell/Baculovirus platform). Through homologous and non-homologous recombination events taking place in the production cell, a pseudo wildtype or replication-competent virus can be reconstituted, which is then co-amplified as contaminant of your viral vector batch. The limitation of the current standard industry assay is the comparably low assay sensitivity with a detection limit of about 1–10 rcAAV in 1e8 vector genomes. We have developed in-house tools that provide significantly higher sensitivity and have developed methods to characterize replicative forms. When we compare AAV batches manufactured with the more conventional AAV helper plasmids with rep and cap genes sitting on the same plasmid to AAV batches manufactured with our unique, split packaging plasmids, we can detect pseudo wtAAV in batches produced with the classical plasmid design that remain undetected using the industry standard assay. However, we very rarely if ever find functional pseudo wtAAV in AAV batches produced with the Freeline platform.

So I would say in summary that the major limitation at the moment is the lack of industry standard assays that allow an in-depth molecular characterization of the vector, and of any kind of packaged DNA impurities. This also affects other assays that rely on a reported vector dose (e.g., potency or infectivity assays) and reporting of impurities per given dose. These are certainly the areas where we put in a lot of effort to try to improve and generate a better understanding.

There are other important limitations beyond the technical ones we've discussed, which are areas in which we also need to invest and obtain the help and support of device manufacturers. For example, for many assays, you require considerable sample volumes. Gene therapy is currently mainly developed in rare and ultra-rare diseases and for some application areas, such as ophthalmology, you require comparably small doses to treat patients. Therefore, compared to the protein/antibody manufacturing space, AAV batch sizes are often small. In other words, we have limited amounts of material to start with, and then a lot of it is going to

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QC testing and AAV characterization. To illustrate, the subvisible particle assay alone, which is one of the pharmacopeia methods to be used, typically requires 1 ml or more of your precious final product – a huge amount, relatively speaking. There are companies that are now offering alternative technologies: the Halolabs HORIZON® is a device that reduces the sample volume to 25 microliters, which is at least a 40-fold reduction in material consumed.

Assay variability, the requirement for a lot of manual sample manipulation, and throughput are further challenging areas. Automation and development of higher-throughput formats is required, which is especially important for process development and characterization where you have to analyze hundreds of in-process samples with the need for short data turnaround times.

One final point is that CMOs and CROs typically have a lot of expertise with the more standard analytical methods that have been used in biologics testing for decades. However, the application of these techniques to gene therapy, and to AAV specifically, requires a deep understanding of the biology of those vectors in order to apply them appropriately. I think the examples given above make this point. It is a key focus for us – 20% of our company resources go into analytics; you need the deep biology expertise in order to be able to understand the wealth of data/information which these methods can generate!

Q Are there any tools just emerging that shows any promise in helping bridge this innovation gap, for you?

MH: The Illumina next-generation sequencing platform is an important step forward for gaining a better understanding of what sequences you can find in a capsid. That is because you can quantify any piece of DNA in a sequence-independent manner. With the more classical qPCR and ddPCR methods, you have to design amplicons for each sequence of interest – this not only comes along with a lot of development and validation work, but also means significant cost for QC testing, and you can also only quantify a handful of known sequences of interest. Therefore, Illumina NGS gives you a much more comprehensive picture of the overall nucleic acid composition of a given vector batch. However, Illumina NGS relies on sequencing of rather short DNA fragments generated during library preparation, and so, as is the case with PCR, it does not provide important functionality data such as vector integrity and impurity fragment size distribution profiles, nor does it offer a comprehensive view of undesired genome re-arrangements.

Long-read, single molecule sequencing platforms (LR-NGS) do in theory have the potential to look at those attributes, as complete DNA molecules can be sequenced without any need for DNA fragmentation during NGS library preparation. The issue there is that single stranded AAV is quite special in terms of structure, especially the genome ends having a very high secondary structure that makes it very difficult for this kind of DNA to generate a library reflecting the size of packaged single-stranded DNA that we can then subject to long-read sequencing. I can't tell you how we did it, for obvious reasons, but I think we are the first group worldwide to have resolved this problem, in collaboration with a CRO. Even though we still have some development work in front of us, especially with respect to

bioinformatics automation, we now have access to a platform that gives us a much deeper insight into the molecular composition of our AAV vector batches. This not only allows us to provide more functionality data, but we can also make use of the data to further improve our plasmid and vector design as well as our upstream process. Therefore, we think that with LR-NGS we have a really big and very important asset in the company.

Q Continuing to look to the future, how can we as a sector become more sophisticated in addressing these issues moving forward?

MH: Firstly, I think it's vital to assemble expertise in all the key areas – from cell biologists and virologists, to engineers trained in process and analytical development appreciating the possible impact of even minor process parameter changes on product quality, to physicians who understand the medical needs and potential impact of impurities on patient safety. If you don't bring together experts from all these areas, you will always miss the whole picture, and you will consequently develop a product that is suboptimal. We have a vast responsibility to our patients and so we should always aim to develop the safest and most efficacious products possible.

A continuous dialogue with the regulatory agencies is absolutely mandatory to make sure that new guidance documents are always well aligned with the steadily increasing scientific and technical insights accumulating from experts working in the gene therapy space. Host cell DNA packaging and potential concerns linked to that is a great example. For classical biologics, there is guidance on residual HCD quantity (10 ng/dose) and size (≤200 bp) limits, but this guidance does not apply to vectors where host cell DNA is packaged into a capsid that has actually been selected based on its capability for highly efficient transfer into a patient's cell. Therefore, the right choice of a manufacturing cell is important, but the current discussions mainly focus on specific oncogenes used to derive such cell lines rather than taking a more holistic approach, balancing the risk of packaging a single, known oncogene against the risk of packaging of a lot of potential proto-oncogenes that are inherent to every manufacturing cell. Engagement with regulators is key as the technology/field is so formative and new standards appropriate to this area need to be developed. The whole gene therapy community shares the responsibility of supporting the regulatory agencies in publishing new and relevant guidance, which in turn helps companies to develop safe products.

A related issue is that we need an incentive to generate more reference material, which is then used in all analytical laboratories – whether CMOs, CROs or in-house labs – to harmonize and standardize methods. At the moment, you really cannot compare anything lab-to-lab, which is maybe one reason why companies are very hesitant to publish and disclose any absolute quality data. We can all report vector genome yields of a given manufacturing platform, but reported data is highly dependent on the method (or even a specific piece of a method) used. You can find yourself reporting two-fold, three-fold, even five-fold different results depending on which piece of a sequence of interest you are covering with your PCR amplicon. Furthermore, even Illumina NGS data or alkaline gel analysis separating packaged DNA in an electric field can delude analysts into thinking that a vector batch represents a homogeneous product. However,

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we know from our own data that this is certainly not the case.

So you can imagine that if we consider intrinsic assay variability on top of the fact that every analyst is setting up assays in different ways, or choosing different methods, we will never be able to really compare the yield and quality data of one lab to that of another. If you could have standardized reference materials and standardized methods brought into every company and academic group, it would really help to generate a better understanding of AAV potency, safety and immunogenicity to the benefit of all.

> "...we need an incentive to generate more reference material, which is then used in all analytical laboratories ... to harmonize and standardize methods."

There are other challenges in viral vector gene therapy that are still tough to overcome. For example, it is obvious that most of the viruses used as gene transfer vehicle interfere with the immune system in patients. AAV has been selected as one of the most promising vector systems for *in vivo* gene therapy because this virus family has comparatively low immunogenicity, but on the other hand, a significant percentage of the human population is infected with any of the different AAV serotypes from early in life and, therefore, develops an adaptive immune B- and/or T-cell response. Understanding the entire complexity of the immune system and of vector batch components that could contribute to the immunogenic profile, and developing assays that allow the careful monitoring or even prediction of immune responses, are challenges that can only conceivably be addressed through combined efforts: we need to build consortia to work on such complex topics as a team rather than as competitors, in my view.

Companies must understand that there are obviously areas where you need to protect your IP and your knowhow, but there are other areas where you can only succeed if you combine efforts and bring all the key stakeholders together. Vector immunogenicity is one such example. Until we have a much deeper understanding, clinical management of vector immunogenicity will be key to the success of gene therapeutics, as Professor Amit Nathwani, CSO of Freeline, has impressively demonstrated through his pioneering AAV8-FIX gene therapy trial, initiated about a decade ago.

Q What are Freeline's chief goals and priorities over the short-to-mid-term, and how do you foresee your own work evolving further in step with this planned progress?

MH: Freeline Therapeutics' overriding goal is to get what we believe to be the best-in-class hemophilia B gene therapy program onto the market and available to patients.

I've talked about having the most potent AAV vector for treatment of Hemophilia B patients in the clinic, which allows us to be focused on our product's potential as a curative

agent, not just disease modifying. We aim at normal FIX clotting activity levels in the 70–150% range at comparably low vector doses and our clinical data published recently clearly underscore our ambition. This program will certainly also validate our AAV capsid, molecular vector design and CMC platform as a powerful engine for rapid and lean development of our pipeline programs, with one program for Fabry's disease already in clinical development, two further programs entering the clinic within the next 18–24 months, and a whole panel of further programs currently in the research/preclinical development phases. Freeline will remain focused on liver gene transfer to address monogenetic diseases but also multifactorial diseases, thereby exploiting the liver as an *in vivo* bioreactor for the production of secreted proteins. Focusing on liver gene transfer will allow us to focus our resources on the development of high quality programs and to continuously improve our vector platform without the need to replicate efforts from capsid tox to biodistribution/shedding studies, or to completely reinvent manufacturing processes and analytical tools. Treating patients suffering from different diseases using the same vector and manufacturing platform will also allow us to generate a larger clinical data base, which we can exploit to better understand the impact of vector design and quality on patient safety and potency.

Our near- and mid-term goals revolve around further development of the analytical platform technologies addressing currently less well understood quality attributes of AAV batches. This will further increase our understanding of underlying biology, which will in turn guide us in the continuous optimization of our manufacturing platform in a reiterative process – and also of our capsid development initiative. Additionally, we've started to identify and aim at further identification of vector-/program-specific components of our manufacturing platform to help us reduce effort for CMC feasibility studies, and to further accelerate lean development of new pipeline programs into and through clinical development to BLA/MAA.

Our long-term goal is to continue investing in next generation enabling technologies that will enable us to develop safe, one-shot medicines for patients suffering from diseases in indications that are currently inaccessible for gene therapy. This includes intelligent vector design and investment in disruptive approaches for manufacturing platform and analytical development to move into much broader diseases with high patient numbers and more narrow therapeutic windows. Going back to my earlier comments, our aim here is not just to increase our capacity, plugging in hundreds of fermenters into our GMP manufacturing facility and moving to huge volume bioreactors, but improving the cell-specific productivity and working on process intensification, thereby minimizing product and process-related impurities and improving overall vector potency.

Q Finally, can you share your vision for what AAV vector quality control might ultimately look like at commercial scale?

"A significant percentage of assays involve methods to quantify the vector and selected DNA impurities without delivering important functionality data."

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MH: There's clearly an issue today in that you need 30–40 assays in order to release a batch of AAV vector, whether for clinical development or commercial use. A significant percentage of assays involve methods to quantify the vector and selected DNA impurities without delivering important functionality data. Also, vector release testing is very time consuming – we talk about typically 3 months or more before you have all the QC data back on a given batch and can then look to release it for use in humans. This drives not only costs, but is also not acceptable if you consider all the speed advantages of gene therapy development: relatively small clinical trials, often clearly defined clinical end points, expedited development pathways, etc. Short clinical development timelines put tremendous pressure on CMC to keep pace. If you then have to wait 3 or 4 months (or more) for batch release, it obviously can negatively impact product commercialization timelines.

My vision, then, is that we can significantly reduce the number of assays – e.g. by replacing all the individual PCR assays for molecular characterization of vector and DNA impurities with a single, more universally applicable platform such as LR-NGS that can also deliver important functionality data. This is what I hope to see happening in the next 5 to 10 years, not only to make QC testing simpler, but to provide us with a much better understanding of our products. More universal platform methods like LR-NGS will also help to unify and harmonize methods within the gene therapy industry and to generate more consistent data for a given batch. Automation of standard industry assays will not only positively impact costs but will reduce time and assay variability by reducing error-prone manual steps.

One other aspect we need to focus more on, which is already standard in the antibody manufacturing field, is the application of Quality by Design (QbD) principles. If you do your homework regarding QbD of your manufacturing platform, you will be able to reliably generate vector batches of consistently high quality for a product's entire lifecycle. The earlier and the more data you can provide on batch-to-batch consistency, the higher the likelihood that you can perhaps remove certain assays from the release testing panel for a commercial product.

Lastly, I'll just mention stability testing. Currently, it consumes a lot of material. We really need to make it a little more viable, to help ensure our final product goes to patients and not to the QC process.

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

VIRAL VECTOR BIOPROCESSING & ANALYTICS:
TODAY'S KEY TOOLS AND INNOVATION
REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

INNOVATOR INSIGHT

Development and validation of quantitative real-time PCR for the detection of residual HEK-293 host cell DNA

Kara Norman

The presence of residual DNA in therapy products may lead to an increased risk of oncogenicity, immunogenicity, and other toxicity. Current regulatory authorities (including the US FDA, EMA and WHO) limited the accepted amounts of residual DNA in biological products making it extremely important to have a sensitive method of quantifying residual host cell DNA. Among the methods of detecting residual DNA, quantitative polymerase chain reaction (qPCR) is the most widely used for residual DNA quantitation due to its sensitivity, accuracy, precision, and time-saving capability. This article examines the development and validation of a new, highly sensitive and accurate integrated solution for detection and quantitation of low level HEK-293 DNA to help meet regulatory requirements.

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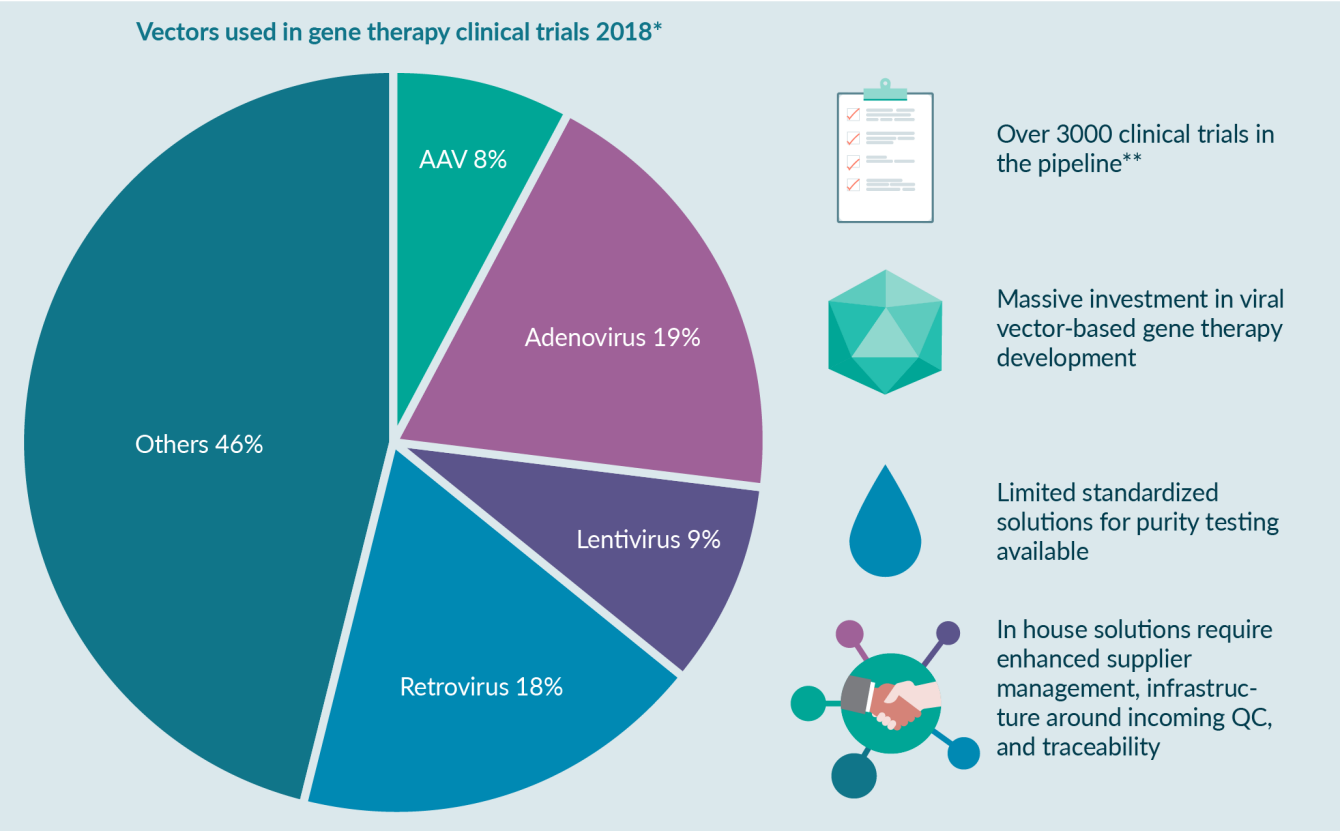
INTRODUCTION

With over 3,000 clinical trials currently in the pipeline, the gene therapy field is moving rapidly. A variety of viral vectors are used in these therapies, including adenovirus, lentivirus and adeno-associated virus (AAV) (Figure 1). Despite massive investment in viral vector-based gene therapy development,

FIGURE 1

The field of gene therapy is advancing rapidly.



*Sourced from *The Journal of Gene Medicine*, 2018 John Wiley and Sons Ltd.
**Global Cell and Gene Therapy Market: Focus on Products, Applications, Regions and Competitive Landscape - Analysis and Forecast, 2019-202.

the speed at which the field is developing means that there are limited standardized solutions currently available for purity testing and analytics. This unmet need has led some groups to turn to the development of in-house solutions. However, these come at a considerable cost.

Residual nucleic acid is a common process impurity tested in viral vector production, and is regulated by bodies such as the FDA and EMA: they require that the DNA content in the final product is less than 10 nanograms per therapeutic dose. This can be a challenge when using AAV in particular, as these vectors can package a large amount of plasmid or cellular DNA inside the viral capsid. This article provides an overview of analytical testing in bioproduction processes and describes the development of a novel residual DNA assay.

DEVELOPMENT OF A NOVEL RESIDUAL DNA ASSAY FOR HEK-293 GENOMIC DNA

The downstream vector purification step is the point at which an analytical technique must be employed to ensure there is a minimum amount of residual DNA present in the final product.

The Applied Biosystems resDNASEQ Quantitative HEK293 DNA Kit is a qPCR-based system that is optimized for detection of host cell DNA from HEK-293 cell lines. Thermo Fisher Scientific has a long history of enabling labs in testing residual DNA, including assays for Chinese hamster ovary (CHO), *E. coli*, human, Vero, MDCK, Pichia pastoris, and NS0 DNA. Since HEK-293 cells are used in the development of viral vectors for both gene therapy

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and other biotherapeutics, the need for a specific kit has become increasingly urgent. This is not only an assay but encompasses an end-to-end workflow solution, from sample extraction to data analysis, with additional support provided for implementation and validation for the whole workflow.

Manual versus automated DNA extraction

Table 1 includes some important considerations when choosing between manual and automated sample preparation methods.

Two sample preparation methods were tested with the HEK-293 residual DNA assay. The manual sample preparation method used was the manual protocol from the PrepSEQ Nucleic Acid Extraction Kit. This is a low-throughput method allowing for 16 extractions per day. The automated method tested also uses the PrepSEQ Nucleic Acid Extraction Kit, and leverages the Kingfisher Flex System by Thermo Scientific; this is a high throughput system that extracts 192 samples per day, requiring considerably less hands-on time (less than 1 minute per extraction).

The PrepSEQ Nucleic Acid Extraction Kit is a universal solution for nucleic acid extraction. The kit works on a variety of host cell DNA types and has also been tested on mycoplasma, as well as more than 60 different viruses, including double stranded and single stranded DNA viruses, double stranded and single stranded RNA viruses, with or without envelopes. PrepSEQ has also been tested under a variety of conditions such as low pH, high salt, and high protein.

The method (Figure 2) starts with the addition of a lysis buffer to the sample, and then magnetic particles are added together with a solution that allows for optimal efficient binding of nucleic acid to the particles. The magnetic particles are collected, followed by a series of wash steps. Finally, nucleic acid is eluted off the magnetic particles. The result is a PCR-compatible nucleic acid extract significantly reduced in inhibitors.

TaqMan Real-Time PCR Assay

Real-time PCR was improved by the introduction of TaqMan DNA polymerase that leverages the 5' nuclease activity of the enzyme, along with fluorogenic labelled probes. Applied Biosystems™ TaqMan® Assays are the industry-leading choice for 5' nuclease qPCR assays. The kit also comes with a DNA control for calibrating assay results. This control consists of precisely quantitated, highly purified genomic DNA from an established HEK-293 cell line.

The assay was validated on two systems from Thermo Fisher Scientific. The

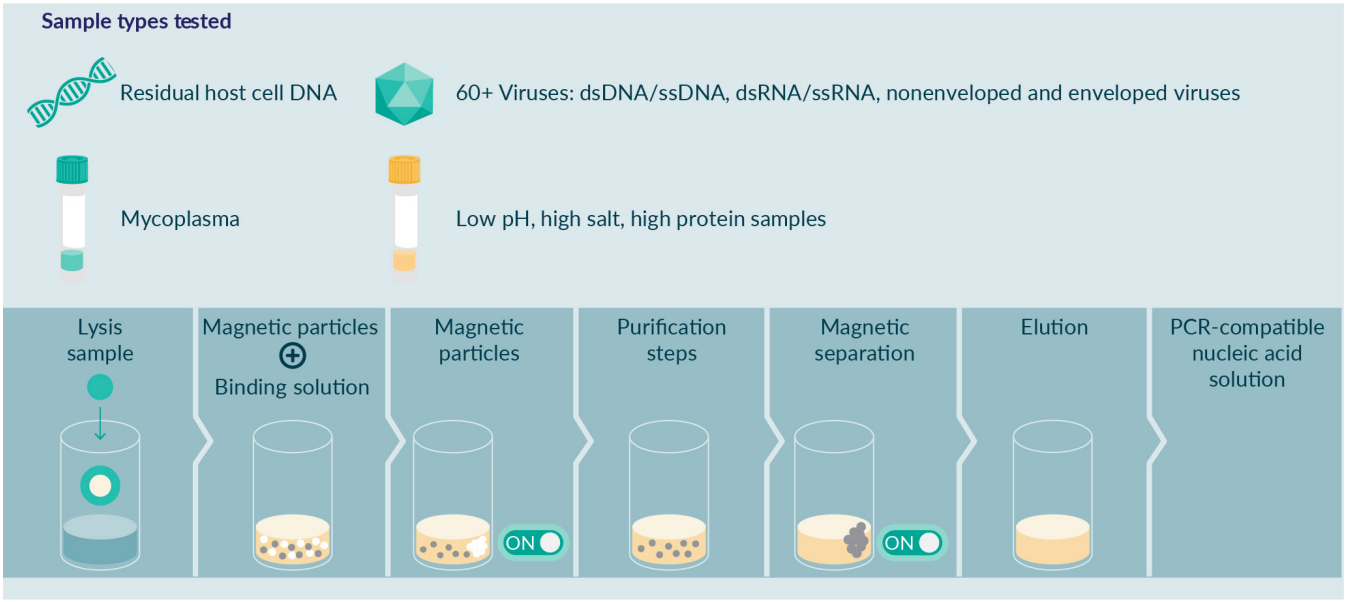
TABLE 1

Key pros and cons of manual versus automated sample preparation systems.

	Pros	Cons
Manual sample preparation system	▶ Requires minimal up-front equipment investment	▶ Additional risk and variation due to human interface - more susceptible to errors and contamination ▶ Higher labor cost ▶ Lower throughput
Automated sample preparation system	▶ Reduced hands-on time leads to minimal variation and susceptibility to errors over long-term ▶ Lower labor cost ▶ Much higher throughput	▶ Requires a large upfront equipment investment ▶ More complex, time consuming and costly implementation

FIGURE 2

Method background: PrepSEQ sample preparation kits.



workhorse system for the residual DNA portfolio is the Applied Biosystems 7500 Fast Real-Time PCR System (7500 Fast). All seven resDNA assays run on this system and are used worldwide. More recently, the QuantStudio 5 was also introduced as a validated system for the residual DNA portfolio. This addition provides precise quantification with 1.5-fold discrimination. For assays in general, the Quant Studio demonstrates excellent reproducibility, and up to a 10-log dynamic range. This improved accuracy and sensitivity enables this platform to be used across a broad range of applications in addition to residual DNA testing, including analysis of gene expression, and micro RNAs.

AccuSeq Analysis Software

The last part of the system, which wraps the entire qPCR workflow, is the AccuSEQ real-time PCR detection software. AccuSEQ software integrates with the QuantStudio 5 and the 7500 Fast instruments, and has been developed with security, audit and e-signature capabilities to enable 21 CFR Part 11 compliance. Figure 3 shows an example of the traceability this software can provide: every change in experimental properties is tracked and recorded in an audit trail. For example, when data are analyzed or a sample is run, the software records what was executed, when, and by whom.

ASSAY RESULTS & STUDY DESIGN

The objective of this study was to determine the performance of the resDNASEQ Quantitative HEK-293 kit. Several parameters, including linearity and PCR efficiency, were tested to ensure accurate quantification of residual DNA. Precision was also tested to ensure the data produced were consistent and reliable. Limit of Detection (LOD), Limit of Quantitation (LOQ) and assay range were also tested to ensure optimal sensitivity and to help support regulatory compliance in measurement of residual DNA. The study design included 3 operators, 2 manufactured lots of the kit, 2 sample prep methods (manual and automated) and 2 instruments: the 7500 Fast and the Quant Studio 5. All measurements were run in triplicate.

Starting with sample extraction, manual and automated sample prep methods were used to extract from a variety of matrices

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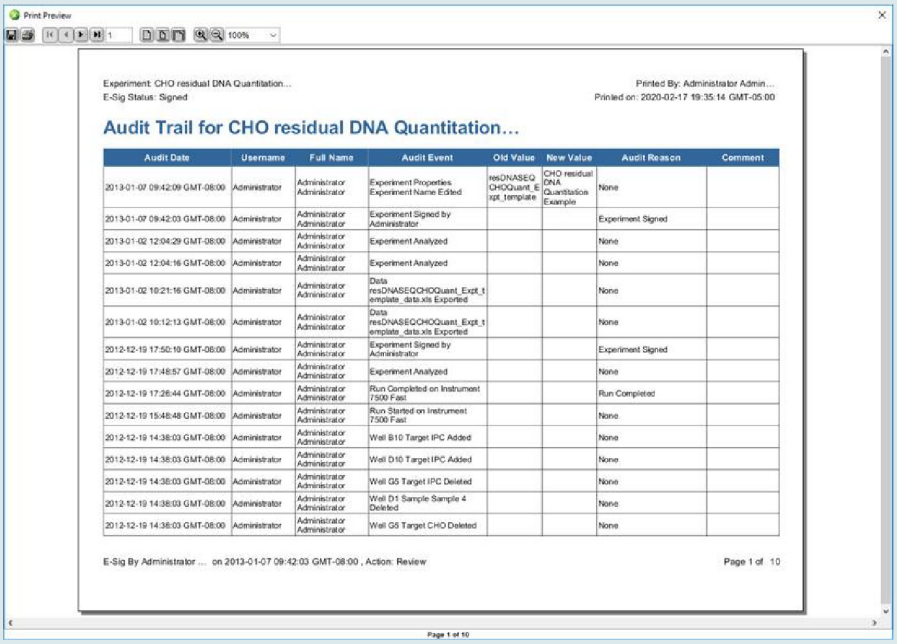
FIGURE 3

Analysis: help enable 21 CFR Pt 11 compliance.



AccuSEQ™ real-time PCR detection software

Integrates with the Applied Biosystems QuantStudio 5 and 7500 Fast Real-time PCR instruments

Developed with security, audit, and e-signature capabilities to help enable 21 CFR Pt 11 compliance

Features ensure full traceability

common to gene therapy and bioproduction workflows, outlined in Table 2. Once obtained, the extracts were spiked with an internal positive control allowing determination of whether inhibition is taking place in the sample: this serves as a measure of reliability of the assay. Internal positive controls were detected under all conditions, indicating that the PrepSeq reagent effectively removed inhibitors from these matrices.

Comparing instruments

Firstly, standard curve performance was tested on the 7500 Fast. Two lots of HEK-293 residual DNA assay were tested for linearity and PCR efficiency. R-squared of the standard curve was 0.999 for both lots, and PCR efficiency was 102% for Lot1 and 101% for Lot 2. This high linearity and efficiency enable the assay to measure

TABLE 2

Results: sample extraction.

Gene therapy matrices tested		Assay performs in extracts from samples containing	
Sample derived from a bioreactor at harvest	✓	Benzonase	✓
Sample after chromatography	✓	Excess DNA of other species	✓
Sample after final purification	✓	Detergent	✓
		Cell culture media	✓

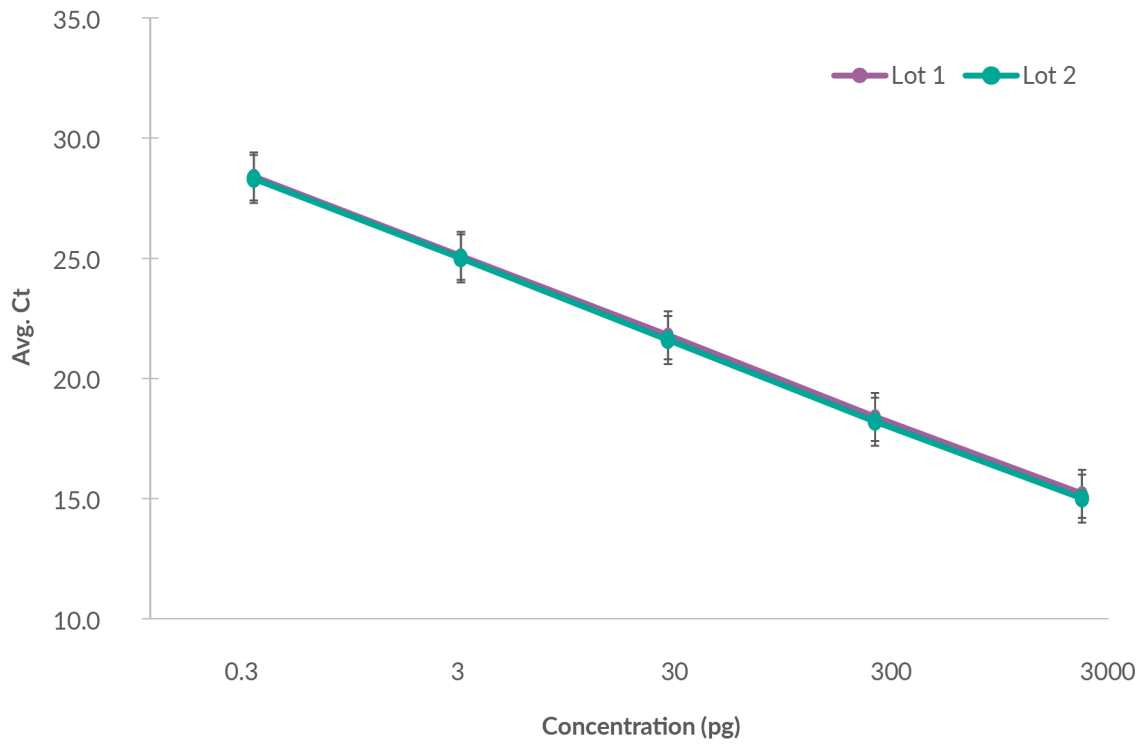
Sample matrix:

- Manual and automated sample prep methods were used to extract from matrices outlined below
- Extracts were spiked with the internal positive control (IPC)
- IPC was successfully detected across all sample types

Results show that sample prep successfully prepared samples from a variety of matrices common to gene therapy bioproduction workflows.

FIGURE 4

Standard curve performance (Applied Biosystems 7500 Fast Real-Time PCR Instrument).



Results demonstrate high linearity and efficiency to enable quantitative results across a broad range of DNA concentrations. PCR Efficiency = 102% (Lot1) and 101% (Lot 2) R^2 = 0.999 (Lot 1) and 0.999 (Lot 2), from 0.3 to 3000 pg.

TABLE 3

Precision measurement (Applied Biosystems 7500 Fast Real-Time PCR Instrument).

	Intra-run					
	HEK293 (Lot 1)			HEK293 (Lot 2)		
DNA spike amount	Avg Ct	Standard deviation	%CV	Avg Ct	Standard deviation	%CV
3,000 pg	15.2	0.08	0.51	15.0	0.04	0.30
300 pg	18.4	0.07	0.38	18.2	0.05	0.29
30 pg	21.8	0.08	0.38	21.6	0.04	0.18
3 pg	25.1	0.08	0.33	25.0	0.03	0.13
0.3 pg	28.4	0.09	0.31	28.3	0.08	0.29
	Inter-run					
	HEK293 (Lot 1)			HEK293 (Lot 2)		
DNA spike amount	Avg Ct	Standard deviation	%CV	Avg Ct	Standard deviation	%CV
3,000 pg	15.2	0.04	0.26	15.0	0.01	0.04
300 pg	18.4	0.02	0.12	18.2	0.03	0.14
30 pg	21.8	0.05	0.22	21.6	0.03	0.16
3 pg	25.1	0.09	0.36	24.9	0.03	0.11
0.3 pg	28.4	0.14	0.48	28.3	0.08	0.28

The standard curve was tested across 6 runs on the 7500 Fast.
Intra-run precision across all concentrations was less than 1% CV.
Inter-run precision across all concentrations was less than 1% CV.
High precision observed at as low as 0.3 pg per reaction.

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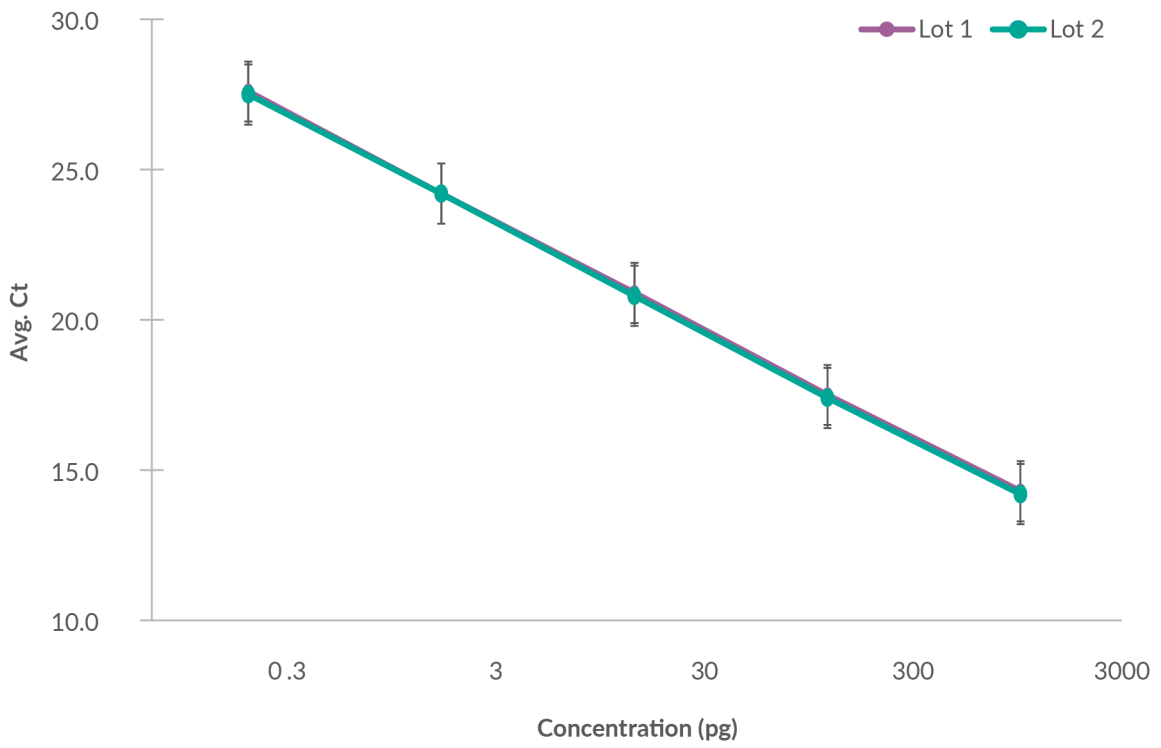
FIGURE 5

Standard curve performance (QuantStudio 5 Real-Time PCR Instrument).



PCR Efficiency = 103% (Lot1) and 101% (Lot 2) R^2 = 0.999 (Lot 1) and 1.000 (Lot 2), from 0.3 to 3000 pg. Results demonstrate high linearity and efficiency to enable quantitative results across a broad range of DNA concentrations.

TABLE 4

Precision (QuantStudio 5 Real-Time PCR Instrument).

	Intra-run					
	HEK293 (Lot 1)			HEK293 (Lot 2)		
DNA spike amount	Avg Ct	Standard deviation	%CV	Avg Ct	Standard deviation	%CV
3,000 pg	14.3	0.06	0.39	14.2	0.02	0.13
300 pg	17.5	0.04	0.22	17.4	0.02	0.10
30 pg	20.9	0.05	0.23	20.8	0.02	0.10
3 pg	24.2	0.06	0.24	24.2	0.03	0.12
0.3 pg	27.6	0.06	0.20	27.5	0.02	0.08
	Inter-run					
	HEK293 (Lot 1)			HEK293 (Lot 2)		
DNA spike amount	Avg Ct	Standard deviation	%CV	Avg Ct	Standard deviation	%CV
3,000 pg	14.3	0.01	0.06	14.2	0.04	0.27
300 pg	17.5	0.08	0.43	17.4	0.07	0.39
30 pg	20.9	0.05	0.23	20.8	0.05	0.24
3 pg	24.2	0.06	0.25	24.2	0.04	0.16
0.3 pg	27.6	0.05	0.19	27.5	0.08	0.30

The standard curve was tested across 4 runs on the QuantStudio 5. Intra-run precision across all concentrations was less than 1% CV. Inter-run precision across all concentrations was less than 1% CV. High precision observed as low as 0.3 pg per reaction. Results demonstrate high precision, indicating that data are consistent and reliable within runs and between runs, even when quantitating very low levels of DNA.

DNA quantitatively across a broad range of concentrations.

Figure 4 demonstrates the type of standard curve obtained from both lots. From lot-to-lot, the data are extremely consistent between 0.3 and 3,000 picograms (pg). Standard curve performance was then compared across six runs on the 7500 Fast. Intra-run precision across all concentrations was less than 1% coefficient of variation (CV), and run-to-run precision was also very tight at less than 1% CV. These results demonstrate extremely high precision, indicating that data will be consistent and reliable both within runs and between runs. (Table 3).

Finally, detection at very low levels of DNA was tested. For this example, a no template control (NTC) was run in parallel with DNA samples at 30 pg. The NTC values were consistently higher than control template values, demonstrating the capability of detecting 30 pg in an extract.

For the QuantStudio 5, standard curve performance was again investigated on two lots of the HEK293 residual DNA kit. Linearity was once again very high with an R-squared value of 0.999 for Lot 1, and 1.0 for Lot 2 (Figure 5). PCR efficiency was 103% for Lot 1 and 101% for Lot 2. Together, these data show that quantitative results may be achieved across a broad range of DNA concentrations on the QuantStudio 5. Results also demonstrated extremely consistent standard curves from Lot to Lot. Intra-run precision was less than 1% CV across four runs of the standard curve. Intra-run precision across four runs was also less than 1% CV in both assay lots. Finally, a 30 pg sample run in parallel with an NTC demonstrated a distinguishable difference, indicating reliable detection at this low DNA concentration. (Table 4).

CONCLUSION

These data show that HEK293 resDNASEQ is a comprehensive system that can provide consistent and reliable data even when quantitating very low levels of DNA. It also provides a rapid workflow, with a time to results of less than 5 hours, including optimized sample preparation. The development of solutions such as resDNASEQ is needed in order to ensure that gene therapy manufacturers are able to meet the strict limitations on residual DNA required by regulators.

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VIEW RESOURCES

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REQUIREMENTS TO MEET FUTURE DEMAND

SPOTLIGHT

INTERVIEW

Vector production in academia: obstacles and opportunities

DANIELA BISCHOF is an Assistant Professor of Clinical Medical and Molecular Genetics, and Director of Vector Production at the Indiana University School of Medicine. She has almost 20 years of experience in the field of gene therapy and has been involved in the production of clinical grade gene therapy lentiviral and retroviral products since 2007.

EMILY HOPEWELL is the Director of Cell and Gene Therapy Manufacturing and an Assistant Professor in Clinical Medical and Molecular Genetics at Indiana University, and is charged with implementing and building manufacturing for cell, gene, and immune therapy at Indiana University.

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Q What are your respective roles and activities at Indiana University (IU)?

EH: I joined IU in July 2018. I'm the Director of Cell and Gene Therapy Manufacturing, which comprises vector production, as well as our cell immunotherapy and transduction

"Academic institutions that are already manufacturing for Phase 1 and 2 clinical trials have the experience and are able to help reduce a massive bottleneck for manufacturing..."

facility, and the bioprocess development lab. The cell immunotherapy facility and the bioprocess development lab were initiated in 2017–2018.

I spend a lot of time talking with investigators who are interested in initiating trials. But my main goal is to develop a robust Good Manufacturing Practice (GMP) team, centered around a common quality management system and facility operations team.

DB: I am the Director of Vector Production that is part of Cell and Gene Therapy Manufacturing. We have a cleanroom facility that contains four separate suites in which we manufacture GMP lentiviral (LVV) and retroviral (RVV) vectors for use in Phase 1/2 gene therapy clinical trials. To that end, we also generate certified cell banks that are used in the production of viral vectors.

My responsibilities include managing all the tasks associated with vector production, beginning with liaising with customers to determine their requirements so that we can manufacture customized products to meet their needs. I ensure that the facility, equipment, processes, raw materials, documentation, and so on are current and ready for use. I also manage financial facets of projects together with our accountant, and legal aspects jointly with the Indiana University legal office.

Q Can you tell us more about the cell and gene therapy manufacturing capabilities and capacities at IU?

DB: The Vector Production Facility was established by Dr Kenneth Cornetta in 1995. It started as a means of enabling scientists and physicians funded by the NIH in initiating Phase 1/2 gene therapy clinical trials. Less than 10 years later we started producing GMP lentiviral vectors. I joined the group towards the end of 2007, and at that time we were in an old facility that had been built into the existing building, which was not ideal as it presented space and design challenges. With the assistance of an NIH Construction Grant (NCRR C06-RR020128-01), a new GMP cleanroom was incorporated into the design of a new research building being developed for the Indiana University School of Medicine. The facility was released to us in 2009.

Initially, there were two staff members in the new facility. We produced 2-4 GMP LVVs and approximately 4 GMP RVVs per year – RVVs were the main product we manufactured at that time. We also generated about 1 GMP-comparable LVV a year. (By GMP-comparable, I mean that these products are generated in the same manner as GMP LVVs, but they're

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> "A disadvantage of being a small group is that personnel have to perform multiple tasks, including procuring and qualifying raw materials, preparing them, maintaining the facility and equipment, qualifying processes, and manufacturing products … It takes close to a year to train staff to be competent in all these tasks."

not produced in a cleanroom setting; they are usually on a smaller scale, and the certification is abbreviated). We were also generating 1–3 cell banks per year.

During this period our maximum scale was 20-liter productions but as we recruited more staff, we transitioned to 30-, 40- and 60-liter productions. We are now averaging about 6–10 GMP LVVs, 3–5 GMP RVVs, and 2–3 GMP-comparable LVVs a year – plus 2–5 cell banks. (I do need to stress these are all approximate numbers, as it varies from year to year).

There is currently a very high demand for vector products, and we are in the process of hiring and training more personnel. Depending on demand, we aim to eventually manufacture at maximum capacity, which is around 16 GMP LVVs, 10 GMP RVVs, 2–4 GMP-comparable LVVs, and 10–14 cell banks.

EH: On the cell side, we have a suite with two cleanrooms. We aren't actually manufacturing cells yet – we are still getting the facility up and running – but we have three projects at a similar preclinical level, and we plan to begin manufacturing in the second half of 2020.

Here at IU, cell manufacturing for INDs has not been done for some time. However, in 2016, Dr Anantha Shekhar, Executive Associate Dean for Research Affairs, received the first Grand Challenges Award from IU for a precision health initiative. The goal is to improve outcomes through precision medicine, and it's been operationalized by weaving areas of technical expertise with targeted disease states. One of the pillars of precision health is cell, gene and immune therapy, so there was a windfall of resources. This is part of the reason why they have decided to invest in vector and cell manufacturing. Around the same time, IU alum, Dr Don Brown, made a sizeable donation to IU to create the Brown Center for Immunotherapy. I'm very lucky to have come in at a time when we've got really good investment in resources and leadership.

Q What are your thoughts on academic facilities expanding and moving into commercial manufacture?

EH: I came from the Moffitt Cancer Center in Tampa, Florida, and last year Moffitt launched a CRO focused on immunotherapy. The trend is definitely there, although I am not sure if it will continue.

The goal of many academic facilities is to support their investigators, but there is no reason they shouldn't capitalize on their areas of expertise, too. This benefits the academic institution as well as the plethora of start-up companies that can't afford to build their own clean room facilities or are too early in development to want to invest. Academic institutions that are already manufacturing for Phase 1 and 2 clinical trials have the experience and are able to help reduce a massive bottleneck for manufacturing, especially in the early phases.

Q What are the key challenges faced in viral vector manufacturing in the academic setting today?

EH: Staffing is one of our major hurdles. It is very difficult for us to find and retain staff with such high demand in the industry for people who have manufacturing experience. We spent 6 to 8 months after I arrived working with HR to adjust compensation appropriately so that we would have a better ability to hire and keep good quality staff. We're also working on optimizing our training to get them up to speed more efficiently.

DB: As Emily mentioned, hiring suitable technical personnel for manufacturing is definitely a challenge. Academia simply cannot compete with commercial entities when it comes to salaries. A disadvantage of being a small group is that personnel have to perform multiple tasks, including procuring and qualifying raw materials, preparing them, maintaining the facility and equipment, qualifying processes, and manufacturing products. This entails a lot of work and numerous procedures that staff need to be trained on. It takes close to a year to train staff to be competent in all these tasks. Also, in a university setting, obtaining enough space to accommodate growing staff and supply needs tends to be a challenge.

Q What specific supply chain related challenges have you encountered, and what steps are you taking to address them?

DB: All of our processes were qualified using specific materials – again, as a small group we did not have the personnel to qualify processes using multiple sources of material. Additionally, at that time, specialized supplies were often available only from one vendor. As a result, we now face backorder issues that can affect our ability to complete projects in a timely fashion. Some of the material qualifications are also

"It is very difficult for us to find and retain staff with such high demand in the industry for people who have manufacturing experience."

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rather time-consuming. For example, qualification of our current tangential flow filtration (TFF) system took almost 2 years to optimize and get ready to incorporate into our GMP manufacturing processes.

> "Developing a collaboration with industry would definitely help us to improve and grow our processes."

As much as possible we have tried to find equivalent materials from alternate vendors for critical supplies. Additionally, we order supplies well in advance, but inadequate storage space means we cannot order materials for a year, or even 6 months, for example. There are also some materials that have a relatively short shelf life.

With gene therapy on the rise and more groups starting to manufacture vector products, we have experienced extended backorder issues with some supplies. We also order custom products from certain vendors, and we have had cases where agreed upon lead times by the vendor were suddenly extended, and we weren't notified. This has caused problems for us. We are now in the process of drafting quality agreements with these vendors in the hopes of alleviating this problem for critical materials.

Another issue is the plasmids we use to generate LVVs – it frequently takes 4–6 months to obtain plasmids. I recently read that some plasmid production companies have a backlog of up to 12 months. As this is obviously a problem, we have to plan well in advance to ensure the plasmids are available when needed.

EH: Procurement is certainly a major bottleneck and related to that is the quality of the plasmids and shortages for production of plasmids. It will be interesting to see what regulations the FDA will require in this regard, moving forward. Phase 1 has a bit more flexibility, but as trials progress you obviously need higher quality raw materials.

Q Can you tell me about your work in terms of scaling up vector bioprocesses, and how that's evolving?

DB: Just under a year ago, the department hired a faculty member to lead a Bioprocessing Development Laboratory (BDL) for Cell and Gene Therapy Manufacturing. Prior to this time, we tried to perform optimization studies during short 'down' periods, but this is not an efficient manner to perform such studies – it was a challenge. In collaboration with Vector Production, BDL is tasked with scaling-up vector manufacturing utilizing a suspension cell system in a bioreactor setting. Alternative methods for transfection during vector production are also being explored – we're always looking to optimize processes, get better titers and increase the efficiency of processes.

EH: The skillset required for process improvement, optimization, scalability and so on is different to the skillset required to manufacture the vector. You need to have

a knowledge of manufacturing requirements, but you also need to have a more robust scientific understanding and scientific process.

There was another faculty member brought on board, outside of our group, who is going to be implementing a preclinical vector core facility that will help investigators at IU who want to use viral vectors for research. In turn, this will assist us in understanding what the investigators need much earlier on, to help streamline the process of moving from preclinical- to clinical-scale manufacturing.

Q What would be top of your respective wish lists in the way of new innovation from the enabling tool and device providers?

DB: As we are a small group with limited financial resources, I would like to collaborate with industry partners who have the kind of systems in place that we are looking to develop, such as bioreactors for scale-up, or continuous flow centrifuges for vector concentration – right now, the expense for such processes is prohibitive for us. Developing a collaboration with industry would definitely help us to improve and grow our processes.

Q What are your chief goals and priorities for the future?

DB: As discussed earlier, top of the list is hiring adequate personnel, so that technicians can become more specialized in a subset of tasks. This would permit us to maximize our production capacity. Right now, the wait period to have a GMP product made is approximately 2 to 2 and a half years. We would like to reduce that substantially, so customers can get their product sooner. This in turn will allow customers to move their gene therapy clinical trials forward at a quicker pace.

EH: One of my priorities is to make sure that we have a robust and sustainable program that we can use for manufacturing of both cell and vector products for use in clinical trials. And as Daniela said, so much of your success is down to building the right team.

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INTERVIEW

EXPERT INSIGHT

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Investor Insight

-

INTERVIEW

Venture creation for cell and gene therapy and beyond

BRUCE BOOTH is a partner at Atlas Venture and focuses on the discovery and development of novel medicines and therapeutic platforms. Bruce is currently chairman of Arkuda Therapeutics, AvroBio, Hotspot Therapeutics, Kymera Therapeutics, Nimbus Therapeutics, Quench Bio, and Unum Therapeutics, and also serves on the boards of Lysosomal Therapeutics and Magenta Therapeutics. He previously served on the boards of past Atlas companies Avila (acquired by Celgene), Padlock (acquired by BMS), Prestwick (acquired by Biovail), Rodin Therapeutics (acquired by Alkermes), Stromedix (acquired by Biogen), and a number of other ventures. Bruce serves as an advisor in various capacities to UCB, Takeda, and the Gates Foundation. He also serves on the boards of the National Venture Capital Association, the Pennsylvania State Research Foundation, and New England Disabled Sports, a charity dedicated to adaptive sports. Bruce blogs about biotech and venture capital topics at LifeSciVC.com, which is syndicated on Forbes. Prior to joining Atlas in 2005, Bruce was a consultant at McKinsey & Company, as well as an investor for Caxton Health Holdings. As a British Marshall Scholar, Bruce received a DPhil (PhD) in molecular immunology from Oxford University. He enjoys running, skiing, hiking, and fly fishing. He lives in Wellesley, MA and has three wonderful kids.

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Q What's occupying your time at Atlas Venture at the moment?

BB: We're an early stage venture capital firm focused primarily on venture creation, so we start new biotechnology enterprises out of academic labs, working in concert with entrepreneurs. Some are spinouts from existing companies, but the vast majority of our new investments are ones we create in our office.

We've also got a portfolio of about three dozen emerging biotechnology companies; everything from raw start-ups through to public companies. Our role on the boards of these companies occupies a significant amount of time as well.

Q What defines and differentiates Atlas Venture's approach to life science investment?

BB: We've been doing this for a long time, and that has honed our team's capabilities to be able to occupy what we believe is the sweet spot of venture creation for new therapeutics.

Some people define themselves by what they're not. We're not broad life science investors – we don't invest in diagnostics or devices, research tools, or healthcare IT. We focus purely on therapeutics, and we think that the pattern recognition developed by focusing on one area gives us the confidence to know what we're doing.

Within the space of early stage therapeutics, we like the venture creation model, which is in-house partnership with entrepreneurs. We build and caretake firms during the early seed stage, essentially the stealth mode of young start-ups, to get the confidence that these companies are ready to build and scale. They then fly the nest and move out of Atlas once they've achieved a certain scale or critical mass. We think this early process involving both the curation of the science as well as the recruitment of the talent to lead these enterprises is an important part of our model.

We are also a flat and equal partnership. The five investment partners are exactly the same in terms of voice within the firm. That culture pervades the rest of the firm regarding the culture we take towards group dynamics and working on problems together. There is no emperor who has no clothes – we are able to champion and challenge each other in a healthy way around these investment decisions.

Q Atlas has been involved in the cell and gene therapy space longer than most – how has your focus evolved over the years, and what is exciting you about your current portfolio?

BB: In general, people think about cell therapy as a relatively young field. But bone marrow transplants started over 50 years ago. For the first 30 or 40 years they were largely done the same way, but they had curative intent. I think that's the great part of cell and gene therapy: we're working towards cures rather than just ongoing treatments. That's the power of this space.

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"Within the space of early stage therapeutics, we like the venture creation model, which is in-house partnership with entrepreneurs. We build and caretake firms during the early seed stage, essentially the stealth mode of young start-ups, to get the confidence that these companies are ready to build and scale."

In the early years we had bone marrow transplants, and adeno-associated virus (AAV) and *ex vivo* retroviral gene therapies really started to emerge in the 1990s. There were a number of clinical trials, but at the end of the '90s the field hit some serious roadblocks. For example, the death of Jesse Gelsinger during a clinical trial happened just a little over 20 years ago, and that was a real tragedy. There was also fear of retroviral integration causing leukemia. The questions raised about gene therapy at the turn of the century essentially put the field on ice from a commercialization perspective and sent it back to academia for more work.

Credit must be given to the various academic institutions that kept advancing the field in the 2000s, because the early clinical data that started to come out regarding next generation approaches about a decade ago started to look positive. That's when industrial and commercial interest and venture capital flows returned. On the gene therapy side as well as for engineered cell therapies like CAR-T, the early publications were in 2011/2012. That was really when the field started its next inflection.

In the last 8 or 9 years it has been incredible to witness the advances in this space, and we've participated across the spectrum, with different types of companies in different areas. I think this wave of gene and cell therapy was reinforced by genetic technologies such as gene editing advancing, too. It has been a culmination of a broad set of technology waves that had all incubated in academia for the last decade or two, that has now hit full force.

We started a number of these companies, like AVROBIO – an *ex vivo* lentiviral therapy company, which is searching for cures in lysosomal storage diseases including Fabry, Gaucher, Cystinosis, and really making great progress. There's also Magenta Therapeutics, which is looking at ways to make transplants safer so you can extend them into autoimmune diseases.

We have CAR T and CAR T-like cell therapies in the cancer space, with both Unum and Obsidian. Obsidian is working on tunable or controllable switches in gene therapy, which is an area of great interest. A number of our companies are also working on different AAVs, whether it be individual product or platform applications. Then of course we have non-viral gene therapy, with companies like Generation Bio working to see if we can move beyond having to use a virus as a chassis for delivering transgenes, and they are also making good strides.

Q As a seasoned investor in the space, what do you see when you look at the financing environment for the sector as a whole? Are there any areas of opportunity that stand out, or any warning signs you see for the future?

BB: Over the last few years we've seen a tidal wave of interest from the investment community, and many companies have been financed and are advancing through preclinical and early clinical trials. Around 900 Investigational New Drug (IND) applications have been filed in the cell and gene space – a lot of them from smaller companies, which is exceptional and great to see.

Something that concerns me is the overfunding and hypercompetition we are seeing in certain areas. The current number of CAR T-like constructs targeting CD19 and BCMA mean that these are incredibly crowded spaces right now, and it's not clear what differentiates them all. Starting to look at capital allocation questions using a competitive lens will be an important part of the investment cycle going forward.

Q What are the most important considerations for you when assessing a potential cell or gene therapy investment now, as opposed to 10 or 15 years ago?

BB: As I mentioned earlier, the differentiation of the particular approach is crucial. There are a lot of 'me too' gene and cell therapies being funded today. This is especially important if we're in the arena of starting new companies – when you're in discovery or preclinical you better have a really good sense of how this differentiates from the current generation of clinical approaches. You then couple that with an experienced, realistic, and inspiring team. It's a combination of differentiated scientific thesis coupled with a team that is seasoned enough to understand both the promise and pitfalls of the space; this is what we tend to focus on early in our assessments.

Q What elements of the overall business model remain particularly problematic for cell and gene therapy start-ups, either due to lack of resources or because they are overlooked? And what would be your advice?

BB: A big issue is the cumbersome manufacturing aspects of getting GMP grade vector made. And in the cell therapy space, there's autologous cell therapy manufacturing, for example. You have to consider

"...we like to think about the patient first and then consider which modalities should be used to address the problems presented to us."

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how you are going to assemble and build your business to address manufacturing challenges: what are you going to do in-house and what are you going to work through partners for? If you're working with partners, what are the contingency plans if one partner has issues? An infection at a contract manufacturer can shut the whole place down and send significant delays rippling through the ecosystem.

> "...when you're in discovery or preclinical you better have a really good sense of how this differentiates from the current generation of clinical approaches."

You don't want to reinvent the wheel with some of these manufacturing technologies, or build a significant amount of scale if you're not going to be able to utilize it. Thinking through the staging of those kinds of investments and the relative risks you're willing to take in terms of emphasis on internal versus external questions, is critically important.

As you advance into the clinic, you should consider the registration pathways and what you need to show to get your therapy approved. The FDA has guidance regarding what duration of follow-up you need to do with your patients, and I think there are important business model considerations around establishing a way to follow your patients for 10–15 years. Lastly, on the commercial side, you need to think about pricing. There's some precedent now, with some approvals for taking an upfront payment and amortizing it over 5 years, for example. I think those models make a lot of sense.

Durability is one of the big unknowns with a lot of the cell and gene therapies, because we're really in the first few years in terms of treating individual patients. We need to consider the long-term. Are we in certain settings truly curing these diseases – especially in monogenic diseases, where we're adding a protein that's missing due to a loss of function mutation? We need to know if we are really curing them in the long run.

Q What are your hopes and expectations for the cell and gene therapy space over the next decade?

BB: The idea that we may be able to cure people with gene and cell therapies is part of the inspiration that gets us all motivated to advance these therapies. I think in 10 years we'll have a good sense of that, and I'm very hopeful that we will indeed be curing patients.

If in 10 years we are still talking about AAV and retroviral vectors, I suspect something hasn't worked in terms of next generation approaches. The new programs entering into the R&D process will be versions 2.0 or 3.0, that are not going to have the immunogenicity that known viruses and known capsids have, or the risks of oncogenic integrations and so on. I predict we're going to move beyond this with non-viral, more synthetic gene delivery techniques. I'd like to see that happen so that we aren't still talking about these rather clunky, although wonderful, version 1.0 technologies.

On the other hand, if you rewind by 10 years, no one could have predicted that we would have 900 gene and cell therapy INDs, and that the biotechnology landscape would be full of

start-ups working in these spaces. It's been a wonderful explosion to watch, and I'm sure the next decade will bring even more surprises.

Q What are your chief goals and priorities for the foreseeable future?

BB: It comes back full circle to what I said at the start – our core focus is venture creation, and we're going to continue to focus on that and probably start half a dozen new companies a year for the next 3 to 5 years, as we think about deploying our current funds.

We're in the business of trying to build great companies that are going to have a positive impact on patients. One of the pieces of this will be cell and gene therapy but of course, we invest more broadly, too. What is really wonderful about the current arena of innovation is that there's this vast modality toolkit that we can utilize to address and treat a whole host of human diseases – you have small molecules, biologics, oligo-based technologies, gene and cell therapies, the whole area of engineering genomes, and you even have digital therapies. At Atlas, we like to think about the patient first and then consider which modalities should be used to address the problems presented to us. This is truly a golden age of biology and science, and we hope to continue to play a role in it.

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CELL & GENE THERAPY INSIGHTS

Investor Insight

-

EXPERT INSIGHT

Addressing the current limitations of AAV gene therapies

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In vivo gene therapy of human disease using adeno-associated virus (AAV) as a vector has become an established therapeutic modality in the past 6 years. With three approved drugs and a substantial number of Phase 3 clinical trials, the sector is progressing rapidly to taking its place in mainstream medicine. However, critical technological limitations have kept the approach confined to a relatively narrow spectrum of indications thus far. In this Investor Insight, we explore the directions of travel towards the follow-on gene therapies that have the potential to expand the reach of the platform towards broader and more complex indications. We review the ongoing efforts to expand the repertoire of tissues addressable with AAV gene therapy; circumvent the limitations of AAV carrying capacity; and to introduce logic and control mechanisms into *in vivo* gene therapies. There are two other important factors limiting the use of AAV: the ability to re-administer and manufacturing at scale. These are well reviewed elsewhere and are not the focus of this Investor Insight.

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INTRODUCTION

The first known use of AAV as a vector *in vivo* resulted in stable transduction of rabbit airway epithelium with the normal cystic fibrosis transmembrane conductance regulator cDNA, with expression detectible for up to 6 months after administration [1]. The possibility of AAV use in the brain was demonstrated soon thereafter, with transgene immunoreactivity detectable in both neurons and glia for up to 4 months and substantial behavioral

effects observed [2]. Significant gene transfer and long-term expression was also observed in cardiomyocytes, with the use of a CMV promotor [3]. Since then, AAV has been used successfully to transduce a large variety of cells. Three gene therapy products utilizing AAV as a vector have been or are currently approved – Glybera® for the Crigler-Najjar syndrome, Luxturna® for RPE65-associated retinitis pigmentosa, and Zolgensma® for type 1 spinal muscular atrophy.

Typical AAV vectors contain a promotor (often specific for the particular tissue or cell type), a gene of interest, a terminator sequence, and the ITRs. The AAV2 ITRs are used in the majority of engineered vectors. As there is evidence that codon choice influences expression rates in mammalian systems (although not as strongly as in bacterial ones) [4], AAV vectors are typically codon optimized for translation efficiency [5].

Packaging limit represents a significant hurdle, given at least 30% of genes responsible for monogenic disorders do not fit [6]. There is good evidence that the use of oversized vectors [7] or overstuffing a normal vector leads to significant decreases in the viral yield and transduction efficiency [8]. A possible workaround includes the use of modular, overlapping or dual vectors to carry large transgenes such as dystrophin [9]. Various strategies to overcome this are discussed below.

Systemic administration of AAV causes occurrence and maturation of a significant immune response both in patients and in model systems [10]. In some cases, the impact of pre-existing neutralizing antibodies towards the particular capsid type appears to be limited [11]. Despite this, successful re-administration of gene therapy was only achieved in immune-privileged sites such as the retina [12]. The resulting limitation to a single treatment remains a significant barrier for gene therapies, preventing dose titration. Apart from various strategies for immune evasion, better control of transgenes will be useful to overcome this limitation; approaches to this are discussed in this Investor Insight.

CARGO CAPACITY & COMPLEX TRANSGENES

The main premise of most gene therapies today is the correction of single-gene defects. According to the Wiley Gene Therapy Clinical Trials Worldwide database, 259 clinical trials, or 10.5% of all performed, were in monogenic diseases [13]. However, monogenic diseases represent a small proportion of overall potentially treatable gene defects. The main reason behind the focus on the monogenic subset is the ease of engineering, but there is another one: the load constraints.

AAV vectors have transfer capacity of ~4.8kb. For certain diseases, this packaging limit means that full-length transgene cannot be delivered to patients. This has been a continual issue when developing gene therapies for inherited retinal diseases (IRDs) which are caused by mutations in genes whose coding sequence exceeds 5.0 kb.

There have been two main approaches to delivering large genes via AAV vectors: a single oversized AAV vector and a dual vector approach. A single oversized vector has proved not to be a realistic option when taking a gene therapy to the clinic. One study has shown that AAV vectors are able to package recombinant genomes as large as 6.0kb, but they typically have lower transduction capacity issues as the larger genome-containing virions are degraded by the proteasome [14]. AAV dual vector strategies can be split into four categories: fragmented, overlapping, trans-splicing and hybrid. Fragmented AAV dual vectors have been shown to have more disadvantages than advantages due to a lack of transgene packaging control as each capsid carries an incomplete fragment of the transgene after becoming truncated during the process [15]. Proof of concept data for the dual vector approach has been generated in IRD mouse models, including Stargardt disease (STGD1) and Usher syndrome type 1B (USH1B) [16]. Auricchio *et al.* found that dual AAV trans-splicing or hybrid vectors were able to transduce mouse photoreceptors leading to improvement of the retinal

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phenotype in these animals. They did note that the transduction levels were lower than if the experiments were performed with a single vector but given the typical size the mutated genes in IRDs this is an important discovery. Moreover, there are examples of affecting neighboring cells in the retina and interfering with the disease without modifying the causative mutation [17]. With the increased start-up activity in the ophthalmology and otology space, we anticipate further advances in the treatment to drive wider use of fragmented vectors.

Diseases that have multiple affected genes need a multiple transgene approach when developing AAV gene therapy. In some cases, a complex response to the pathological mechanism utilizing several proteins might also be beneficial. Exploratory triple transduction studies have been performed in parkinsonian rats [18]. Ozawa *et al*. hypothesized than PD patients may benefit from co-expression of three therapeutic proteins: tyrosine hydroxylase (TH), aromatic-L-amino-acid decarboxylase (AADC), and GTP cyclohydrolase I (GCH) to enhance dopamine production. They simultaneously delivered each protein via three separate AAV vectors. This approach resulted in enhanced dopamine production in denervated striatum of parkinsonian rats and improved on previous studies where the group took a double transduction approach, although the clinical dataset was mixed [19].

Carrying capacity of other vectors was engineered successfully for gene therapy purposes. In particular, the pioneering work of Oxford BioMedica on the engineered lentiviral Equine Infectious Anemia Virus (EIAV) vector demonstrated the ability to engineer the capacity in principle, leading to several clinical trials [20,21]. Therefore, authors remain largely positive regarding engineering carrying capacity in the future.

Dual AAV vector approaches and AAV-mediated delivery of multiple transgenes still need further optimization. However, these initial pre-clinical proof of concept studies indicate that the limited packaging capacity of AAVs can be overcome to help expand gene therapy into more diseases.

TISSUE TROPISM & TARGETING SPECIFIC CELL TYPES

AAVs rapidly emerged over the past several years as a dominant vector used for *in vivo* gene therapy. It has demonstrated versatility, safety and efficacy in multiple human trials, culminating in several drug approvals. However, AAV has inherent biological features that place severe limitations on the applicability of the technology today. First and foremost is its tissue tropism.

Most serotypes of AAV demonstrate a strong tropism for hepatocytes after systemic injection [22]. Some subtypes, like AAV9, demonstrate good transduction rates in nervous cells [23]. Currently approved therapies rely heavily on using these tropisms to guarantee optimal transduction rates in their selected tissues (Table 1).

The control of the tissue tropism remains fairly limited. There are also early indications of the tropism details being understood more poorly than originally thought. The entry of the virus into human cells is poorly understood both for wild-type and engineered vectors. Some of the serotypes, including the most commonly used AAV2, attach to cells using heparan sulphate [24]. Incidentally, this makes them less than ideal in all diseases involving heparane sulfate metabolism, such as the type III mucopolysaccharidosis (San-Filippo disease). Based on genome-wide screening in haploid human cells [25] a previously unknown human protein KIAA0319L (adeno-associated virus receptor, AAVR) was established as the essential host factor for AAV2 infection [26]. In addition to the primary carbohydrate interactions, secondary receptors also play a role in viral transduction and contribute to cell and tissue selectivity of viral variants. AAV2 uses the fibroblast/hepatocyte growth factor receptor [27] and the integrins αVβ5 and α5β1 [28]; AAV6 utilizes the epidermal growth factor receptor [29];

TABLE 1

Currently approved AAV gene therapies, their vector serotypes and tissue specificity.

Commercial name	Technical name	Vector serotype	Tissue
Luxturna®	Voretigene neparvovec-rzyl	AAV2	Retina
Zolgensma®	Onasemnogene abeparvovec-xioi	AAV9	Neurons
Glybera®	Alipogene tiparvovec	AAV1	Liver

and AAV5 utilizes the platelet-derived growth factor receptor [30]. AAV9 utilizes galactose binding, which enables it to cross the blood-brain barrier in some cases, uniquely among the parvoviruses. It has been proposed as a vector for brain gene therapy with a possibility of intravenous administration, although it is yet unclear what the transduction efficacy in humans is with this mechanism [31].

There have been several published attempts to manipulate the tissue specificity of AAV derived from both the academic and industrial communities. Reconstruction of ancestral AAV sequences and the application of directed mutagenesis to the derived ancestral vectors was shown to allow construction of vectors with very broad infection spectra [32]. Application of directed evolution to ancestral variants yielded a recently presented vector 4D-C102 (4D Molecular Therapeutics) which demonstrated rapid and durable transgene activity in the heart, significantly better than that of AAV serotypes 1, 8 and 9 [33]. Sequential directed evolution also yielded several capsids with significantly increased liver transduction rates [34], significantly increasing the viability of AAV-driven gene replacement in diseases which require affecting a significant proportion of the liver cells [35]. Selective evolution of AAV produced other useful capsids, including those for transducing photoreceptor cells [36] as well as other retinal cell types [37] following an intravitreal injection, albeit with limited translation to clinical application thus far.

Several direct ancestral variants have also been successful in expanding the potential tissue repertoire of gene therapy. One example, Anc80, efficiently transduced kidney cells [38] as well as several cell subtypes in the mammalian inner ear [39], where it demonstrated pre-clinical therapeutic efficacy in disease models [40].

It is also important to consider the limitations of the available model systems when assessing the engineered capsids and their tissue specificity. Use of Cre-dependent selection generated a capsid named AAV-PHP.B which was successful in penetrating the blood-brain barrier (BBB) and transducing multiple CNS cell types effectively in C57BL/6J mice [41]. Variants of this vector also succeeded in rats [42] whilst yielding very different outcomes in other species [43]. The controversy is partially explained by the fact that some mouse strains such as BALB/cJ lack LY6A, the receptor responsible for BBB crossing in other strains [44,45]. This was only established using whole-exome-sequencing following the challenges with transgene delivery using AAV-PHP.B in non-c57BL/6J mice. The inventors of the original AAV-PHP.B capsid have recently proposed other capsids derived from multiplexed Cre-dependent selection that are apparently capable of transducing neurons following intravenous administration in several model organisms, thus overcoming the earlier limitations [46].

Overall, the reach of the AAV vector remains restricted to several key tissues. In the liver, several different vector systems demonstrated clinical efficacy, and the improved versions are advancing towards the clinic. The retina and, to an extent, the brain, both have validated vectors capable of robustly transducing functionally and genetically defined cell populations. To achieve significant gains in terms of the addressable diseases, we need capsids able to transduce the key organs, including the heart, the lung, the kidney and the pancreas – preferably from the bloodstream – while preserving liver function. This remains a significant engineering challenge. Moreover, improving the efficacy of transduction is crucial to allow both tissue targeting

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and dual vector strategies, as gene therapy has to contend with relatively low target cell counts and low probabilities to get two functional constructs in the same cell.

BETTER CONTROL OF TRANSGENE FUNCTION

Greater control over AAV gene therapies has become a focal area of research in the hope of improving safety and efficacy. One of the main risks of using gene therapy is that they cannot be switched off or modulated once they are delivered to a patient's cells, and the complete removal of the therapeutic agent is nigh but impossible. The introduction of an on/off switch in AAV vectors would enable inhibition of transgene expression in case there is an issue with gene transfer or an off-target effect.

One of the more novel approaches to control gene expression of AAV-delivered therapeutics was published recently. Guocai Zhong and colleagues at the Scripps Research Institute in Florida published a study demonstrating that they could control *in vivo* regulation of AAV-delivered transgenes using an engineered RNA 'on-switch' [47]. The team engineered a type III hammerhead ribozyme to improve its enzymatic activity making it 60- to 80-fold more active in reducing the expression of a transgene. These ribozymes cut themselves in two when they are copied into RNA from the DNA that encodes them. The AAV 'on-switch' is activated when the self-cleaving activation of the ribozymes is switched off in the presence of morpholinos and the transgene. This is a novel approach, but we expect further development needed, including optimization of morpholino delivery.

Other platforms with 'on-demand' activation remain confined to excitable tissues but are nonetheless quite powerful and revolve around controlling the activity of the transgene rather than its expression. The first one to emerge is optogenetics, or the use of light-sensitive proteins such as bacterial or viral opsins to activate or inhibit membrane potential sensitive cells. Several start-up companies explored this modality, with varying results. Applied Genetic Technologies Corp developed a combination of an optogenetic therapeutic targeting retinal nerve cells with a bionic vision goggle, pioneered by Bionic Sight [48]. Retrosense Therapeutics (acquired by Allergan) developed an approach of transducing retinal ganglion cells with the algal Channelorhodopsin 2 in patients with late-stage Retinitis pigmentosa [49]. Gensight Biologics explored a similar approach to targeting retinal ganglion cells (currently in clinical evaluation, ClinicalTrials.gov Identifier: NCT03326336 Dose-escalation Study to Evaluate the Safety and Tolerability of GS030 in Subjects with Retinitis Pigmentosa [PIONEER]). The optogenetic platform is a widely used and powerful research tool but remains largely untapped as a therapeutic due to the difficulty of delivering consistent and powerful light sources to the therapeutically relevant tissues [50].

Another rapidly advancing approach is chemogenetics, where the activity of an engineered receptor or channel is controlled with a small molecule. The current repertoire includes the 'designer receptor exclusively activated by a designer drug (DREADD)' platform which utilizes engineered GPCRs [51] and has shown pre-clinical efficacy in epilepsy [52] as well as alcohol use disorder [53] and other indications. The more recent 'pharmacologically selective effector molecule (PSEM)' platform relies on mutant chimeric ligand-activated ion channels. Whilst originally demonstrating relatively low ligand sensitivity [54], it has been optimized and can now achieve effective neuromodulation using a sub-therapeutic concentration of an approved drug [55] and is being developed in several neuroscience applications by New York-based Redpin Therapeutics.

Other actuators of controllable receptors for excitable tissues have been tried as tools, and sometimes pre-clinically. Activation of genetically engineered neuronal cells with magnetism has shown some promise, but

reproduction of the successes of the original technique [56] has met with mixed success [57]. Another possible approach utilizing physical forces is thermogenetics, which relies on the thermosensitive transient receptor potential (TRP) channels. It achieved rapid, robust and reproducible repeated activation of snake TRPA1 channels heterologously expressed in non-neuronal cells, mouse neurons and zebrafish neurons *in vivo* by infrared (IR) laser radiation [58].

Overall, the development of controllable gene therapies delivered substantial benefits for the lab-based research methods (optogenetics in particular), whilst the transition to pre-clinical development took a significant amount of time. However, the convergence of more sophisticated bioinformatics techniques for promotor design and development with the next-generation controlled receptor systems holds significant promise for achieving better control over one-shot gene therapies.

DISCUSSION

AAV has several notable advantages as a gene therapy vector. It does not integrate, forms robust, long-term episomes that provide durable transgene expression, is relatively easy to engineer and appears generally safe and well tolerated from over 200 clinical trials it was tested in.

One particularly attractive feature of *in vivo* gene therapy is its 'one and done' approach. A single administration of an efficacious concentration of an AAV vector appears to be capable of significant phenotypic correction in multiple therapeutic areas, including hematology, ophthalmology, metabolic diseases and neurology. However, this virtue is one born of necessity: we have no capability to re-administer AAV outside the eye. Moreover, many clinical protocols now opt for prophylactic use of steroids in order to manage the expected immune-related side effects of the therapy. A robust way of immune evasion, enabling re-administration, will be potentially necessary to resolve many conditions where large-scale coverage is needed. The same would also allow the treatment of patients with pre-existing neutralizing antibodies. On top of that, further improvements in tissue specificity and carrying capacity are needed. Enhancing transduction efficacy is necessary to avoid applying excessive doses of the virus which can lead to complement activation and other severe immune responses.

Overall, given significant progress on these fronts in the past decade, AAV is likely to remain the vector of choice for *in vivo* gene therapies until such time that non-viral alternatives are available and tested in the clinic. Any technological improvements representing significant progress in carrying capacity, immune evasion, transduction efficiency, ease and throughput of manufacturing, tissue and cell type specificity and transgene control of these vectors are likely to attract significant interest of investors and pharmaceutical companies alike.

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CELL & GENE THERAPY INSIGHTS

April 2020

Clinical Trends

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April 2020
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COMMENTARY

INTERVIEW

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COMMENTARY

Trends in cell and gene therapy clinical development for rare and ultra-rare diseases

Sven Kili shares his regular analysis of current clinical trends, this time focusing on the key area of rare diseases. As pressure mounts on patient recruitment, and the relatively few specialist clinical centers currently equipped to deal with advanced therapies, the clinical development community is seeking novel trial designs, endpoints and approaches to generate compelling, robust data from a minimal number of patients. Meanwhile, Covid-19 is presenting a further, hugely significant challenge to the entire field.

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PATIENT & CLINICIAN COMPETITION FOR RARE DISEASE CLINICAL TRIALS

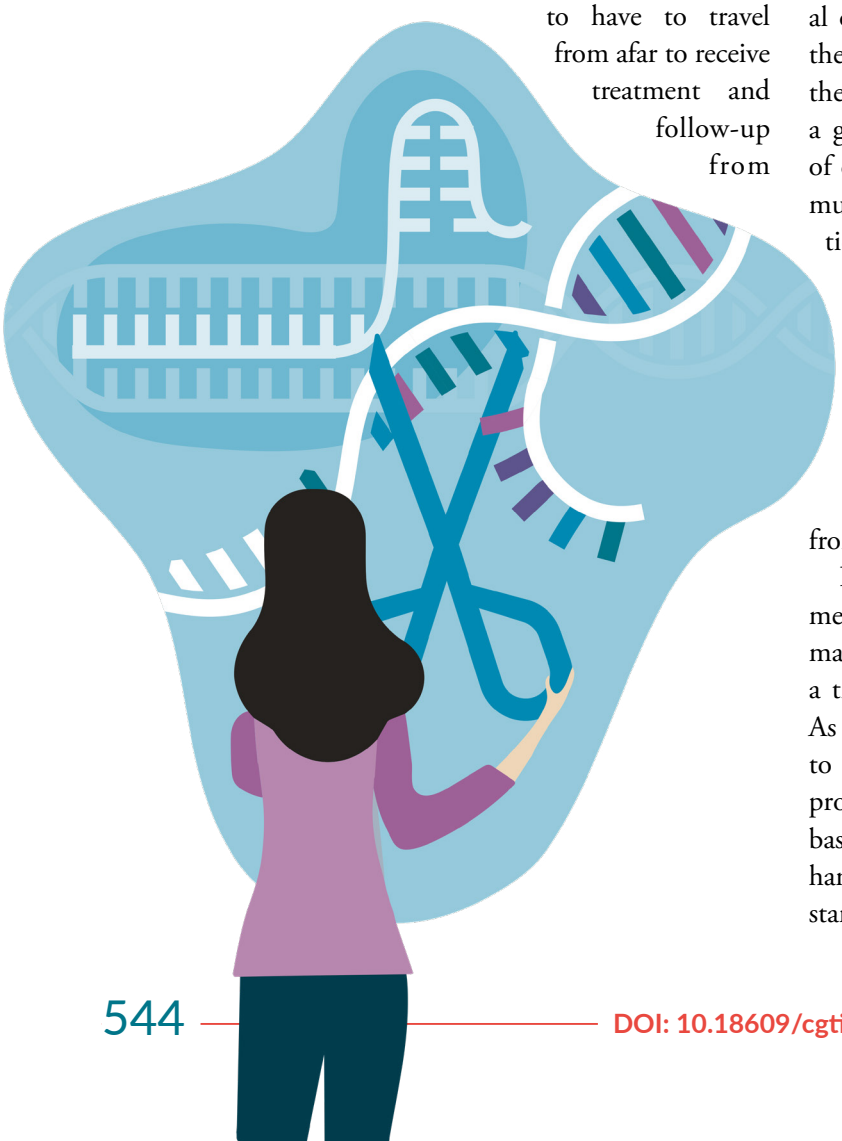
As more and more cell and gene therapies are being developed for small populations of rare disease patients, we are starting to see increasing pressure not only on patient recruitment, but also on the clinical trial centers capable of delivering these therapies.

Regarding recruitment, the inherent nature of advanced therapies exacerbates the clinical development challenges faced throughout the rare diseases area. In contrast to most of the more traditional types of therapies, which are typically administered on a repeat basis, cell and gene therapies tend to be administered only once, meaning the possibility of 'cycling' patients

through more than one trial is removed. Furthermore, in the case of gene therapy, this single dose tends to lead to a permanent 'correction' or change to the patient's genetic condition, hopefully removing their eligibility for other future trials.

Many cell and gene therapies rely on treating patients in the early stages of their disease, especially for those diseases with a metabolic component where the damage may be irreversible. This makes early patient identification especially important. However, patient identification is something that is often very challenging with these diseases; there may be no specific newborn screening or childhood diagnostic tests – e.g., hypercholesterolemia or minimal lumen diameter (MLD) – meaning that patients tend to be identified only once they have suffered a complication – a scenario which potentially makes these patients less than ideal study candidates.

Specialist centers treating these ultra-rare and complex diseases are few and far between. Consequently, patients tend to have to travel from afar to receive treatment and follow-up from teams specialized in caring for them. These centers are the natural locations for novel rare cell and gene therapies to be tested, but they are busy treating patients and so may struggle to participate in multiple clinical trials competing for the same small patient pool. The sheer variety of advanced therapy modalities reaching the clinic today adds a further layer of complexity, as competing products often come with widely differing treatment and follow up requirements. As a result, staff are often unable to assimilate more than one or two complex trial flows.



TRENDS IN DESIGN & ENDPOINT SELECTION FOR RARE DISEASE CLINICAL TRIALS

In response to the mounting difficulty in recruiting sufficient patient numbers to cell and gene therapy trials, investigators and regulators alike have begun to explore new clinical trial designs and even some new functional outcomes. This is not a trend isolated to the cell and gene therapy field – throughout the rare and ultra-rare diseases area, there is a growing need for much greater efficiency of clinical study design in order to obtain as much value and data from these limited patient populations.

A variety of potential approaches have been proposed, including the increased use of natural history studies to better understand a given disease, and even offer a historical control cohort. This approach will help when trying to limit the use of placebo as a treatment comparator, from both design and ethical perspectives.

Prior to Genzyme showing that their treatment could be financially viable, rare diseases management was largely ignored by all but a tiny proportion of clinicians and patients. As a result, most of the information available to the scientific community concerning the progress of many of these diseases is largely based on the personal experiences of a relative handful of key clinicians. This is not an ideal starting point for understanding how these

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diseases develop in patients, nor for being able to demonstrate in a robust way whether one novel therapy is much better than another. As the cell and gene therapy field forges ahead in developing new therapies for these underserved indications, both developers and regulators alike are learning as they go. Natural history studies offer important insights into how patients were and are treated outside the clinical development sphere. Additionally, when developing a therapy for a very small group of patients, it is important that each patient contributes to the data set being generated. The same applies to indications that are fatal, of course – nobody wants to be placed in the placebo group where they may end up dying. Lastly, natural history studies help regulators and payers to better understand the healthcare utilization by these patients, assisting them in defining just how much better (or worse) a given new therapy is going to be than the standard of care.

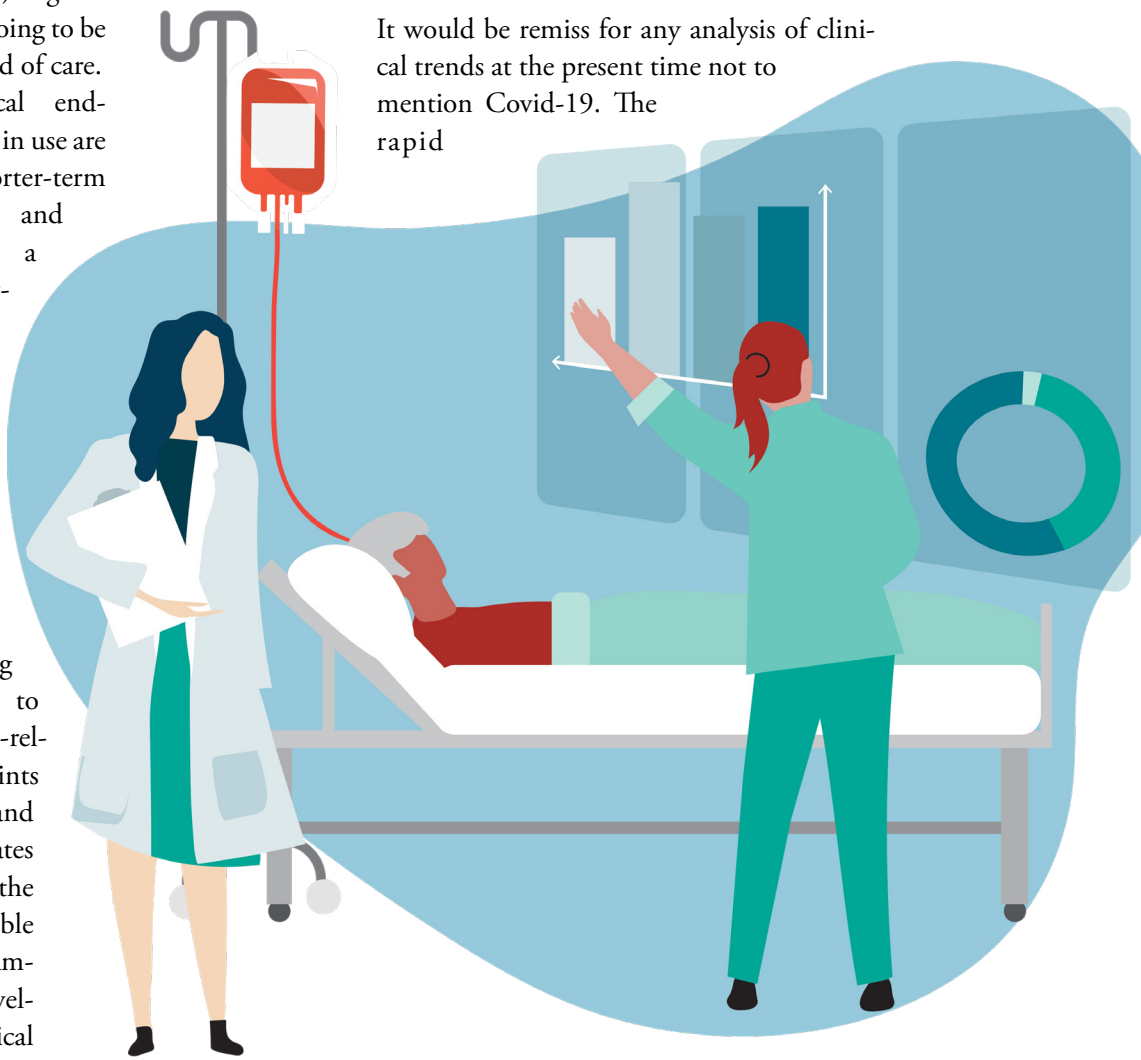
Many clinical endpoints currently in use are related to shorter-term interventions and when utilizing a cell or gene therapy product, it may be more appropriate to consider newer, more relevant surrogate endpoints. Engaging in discussion to define patient-relevant endpoints with patients and patient advocates is advisable at the earliest possible stage, allowing ample time to develop a non-clinical rationale and also to obtain the advice and buy in of the regulators.

A number of trial designs including longitudinal data utilizing repeated measurements (as opposed to scores) have been proposed to reduce clinical trial participant numbers by up to 30%. Other proposals for making trials more efficient include avoiding dichotomizing continuous data.

Meanwhile, some patient groups have suggested making more efficient use of individual patients by prioritizing the order in which they participate in trials, thus enabling them to contribute to as many different studies as possible.

THE IMPACT OF COVID-19 ON PLANNED & ONGOING CLINICAL TRIALS

It would be remiss for any analysis of clinical trends at the present time not to mention Covid-19. The rapid



spread of the virus throughout the world has caused major disruptions to the way healthcare is delivered, but also to how we are developing new cell- and gene-based therapies. As a result of the pandemic, we are seeing varying responses concerning both the delivery and the development of these therapies.

Regarding delivery, many of the diseases for which cell and gene therapies (including stem cell transplants) are being used are very severe or even fatal. Delay in treatment could considerably shorten a patient's life. This leaves treatment centers with no real choice: the treatment must go on! Therefore, in order to still be able to treat patients, these centers are having to adopt new and often even stricter infection control measures in an attempt to ensure the safety of their patients. These include aspects such as isolation before and after procedures.

For even potentially life-saving cell and gene therapies in clinical development, there appears to be a split amongst sponsors on a global basis. Some have opted to suspend clinical trials whilst others are forging ahead with or without additional screening or isolation activities. Only time will tell which was the wiser choice.

Regardless, at this time, we should give thought to the clinical investigators, many of whom are on the frontline day to day dealing with the pandemic. How many of them have the time or headspace to deal with the additional responsibilities and complexities of a clinical trial?

In response to the Covid-19 pandemic, many cell and gene therapy companies are joining the global hunt for treatments, tests, and vaccines for the virus. Areas in focus at the time of writing include:

- Development of patient testing
- Vaccine development
- Non-specific symptom treatment – e.g., ARDS with MSCs
- Specific Covid-19-directed therapies – e.g., Anti-viral antibody or CAR T therapies

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Principal, Sven Kili Consulting Ltd, UK

SVEN KILI provides specialist strategic consulting services to innovative Regenerative Medicine companies. His clients include small and medium sized companies from company formation through to clinical development and commercialisation. He was previously the Head of Development for the Cell and Gene Therapy division of GSK Rare Diseases where he led teams developing ex-vivo Gene Therapies for a variety of rare genetic disorders including Strimvelis®, the first *ex-vivo* gene therapy to be approved for children with ADA-SCID; Wiskott – Aldrich syndrome (WAS); Metachromatic Leukodystrophy (MLD) and Beta-Thalassemia. Prior to this, he was Senior Director, Cell Therapy and Regenerative Medicine for Sanofi (Genzyme) Biosurgery where he led the clinical development, approval and commercialisation activities of the first combined ATMP approval in the EU for MACI®. His team also prepared and submitted Advanced Therapy regulatory filings for Australia and the US, including health technology assessments and he was responsible for late stage developments for Carticel® and Epicel® in the US. Before joining Genzyme, Sven led the cell therapy activities and oversaw all UK & Irish regulatory functions and was the QPPV for pharmacovigilance for the Geistlich Pharma. Sven trained as an Orthopaedic surgeon in the UK and South Africa and since leaving full-time clinical practise has developed expertise Cell and Gene Therapy in clinical development, regulatory compliance, value creation, risk management and product safety, product launches and post-marketing activities. He sits on the board of CCRM in Canada; Xintela – a Swedish Stem Cell company and is the chair of the CGTAC as part of the UK BIA and the VP of the Standards Co-ordinating Body for Regenerative Medicine. Additionally, he still maintains his clinical skills in the UK NHS and serves as an ATLS Instructor in his spare time.

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CELL & GENE THERAPY INSIGHTS

Clinical Trends

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INTERVIEW

Advanced therapy clinical trials for rare pediatric disorders: key challenges and lessons learned

MARIA JOSE DE CASTRO LOPEZ is a consultant in pediatric metabolic diseases at the Clinical University Hospital from Santiago de Compostela, Galicia, Spain, and has been involved in a number of gene clinical trials for inborn errors of metabolism.

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Q What are you working on right now?

MJdCL: I'm a pediatrician and a consultant in inborn errors of metabolism. I take care of patients on a daily basis, but I'm also involved in Phase 1 and 2 clinical trials at my hospital in Santiago de Compostela, particularly for lysosomal storage diseases like Sanfilippo syndrome.

Q How is the situation with COVID-19 impacting your clinical trial activities at the moment?

"You need a team that is well coordinated and available almost all of the time, and the hospital pharmacists have to follow extremely specific rules regarding biological risks."

MJdCL: We are following our regulatory agencies, which have released some guidance on how to continue our trials. But as you can imagine, it's difficult to recruit patients right now and for the moment, international recruitment here has been cancelled.

Of course, we need to balance the potential benefits of the experimental drugs and the disease of the patients. For example, Sanfilippo syndrome is a devastating disease, and the patients are continuing to grow and experience further cognitive deterioration. So right now, we are trying to maintain recruitment in Spain, especially for those patients who may be very close to the end of the potential therapeutic window – prompt treatment will be of critical importance towards saving their cognitive function.

Regarding follow up of clinical trial patients, we are trying to change the requirements in this regard for the safety of our patients. Instead of having these tests at the hospital, we are using a service to perform testing at their homes.

Q Can you tell us about your experience to date in working with advanced therapy medicinal products (ATMPs) in the clinical trial setting?

MJdCL: My experience with ATMPs has been short, but very intense! I started 3 or 4 years ago with my first gene therapy trial, and right now I am involved in six Phase 1 and 2 gene therapy trials for inborn errors of metabolism.

Q What, for you, are the most challenging aspects of working with gene therapies?

MJdCL: The gene therapies I work with are all investigational products in the clinical setting and the trials are very different from this point of view. They are very exhaustive because the schedules are very tight and the data has to be carefully registered. The people involved have to be highly experienced, focused, and have to follow specific procedures that are very closely monitored. You need a team that is well coordinated and available almost all of the time, and the hospital pharmacists have to follow extremely specific rules regarding biological risks. Moreover, it is critical to keep a close contact with the patient and the family.

But even when taking all of this into account, when it comes to the administration of these products on a daily basis, I actually think it's very easy; only one intervention. Our patients are doing really well, because the trials are relatively easy for them.

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"You also need to network with all of the specialist physicians covering a particular rare disease, both in your country and internationally, so you can be made aware where and when a patient has been diagnosed – you can then offer them the possibility of joining your trial ... We have contacted patients regarding our studies here in Spain, but also as far afield as Australia."

Q How have you experienced the clinical trial scene for ATMPs against pediatric disorders evolving over recent times?

MJdCL: Especially for rare diseases in pediatric patients, there is a boom. It seems like everybody is involved in developing this kind of therapy. They are potentially curative and we know that most of these diseases are monogenic, which makes them attractive to therapy developers and the scientific community. So for us, for our patients and their families, these therapies are very appealing – there is a lot of enthusiasm for getting involved with them.

Ten years ago, there were no experimental products and no treatment was available in most of these diseases. Today, many of our patients have a potential trial on the horizon, or sometimes there are even multiple trials for the same disease that are competing to include them.

Q Staying on the topic of patient recruitment, what is your advice for addressing this growing challenge?

MJdCL: PIs and sponsors need to be part of a network. Being in contact with family and patient associations is crucial in order to let them know that there is a trial that could potentially help their members. You also need to network with all of the specialist physicians covering a particular rare disease, both in your country and internationally, so you can be made aware where and when a patient has been diagnosed – you can then offer them the possibility of joining your trial. I'm talking from personal experience here; we have contacted patients regarding our studies here in Spain, but also as far afield as Australia.

From the patient's perspective, it's important to join a patient association. And when you receive information about different potential trials, my recommendation is to rely on the published data. In my opinion, that is the only way to evaluate and compare their potential benefits.

Q What have been the key learnings or best practices in conducting these trials against rare pediatric disorders that you've come across so far?

MJdCL: The first thing that is important for the success of a clinical trial is the commitment of the team. You also need to have experience with the disease – because we are talking about rare diseases, there are not many physicians who really know how these diseases are diagnosed, or how they're going to evolve over time. Experience with these patients is crucial and I think that these kinds of trials should be conducted in referral centers. From the point of view of the pharmacies and laboratories, they must have experience managing these kinds of investigational products and the biological samples.

Also, and a I mentioned before, keeping a close contact with the family is of paramount importance. I truly believe that they are part of the team, and I let them know it!

Q A number of rare disease gene therapies have made it to the market, but their success is often limited. From your perspective at the coal face of clinical care, what can be done to ensure these therapies survive in the market in order to allow future generations of patients to benefit from them?

MJdCL: These drugs are amazing and have huge potential, but we face many challenges with gene therapies, particularly in the field of rare diseases. One of them is that in order to really understand the diseases and better tailor our outcomes, we need more natural history studies. In my view, obtaining clearer outcomes is a very important goal for the future.

For some diseases we also need accurate biomarkers, as some of the current ones are not ideal. I expect that the regulatory agencies are going to experience some difficulties when these potentially unsuitable biomarkers are tied into the ongoing assessment of how well ATMPs are working.

Additionally, we mustn't forget that before these investigational products can become truly routine, we will need to consider how they are going to be funded, because they are very expensive.

Finally, we need to be sure that these therapies are providing a benefit to our patients, and a key part of this effort is looking at how much of that benefit is measurable. We need to ask what specific results we need to see in order for them to be considered successful and become therapeutic options. The key is defining the benefits with the most accurate tools we have. I think we need to go deeper

"...it's difficult to recruit patients right now and for the moment, international recruitment here has been cancelled."

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into this element, and ensure close communication with our patients, so that all our decisions are underpinned by solid rationale.

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CELL & GENE THERAPY INSIGHTS

Clinical Trends

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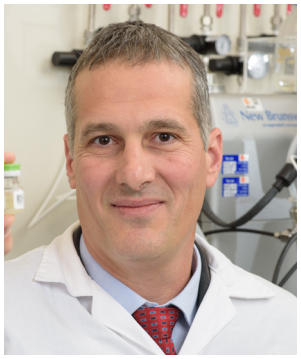
INTERVIEW

Cell therapy's role in defeating Covid-19



YAKY YANAY was appointed as President from February 2014 and as CEO from June 2019, after a period of being Co-CEO since March 2017. Mr Yanay served in variety of executive positions in Pluristem since 2006 including Chief Financial Officer, Chief Operating Officer and Executive Vice President. He is the former Co-Chairman and current board member of Israel Advanced Technology Industries (IATI), the largest umbrella organization representing Israel's life science and high-tech industries. Mr Yanay has founded several activities and organizations over the years to promote and support the Israeli life science industry. Before joining Pluristem, he was the Chief Financial Officer of Elbit Vision Systems Ltd, a public, machine vision, high-tech company. Prior to that Mr Yanay served as manager at Ernst & Young Israel. He holds a bachelor's degree with honors in business administration and accounting and is a Certified Public Accountant in Israel.

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Q These are unprecedented times as Covid-19 sweeps around the world – how is Israel fairing at the moment?

YY: It's definitely a very difficult and challenging time for everyone in the world, including us in Israel.

I think that we are lucky that Israel was prepared somewhat ahead of much of the rest of the world in terms of taking some preventative actions, which have helped in controlling the spread of the disease. I do hope we're not going to see significant increase over this period, which would allow us to continue controlling the disease. I remain optimistic.

Q Pluristem has engaged in the fight against Covid-19 – can you firstly tell us more about the scientific and clinical rationale behind Pluristem's PLX cell platform being applied to treat patients?

YY: Pluristem is a regenerative medicine company developing a cellular product that is derived from placenta after full-term delivery.

This product has been developed extensively in the last decade, and we are in quite late-stage clinical development – in fact, we have several Phase 3 studies running globally. We are targeting indications that involve a high degree of inflammation: ischemic conditions like peripheral arterial diseases, diabetes complications, as well as muscle regeneration.

We know that these cells are great secretors of anti-inflammatory cytokines or proteins, but they also have a very good ability to control and modulate the immune system. If the immune system is getting out of balance – for example, by starting a cytokine storm, which eventually leads to sepsis – our cells are able to control it and prevent it from overreacting.

In the past, we've looked modelled and studied a number of different respiratory disorders, including lung fibrosis, pulmonary hypertension, and acute respiratory distress syndrome (ARDS). Obviously, it became apparent very early on in the Covid-19 outbreak that the main complication and cause of death is respiratory disorders such as severe pneumonia. Additionally, a few weeks ago, we started to see data coming from China reporting the application of cells of a similar class to ours in patients suffering with ARDS.

You can think of these cells as tiny factories, which we inject into the muscle. The cells communicate with the patient's body, as cells do, and based upon the signaling that the cells are able to capture from the patient, they respond by secreting a lot of different proteins and cytokines that can push the body towards not just regeneration, but also controlling and

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balancing the immune system. This is how we believe we can help those patients in intensive care and on ventilators with extremely severe respiratory complications.

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Q Tell us about the collaboration with Charitè University of Medicine Berlin to further test and develop this application – what's the background to the partnership, and how are you overcoming operational challenges given the current state of lockdown around the world?

YY: We are fortunate to work with a lot of different partners and collaborators globally – this was our strategy from day 1: we understood that because we were to develop a very unique, advanced technology, it made sense to do it in collaboration with leading institutes.

Charitè is a very good example. We already collaborate with them on two of our Phase 3 studies, in critical limb ischemia (CLI) and recovery following hip fracture, both of which have been supported and funded by Horizon 2020. We work closely with Professor Hans-Dieter Volk (lead immunologist at the Charitè Hospital) and Professor Petra Reinecke. Once we decided that it made sense to use the PLX cells to treat Covid-19 respiratory complications, it was just natural for us to speak with our collaborators, who are expert in such disorders, to quickly establish the ties to allow us to fight the disease together.

The Charitè is the largest clinical institute in Europe, which gives us great access to the entire European continent. They know our cells very well – they know the mechanism of action, which allows us to move quickly in regulatory and clinical development terms.

It's obviously very difficult to travel at the moment, but fortunately we are well prepared in that we already have in place several logistics hubs around the world, including two in Germany, one in the UK, and two in the USA. These hubs contain our cells and they can easily ship them to any location in the world. Additionally, there are no special restrictions in terms of shipping the actual cell product from Israel, or indeed, anywhere around the world.

We have the advantage that our cells are allogeneic, so there is no match between the donor and the patient – it's an off-the-shelf product ready to distribute and use, without the need for patient-specific preparation. It is also a very scalable platform. From a single donor placenta, we can treat over 20,000 patients.

Q Pluristem received an approval from the Israeli Ministry of Health around allowing per patient compassionate use treatment of

Covid-19 Patients with PLX cells – how does that pathway work, and is it something that is feasible in other countries around the world?

YY: Given our focus is on treating the sickest patients rather than any preventative goal, as you would have with a vaccine, it makes perfect sense for us to start to treat patients as quickly as possible. Obviously, it also makes sense for us to try to start domestically and then to expand. With all the scientific background data we have accumulated over the past decade, and because the regulators in Israel, Europe and the USA are all very familiar with the product and its excellent safety profile, we were able to quickly gain the Israeli Ministry of Health's consent to start treating very late-stage Covid-19 patients.

So we have already started – we recently reported–preliminary data from our COVID-19 Compassionate Use Program, treating seven patients with acute respiratory failure. All treated patients were in Intensive Care Units (ICU) on ventilators and suffered from Acute Respiratory Distress Syndrome (ARDS). We saw 100% survival rate for all seven patients while 4 of the 6 (66%) patients that completed 1 week follow up demonstrated improvement in respiratory parameters.

We also expanded the program to the USA and recently announced our first patient (in the Holy Name Medical Center, New Jersey) under the US Food and Drug Administration's (FDA) Single Patient Expanded Access Program. This is also a compassionate use program, which is part of the US Coronavirus Treatment Acceleration Program (CTAP).

We are in the process of enrolling more patients into this initial compassionate use study. Now our main goal is to convert very quickly from a compassionate use program to a full, controlled global clinical study, which will allow other regulators and their jurisdictions to participate as well.

In the current environment, everything is happening quickly: regulators are responding, hospitals are responding, everyone is working fast. There are no borders – the authorities are trying to remove as much of the bureaucracy as possible.

So we do intend to very quickly reach patients in the USA and Europe, and hopefully further afield, too. We are also having discussions with different Asian countries. In terms of a timeframe, we hope this will be days and weeks, not months. We need to respond now.

Q You've mentioned the state of manufacturing and logistics readiness that you are able to leverage, but what might further expansion look like? What challenges would you anticipate facing, and how would you look to overcome them in order to meet global demand on an even larger scale, potentially?

YY: I think this is an issue we face across the entire cell and gene therapy space: while we're seeing very promising data at a small-scale, how can we convert that success to enable the provision of genuinely large-scale, affordable products?

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This is absolutely essential and is something that is really part of the DNA of Pluristem. Even 10, 15 years ago when we started, we understood that it's not just about the science and technology, or the ability to demonstrate a product's effectiveness in a relative handful of patients.

Of course, cells are so very different from other drugs with which we are familiar. If you put them into a process where scalability is not adequately addressed and which is not tightly controlled, the main risk is losing the product and its potency. This is the reason why Pluristem has invested more than $300 million in order to establish and build perhaps the most advanced manufacturing technology platform in the world. From day 1, we built and designed everything to work at large scales. I think it is probably fair to say we are the cell therapy company with the highest capacity in the world today.

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We know cells can do things that any other agents cannot, because they are native speakers that understand the language of the body. We as human beings still aren't able to understand the entire array of signaling and multi-layered communication the body carries out, but the cells speak the language: they can sense the signaling, they are able to respond and to interact, and secrete multifactorial factors. So the main challenge now is to be able to create a lot of these cells, with high batch-to-batch consistency, but also in a way that is going to be affordable.

This is the situation we find ourselves in today with Covid-19. We need to treat thousands of patients in a very short time period, not dozens or hundreds. This is our thinking and it guides how we are continuing to design and increase our manufacturing capacity. I think that this pandemic will ultimately play quite a significant role in defining what cell and gene therapy will look like in the future, because we need it to be affordable and available on a global scale.

Regenerative medicine is really about working with the body. In our field, we think about how to increase the activity of the immune system, or provide a better blood supply to an organ, or rebuild a piece of tissue. That's a very different approach to what we're used to seeing. So our mission is to be able to fight Covid-19 in the very short-term, but this is actually just one battle in a much larger war. This is the war of aging, of chronic disorders, and of unsustainable healthcare systems.

It is time for regenerative medicine to really come to the fore in supporting this global effort. Covid-19 is one example that we are seeing, and the fact that we are seeing the elderly and the

sick dying is basically telling us that this is something we should consider, understand, and seek to prevent. We are all aging – the main goal is that we should try to age as healthily as we can. That's a very large concept that regenerative medicine is targeting and pushing. Hopefully all of us will be able to join forces together to win both the Covid-19 battle and the war to come.

AFFILIATIONS

Yaky Yanay
CEO, Pluristem

AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

COMMERCIAL INSIGHT: FEB 2020

Commercial Insight: cell & gene therapy

Providing a critical overview of the sector's commercial development: M&As, licensing agreements & collaborations, financial results, IPOs and clinical/regulatory updates, with commentary from our Expert Contributors.

Cell & Gene Therapy Insights 2020; 6(3), 255–266

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CELL THERAPY – Mark Curtis. Director, Manufacturing Partnerships, AVROBIO

Sonoma Biotherapeutics debuted with a $40M series A. Investors in its first round included ARCH Venture Partners, Milky Way Ventures, 8VC, and Lyell Immunopharma. The company will use T cells to target autoimmune and degenerative disorders, an area of high unmet need with a competitive landscape much less crowded than the oncology space. In parallel, researchers from the Wellcome Sanger Institute, New Castle University, and the University of Ghent released a cell atlas of the human thymus providing insight into the signaling pathways that lead to the formation of different types of T cells over the early lifespan of a human. Data from the atlas can be leveraged to better understand how T cell therapies can be developed and manufactured to combat both cancer and autoimmune disease.

GENE THERAPY – Richard Philipson. Chief Medical Officer, Trizell Ltd, UK

The medical importance of gene and cell therapies continues to be recognised by both FDA and EMA, with the expedited review by both agencies of Biomarin's haemophilia A treatment valoctocogene roxaparvovec, Priority Review in the US for BMS's lisocabtagene maraleucel (liso-cel) for relapsed or refractory large B-cell lymphoma and Fast Track Designation in the US for Lysogene's LYS-SAF302 for mucopolysaccharidosis Type IIIA. Results for BMS's cell therapy liso-cel look particularly impressive, with high rates of durable complete responses and low rates of more severe grades of cytokine release syndrome, for difficult to treat patients with relapsed or refractory large B-cell lymphoma. Historically, median life expectancy for patients who relapse or are refractory to current standard of care treatments following multiple lines of therapy is approximately 6 months, so the outcome of median overall survival of 21.1 months in TRANSCEND NHL 001 looks particularly impressive.

Clinical Regulatory

FDA ACCEPTS BLA FILING OF BIOMARIN'S GENE THERAPY

The FDA has granted priority review designation and has accepted Biomarin's Biologics License Application (BLA) for its gene therapy, valoctocogene roxaparvovec. If approved, it will be the first gene therapy product to be approved in the US for any type of hemophilia.

The gene therapy uses an adeno-associated virus (AAV) vector to deliver the human blood clotting gene, *FVIII*, to patients with severe hemophilia A, an X-linked bleeding disorder caused by deficiency of the clotting protein, FVIII.

With a potential price tag of more than US$1 million, valoctocogene roxaparvovec is one of several gene therapies under development that has claimed to cure hemophilia permanently following a one-time gene delivery.

The BLA is based on results from a 3-year Phase 1/2 trial and interim results from a Phase 3 study conducted in hemophilia patients treated with valoctocogene roxaparvovec. Earlier this year BioMarin had published data from the Phase 1/2 trial which demonstrated that a single infusion of valoctocogene roxaparvovec resulted in sustained and clinically relevant benefit in all the 13 patients treated. The study was published in the *New England Journal of Medicine*.

valoctocogene roxaparvovec was produced in BioMarin's gene therapy manufacturing facility located in Novato, California. As part of the marketing authorization application, FDA will also inspect the facility.

In addition to the BLA, the FDA has also accepted the premarket approval (PMA) application for companion diagnostic, an AAV5 total antibody assay intended as a companion diagnostic test for valoctocogene roxaparvovec. It consists of a simple blood test to help identify patients most likely to respond to AAV5-based gene therapy. BioMarin implemented the test in multiple clinical studies evaluating valoctocogene roxaparvovec treatment of hemophilia A patients without antibodies to AAV5. The assay is produced by ARUP Laboratories, a national reference laboratory of the University of Utah.

Dr Hank Fuchs, President of Global R&D at BioMarin commented:

"Valoctocogene roxaparvovec has the potential to be the first gene therapy approved in any type of hemophilia and the acceptance of this application and its priority review status marks a significant milestone for gene therapies in general and for the hemophilia community specifically".

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Expert Pick

Biomarin's treatment for adults with severe hemophilia A – valoctocogene roxaparvovec – has now been accepted for expedited review in both Europe and the US. At the end of 2019 EMA validated the company's Marketing Authorization Application, with accelerated assessment starting in January 2020. Now the application has also be accepted by FDA under Priority Review, with a PDUFA action date of 21 August 2020. Also undergoing regulatory review is a companion diagnostic developed by the company, intended to identify the estimated 20% of patients unlikely to respond to treatment due to pre-existing immunity to AAV5. A recent New England Journal of Medicine report of 3 years' follow-up data in patients treated with a single infusion of valoctocogene roxaparvovec demonstrated sustained improvements in bleeding events and prophylactic factor VIII use. The outcome of the reviews in both Europe and the US will therefore be anticipated eagerly by patients and the company alike. – Richard Philipson

CD229-TARGETING CAR-T THERAPY OFFERS HOPE TO MULTIPLE MYELOMA PATIENTS

Researchers at the University of Utah have developed CD229-specific CAR-T cells against multiple myeloma (MM), a plasma cell malignancy which is the second most common hematologic malignancy worldwide.

Although there are several treatment options available, MM remains an incurable disease. CAR-T therapy has emerged as an effective approach for a number of other hematologic malignancies and recent results from B-Cell Maturation Antigen (BCMA)-targeting CAR T-cell clinical trials in MM showed impressive overall response rates. However, the durability of these responses was limited and even patients with initial complete responses eventually relapsed.

In the present study published in *Nature Communications*, Dr Djordje Atanackovic and team have developed a CAR-T cell therapy against CD229, a surface antigen which is expressed universally in MM patients.

Data showed that the CD229 CAR-T cells were highly active *in vitro* and *in vivo* against MM plasma cells, memory B cells, and MM-propagating cells. This implies that the therapy has the potential to eliminate not only terminally differentiated MM plasma cells, which are also targeted by BCMA CAR-T cells, but also memory B cells, a potential reservoir for clonotypic MM cells and MM-propagating cells.

Researchers are hopeful that this broader nature of targeting of MM cells could lead to more robust and durable clinical responses.

Ones to Watch

CD229: NOVEL TARGET FOR MULTIPLE MYELOMA TREATMENT

Researchers at the Huntsman Cancer Institute in Utah have identified CD229 as a target for multiple myeloma. CAR-T cells targeting CD229 administered to mice were able to kill myeloma cells in vivo. There are a number of notable companies developing T cell therapies targeted to BCMA for multiple myeloma, including bluebird and J&J. However, a potential setback of BCMA targeted T cells is that they may not wipe out more primitive myeloma cells that are capable of sustaining malignancy, which may lead to relapse in patients. CD229 targeted CAR-T cells have been shown to target both multiple myeloma cells and their more primitive parent cells, suggesting that targeting CD229 may allow for development of therapies with improved durability.

–Mark Curtis

LYSOGENE RECEIVES FAST TRACK DESIGNATION FOR ITS GENE THERAPY OF MPS IIIA

The FDA has granted Fast Track Designation to Lysogene's LYS-SAF302 program for the treatment of mucopolysaccharidosis Type IIIA (MPS IIIA/ Sanfilippo A). The Fast Track program was introduced to accelerate the review of new drugs that have the potential to address serious medical diseases. A product that receives this designation is eligible for more frequent interactions with FDA, potential eligibility for accelerated approval, priority review, and rolling BLA review.

MPS IIIA is a rare and lethal inherited neurodegenerative lysosomal storage disorder caused by mutations in the *SGSH* gene, which encodes an enzyme called Heparan-N-sulfatase necessary for heparan sulfate recycling in cells. Lack of SGSH results in a build-up of sugars in the body, particularly the brain, leading to severe neurodegeneration. It affects approximately 1 in 100,000 newborns and currently there are no treatments available.

Lysogene's LYS-SAF302 is an AAV10-mediated gene therapy designed to replace the faulty *SGSH* gene with a functional copy of the gene in the brain. Currently it is being investigated in a Phase 2/3 trial, AAVance, to assess the efficacy of one-time delivery of LYS-SAF302 in improving or stabilizing the neurodevelopmental state of MPS IIIA patients. The trial which Is expected to complete in 2022 will enrol 20 patients at eight sites in the US and Europe. 17 patients have been treated out of the total of 20.

Marie Deneux, Lysogene's Chief Regulatory Officer commented:

"This Fast Track designation demonstrates the regulators' sustained interest in Lysogene's cutting edge in vivo *gene therapy program.*

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LYS-SAF302 has previously received Orphan Drug Designations for the treatment of MPS IIIA in the European Union in 2014 and in the US in 2015, as well as Rare Pediatric Disease Designation in the US. In the complex field of gene therapy for neurodegenerative diseases, a continued communication with FDA is essential."

Ones to Watch

Lysogene continues to make good progress with its AAVrh10 vector, which expresses the gene encoding the enzyme heparan-N-sulfamidase, for the treatment of mucopolysaccharidosis type IIIA (MPS IIIA, also known as Sanfilippo syndrome). This is a second generation vector, which has increased potency and efficacy in a mouse model of MPS IIIA when compared with the first generation vector. It is hoped that direct delivery of the vector to the CNS will provide greater treatment benefit than other routes of administration, as the vector can be transported along neuronal connections to distal sites, and the secreted enzyme can undergo both anterograde and retrograde transport from the injection site. In a previous small study conducted using the first generation vector in 4 children, encouraging signs of improvement were noted, and the potential importance of the treatment is clearly recognised by FDA, with its approval of Fast Track Designation.

– Richard Philipson

BRISTOL-MYERS SQUIBB'S CAR-T THERAPY RECEIVES FDA'S PRIORITY REVIEW

Bristol-Myers Squibb has announced that the FDA has accepted the Priority Review of its BLA for lisocabtagene maraleucel (liso-cel), the company's autologous anti-CD19 CAR-T therapy for treating adult patients with r/r large B-cell lymphoma. The FDA has set a Prescription Drug User Fee Act (PDUFA) goal date of August 17, 2020.

The BLA, submitted by Juno Therapeutics, a wholly owned subsidiary of Bristol-Myers Squibb Company, is based on the safety and efficacy results from the TRANSCEND NHL 001 trial, evaluating liso-cel in 268 patients with r/r large B-cell lymphoma, including diffuse large B-cell lymphoma (DLBCL), high-grade lymphoma, primary mediastinal B-cell lymphoma and Grade 3B follicular lymphoma.

Data presented at the 61st American Society of Hematology meeting showed durable clinical activity and the study had met all primary and secondary efficacy endpoints.

The trial was conducted in 342 patients; 268 patients received liso-cel at 3 different doses and 24 patients received nonconforming product. Primary endpoints were treatment-emergent adverse events (TEAEs) and overall response rate (ORR). Among patients evaluable for efficacy (n=255), ORR was 73% and the Clinical Response rate was 53%. Progression-free survival (PFS) after liso-cel infusion was substantially longer than PFS from the immediate prior therapy. The therapy also showed favorable safety profile in the patients.

Liso-cel was previously granted Breakthrough Therapy and Regenerative Medicine Advanced Therapy designations by the FDA for r/r aggressive large B-cell non-Hodgkin lymphoma and Priority Medicines (PRIME) scheme by the EMA for r/r DLBCL.

Dr Stanley Frankel, SVP of the company's Cellular Therapy Development commented:

"There remains a critical need for additional therapies in large B-cell lymphoma, particularly for relapsed or refractory patients," said "Based on the TRANSCEND NHL 001 data, liso-cel has the potential to expand treatment options for those affected by this aggressive blood cancer who did not respond to initial therapies or whose disease has relapsed. This BLA acceptance and Priority Review designation is an important step as we work to improve treatment for these patients in need."

AVROBIO'S GENE THERAPY PROGRAMS PROVIDE HOPE

AVROBIO has provided updates on two of its gene therapy programs and it's recently launched plato™ gene therapy platform.

AVR-RD-04 for cystinosis

Three-month data obtained from the first patient dosed in the investigator-sponsored Phase 1/2 trial of the company's AVR-RD-04 investigational gene therapy for cystinosis has shown improvements in early measures of efficacy compared to baseline. There have been no reports of safety events attributed to the investigational drug product.

Cystinosis is caused by the accumulation of cystine in lysosomes leading to crystal formation in the lysosomes of cells, causing debilitating symptoms including corneal damage, difficulty breathing and kidney failure, often leading to a shortened lifespan.

Three months following administration of AVR-RD-04, the first patient had a VCN of 2.0. VCN gives a measure of the average number of copies of the lentiviral-vector inserted transgene integrated into the genome of a cell and can be used to help assess the durability of a gene therapy.

AVR-RD-01 for Fabry disease

The company has also provided positive interim data that favors the therapeutic potential of its lentiviral-mediated gene therapy (AVR-RD-01) in treating Fabry disease, an X-linked, rare lysosomal storage disorder caused by a deficiency of alpha-galactosidase A enzyme.

AVROBIO is conducting two lentivirus-mediated gene therapy trials of AVR-RD-01; an investigator-sponsored Phase 1 study and the AVROBIO-sponsored Phase 2 trial (FAB-201).

The therapy uses patient's stem cells and they are genetically modified by adding a functional copy of the *GLA* gene coding for alpha-galactosidase A (AGA). The modified cells are then infused back into the patient via a one-time procedure. The procedure expects to achieve a sustained increase in the enzyme, with the potential to significantly improve patient outcomes and eliminate costly lifetime biweekly intravenous infusions of enzyme replacement therapy (ERT).

The Phase 1 study evaluates the safety of AVR-RD-01 Fabry disease patients who have

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been treated with standard of care ERT for at least 6 months prior to receiving the gene therapy. The Phase 2 trial is evaluating the efficacy and safety of the gene therapy in eight to twelve patients who have never received ERT (treatment-naive). Five patients were dosed in the Phase 1 trial and four patients in the Phase 2 trial.

Data showed that VCN continue to be stable at 32 months following AVR-RD-01 treatment for the first patient in the Phase 1 trial, suggesting successful engraftment, which is critical to the long-term success of investigational *ex vivo* lentiviral gene therapies. The VCN data trend was generally consistent across the seven other Phase 1 and Phase 2 trial participants out six to 24 months.

The first three AVR-RD-01 Phase 2 patients entered the study with minimal endogenous enzyme activity. At 9, 12 and 18 months after dosing, data from these three patients indicate sustained increased leukocyte and plasma enzyme activity, suggesting that they are now producing an endogenous supply of functional AGA enzyme. This enzyme is essential for breaking down globotriaosylceramide (Gb3) in cells; without it, a toxic metabolite, lyso-Gb3, may accumulate, potentially causing cardiac and kidney damage and other symptoms.

The effect of gene therapy on the metabolite levels were also evaluated and a significant reduction in lyso-Gb3, an unwanted metabolite that accumulates in the cells of Fabry disease patients, was observed. The gene therapy was also well tolerated in both the trials.

Three of the five Phase 1 patients have discontinued ERT and all three remain off ERT for 6, 14 and 15 months. The Phase 1 trial is fully enrolled. AVROBIO continues to actively enroll the Phase 2 trial in Australia, Canada and the US.

plato™ gene therapy platform

AVROBIO has also reported on the clinical debut of its plato™ gene therapy platform, a new closed, automated vector system for $CD34^+$ cell-based therapies developed to enable worldwide commercialization of the company's gene therapies. Data showed improved enzyme activity, transduction efficiency and VCN in drug product manufactured using plato compared with drug product produced using the academic platform, as well as higher *in vivo* enzyme activity at 1 month in the first patient treated with plato, as compared to other patients treated using the academic platform.

THYMUS ATLAS COULD BE A VALUABLE TOOL IN DEVELOPING NOVEL T-CELL BASED THERAPIES

Using single-cell RNA sequencing, researchers have mapped thymus, the organ responsible for producing T cells across the human life span. This atlas could help researchers understand how thymus cells change over time and how T cells develop, opening the door to new treatments for cancer and autoimmune disease.

The collaborative research conducted by scientists at the Wellcome Sanger Institute, Newcastle University, Ghent University and the University of Cambridge, have identified more than 50 different cell states in the human thymus. The thymus cell atlas is part of the Human Cell Atlas project, an international effort started in 2016 to map all cells in the human body as a resource for better understanding health and disease.

Using single-cell RNA sequencing, the teams analyzed about 200,000 individual cells from embryonic, fetal, children and adult thymic glands. Using computational methods, they identified where different cell types are found in the thymus and the states in which they exist. They also mapped out how various kinds of T cells develop from their precursors

in the fetal liver and the signals which differentiate immature immune cells to mature T cells.

In addition to providing the information about how the immune system develops to protect our body, findings from the study also shed light on improving T-cell based treatments such as CAR-T therapies. It could also be a valuable tool for developing therapies for diseases characterized by the improper development of T cells, such as severe combined immunodeficiency, or by the turning of immune cells against the body's own tissues, like Type 1 diabetes.

According to Dr Muzlifah Haniffa, a professor of immunology and dermatology at Newcastle University and a senior author of the study,

"With this thymus cell atlas, we are unravelling the cellular signals of the developing thymus, and revealing which genes need to be switched on to convert early immune precursor cells into specific T cells. This is really exciting as in the future, this atlas could be used as a reference map to engineer T cells outside the body with exactly the right properties to attack and kill a specific cancer—creating tailored treatments for tumors."

Licensing agreements & collaborations

GENPREX COLLABORATES WITH THE UNIVERSITY OF PITTSBURGH FOR DIABETES GENE THERAPY

Clinical-stage gene therapy company Genprex has announced that it signed an exclusive license agreement with the University of Pittsburgh for a diabetes gene therapy that may have the potential to cure Type 1 and Type 2 diabetes, which together currently affect approximately 30.3 million people in the US, or 9% of the US population. Diabetes is the seventh leading cause of death in the US.

The technology was developed by Dr George Gittes at UPMC Children's Hospital of Pittsburgh. It works by reprogramming alpha cells in the pancreas into beta-like cells, restoring their function, thereby replenishing levels of insulin. The infusion process uses an endoscope and an AAV vector to deliver *Pdx1* and *MafA* genes to the pancreas. The proteins produced by these genes transform alpha cells in the pancreas into functional beta-like cells, which can produce insulin but are distinct enough from beta cells to evade the body's immune system.

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The diabetes gene therapy has been tested *in vivo* in mice and nonhuman primates. In studies of diabetic mice, the gene therapy approach restored normal blood glucose levels for around 4 months. According to Dr Gittes, the duration of restored blood glucose levels in mice could translate to decades in humans. Following preclinical studies, Dr Gittes and his team plan to begin a Phase 1 clinical trial in diabetic patients, which could be the first gene therapy tested in humans for diabetes.

Genprex will add this technology to its research and development pipeline, diversifying its portfolio and expanding its clinical development programs. The company will continue its focus on developing its immunogene therapies for cancer, including Oncoprex™ immunogene therapy, its lead drug candidate for non-small cell lung cancer, in parallel with development of the new diabetes gene therapy.

Dr Gittes commented:

"One of the biggest advantages of this gene therapy is that it could eliminate the need for insulin replacement therapy for diabetic patients. Lifting this huge burden for the millions of patients who must continuously monitor blood glucose levels and inject insulin daily would be a breakthrough in modern medicine. This therapy has the potential to truly disrupt the diabetes market."

IMMATICS PARTNERS WITH GSK TO DEVELOP NOVEL ADOPTIVE CELL THERAPIES

Immatics Biotechnologies, a clinical-stage biopharmaceutical company focusing on developing T-cell redirecting cancer immunotherapies, has entered into a strategic collaboration agreement with GSK to develop novel adoptive cell therapies targeting multiple cancer indications.

The companies will collaborate on the identification, research and development of next-generation T-Cell Receptor (TCR) Therapeutics with a focus on solid tumors. The parties will initially focus on autologous T-cell therapies with the option to add allogeneic cell therapies. The therapies will be developed using two of Immatics' TCR-T programs, ACTallo® approach and XPRESIDENT® technology.

Under the terms of the agreement, Immatics will receive an upfront payment of 45 million pounds (~$50 million) for two initial programs and is eligible to receive over $550M in development, regulatory and commercial milestone payments for each product as well as additional royalty payments. GSK obtains an option to select additional target programs to include in the collaboration. For each additional program, Immatics is entitled to option, milestone and royalty payments.

Immatics will primarily be responsible for developing and validating the TCR Therapeutics up to designation of a clinical candidate. GSK will be responsible for further worldwide development, manufacturing and commercialization of the TCR Therapeutics with the possibility for Immatics to co-develop one or more TCR Therapeutics including the conduct of the first-in-human clinical trial upon GSK's request.

Harpreet Singh, CEO of Immatics commented:

"We are delighted to enter into this strategic collaboration with GSK – a partner who is already committed to adoptive cell therapies and TCR-T approaches. By combining Immatics' world-leading target and TCR discovery platforms with GSK's advanced manufacturing, development capabilities and a commitment to next-generation TCR-T technologies, both companies are joining forces to enable the development of effective novel therapies for cancer patients with high unmet medical need."

Ones to Watch

Immatics has built an impressive list of partnerships over the last several years centered on its TCR discovery platform and translational capabilities, including Amgen, Celgene, and Genmab. This past month, Immatics announced yet another collaboration, this time with GSK. Under the terms of the deal GSK has selected intracellular targets from Immatics's portfolio of 180 TCR targets. While the deal gives GSK exclusive access to those targets, it also leaves the door open for Immatics to co-develop the assets along with GSK and contribute to translational research and potentially early clinical development as well.

– Mark Curtis

SIRION BIOTECH JOIN HANDS WITH INPROTHER FOR DEVELOPING CANCER IMMUNOTHERAPY

SIRION Biotech, a biopharmaceutical company developing viral vector-based gene delivery technologies for gene and cell therapy has partnered with a Danish start up, InProTher Aps, with a broad licensing agreement which includes coverage of SIRION's adenovirus technologies to cancer vaccines encoding Endogenous Retrovirus (ERV)-derived antigens for active immunotherapy.

In addition, the companies have agreed to the assignment of ownership rights in a patent application for an adenoviral vector capable of encoding a virus-like particle (VLP), which displays an inactive immune-suppressive domain (ISD). This vaccine shows an improved immune response from either or both of the response pathways initiated by CD4 T cells or CD8 T cells. SIRION and InProTher have been collaborating for over 5 years in the fields of HPV vaccine development and ERVs.

InProTher is an immunotherapy company that is applying adenovirus technologies both for cloning large nucleic acids and increasing the yield of replication-incompetent adenoviruses. The goal is to develop the world's first adaptive immune therapy capable of targeting immunosuppressive genes of ancient retroviruses that normally are dormant in the human genome. The retroviral genes are reactivated in cancer and essential for tumor development. InProTher's proprietary combination of novel technologies is designed to break tolerance to this unique antigen family, thus providing broad anti-cancer efficacy.

As part of the agreement, SIRION Biotech will receive shares of InProTher Aps, as well as representation on their Board of Directors. The parties have also agreed on milestones and royalties should InProTher's developments pass clinical development hurdles.

Dr Peter J. Holst, Interim CEO and CSO of InProTher ceommented:

"InProTher's proprietary combination of novel technologies is designed to break tolerance to this unique antigen family, thus providing broad anti-cancer efficacy. SIRION has been a creative, loyal and responsive partner over the years, and their adenovirus technology is ideally suited to our needs."

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Finance

SONOMA BIOTHERAPEUTICS RAISES $40 MILLION IN SERIES A FUNDING

Sonoma Biotherapeutics, a privately held company developing regulatory T cell (Treg) therapies for autoimmune and degenerative diseases, was launched in South San Francisco and Seattle. Additionally, it raised $40 million in its Series A financing. The company was founded by four pioneers of Treg cell biology and cell therapy and is financed by a syndicate of leading biotech investors. By bringing together next-generation research, development and manufacturing capabilities in cell therapy and genetic engineering, Sonoma aims to advance regulatory T cell therapy in autoimmune and degenerative diseases.

The goal of Treg therapy is to restore a state of self-tolerance by halting harmful inflammatory responses in autoimmune diseases such as rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis, along with degenerative diseases including amyotrophic lateral sclerosis (ALS) and Alzheimer's.

Tregs have been proven to play key roles in many of these conditions. These cells' natural ability to migrate to inflamed tissues and control harmful immune responses make them ideal for treating a range of conditions. In addition, the ability to engineer Treg cells to target specific disease-causing antigens reduces the potential for unwanted systemic effects. The role of Tregs in tissue maintenance and repair offers the potential for effective, durable and restorative treatments.

Jeffrey Bluestone, founder and CEO of Sonoma commented:

"With this team and our assembled expertise and technologies, we are in an ideal position to move adoptive cell therapy beyond cancer, to establish safe, effective and long-lasting treatments for a range of conditions where current drugs and biologics are simply not good enough. As the immune system's master regulators of protecting the body against self-destruction, Treg cell therapy is perhaps the ideal means to shut down unwanted immune reactions and provide meaningful treatment for patients."

In this regard, Sonoma Biotherapeutics has entered into a strategic partnership with Lyell that provides both parties with access to technologies and know-how to enhance the durability, stability and specificity of cell therapies in their respective indications of focus. This partnership will further enable Sonoma's rapid translation of Treg therapies from target identification and discovery, through preclinical and clinical development.

AUDENTES TO INVEST $109 MILLION TO BUILD GENE THERAPY MANUFACTURING FACILITY

Audentes Therapeutics, an Astellas company focused on developing AAV-based genetic medicines, has announced its plans to invest $109 million to build a new 135,000 square foot, state-of-the-art gene therapy manufacturing facility in Sanford, North Carolina.

The initial phase of the capital investment is planned to occur over approximately 18 months, and the plant is expected to be operational in 2021. The remaining investment will take place over two additional planned expansion phases. The company plans to create over 200 new jobs in Lee County.

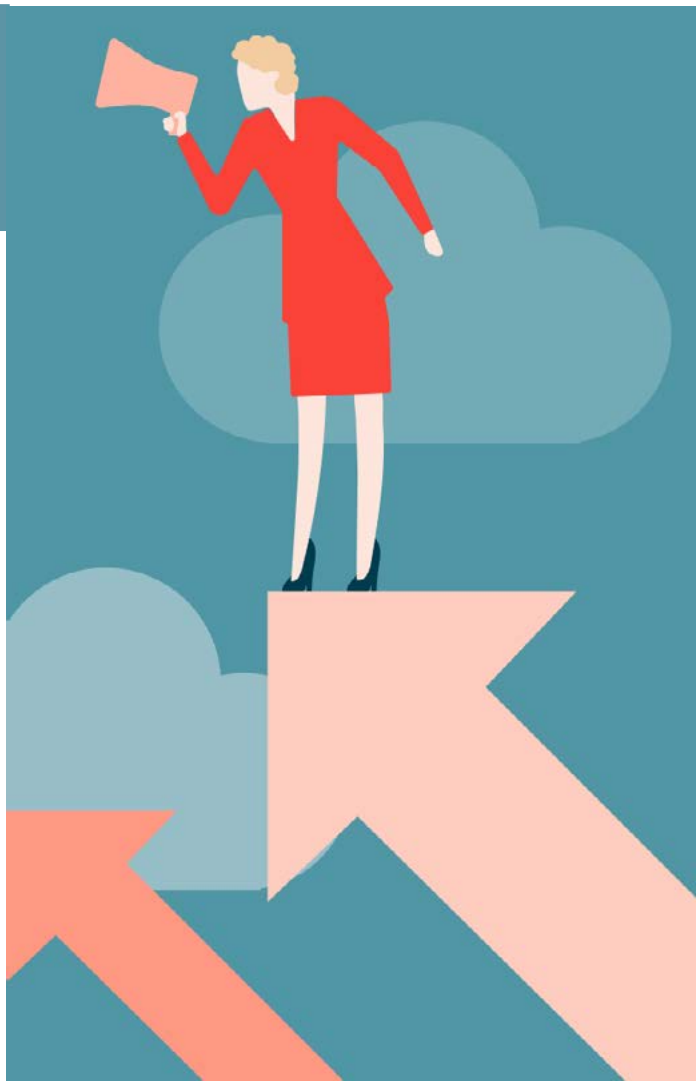
Audentes is specialized in developing gene therapies for patients with rare neuromuscular diseases using its AAV gene therapy technology. The company mainly uses three modes of operation: gene replacement, vectorized exon skipping and vectorized RNA knockdown.

Movers & Shakers

DR GÉRALDINE HONNET JOINS SENSORION AS CMO

Sensorion, a gene therapy company developing therapeutics for hearing loss disorders, has appointed Dr Géraldine Honnet as its Chief Medical Officer.

Dr Géraldine Honnet has over 20 years of experience in the pharmaceutical sector and she joins Sensorian from Généthon, where she spent 9 years as CMO. During her time at Généthon, she was responsible for overseeing international gene therapy clinical trials in multiple rare diseases and for the development of its pipeline from pre-clinical to registration, managing Medical Affairs, Clinical Development, Clinical Operations and Regulatory affairs departments. Prior to that, Dr Honnet worked in Transgene where she was responsible for developing gene therapy products for infectious diseases. Before joining Transgene, she was a Medical Director at Parexel International and International Project Manager for Janssen-Cilag (Johnson & Johnson).



- Written by Dr Applonia Rose, *Cell and Gene Therapy Insights*

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CELL & GENE THERAPY INSIGHTS

INNOVATOR INSIGHT

Key steps to industrialize your process and de-risk your pathway to commercialization

Behnam Ahmadian Baghbaderani

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In order to successfully manufacture clinical-grade GMP products, it's important to take a step by step approach towards their development.

The first step, what we term the manufacturability assessment or diagnosis to de-risk manufacturing, focuses on establishing the baseline process, and identifying the major risks involved in our clients' manufacturing process considering GMP guidelines. This step will help to define the focus and scope of the next step (i.e. development step).

Once there is a good understanding of the scope, then we move to development step and designing experiments centered around process optimization based on the manufacturing design considerations and critical quality attributes. We focus on different challenges, for example scale up or scale out, efficiency and yield, optimization of unit operations (for example, initial starting cell isolation), sensitivity and robustness of the process, development of appropriate analytical methods and assays, implementing appropriate in-process control characterizations in the process, and cell harvest and banking strategy. In addition, it's important to evaluate the raw materials involved in the processes to make sure they fit the GMP guidelines and compliance.

We include our Manufacturing Science and Technology Team (MSAT) early on, even during development, to make sure this understanding of manufacturing design considerations is properly implemented into the process development strategy. Once the manufacturing process is robust and reproducible, we work with our MSAT to transfer this process into a GMP manufacturing setting.

Some of the best practices we recommend in the development of GMP manufacturing processes, to meet clinical and commercial needs, are highlighted in this Figure 1. We focus on minimizing open manual unit operations, optimizing sub-optimal unit operations, implementing appropriate analytics, in-process controls, and in-process decision making points to better understand the process parameters in relationship with quality attributes of the final product and build the relationship between the critical process parameters (CPP) and the critical quality attributes (CQA). And depending on the application, whether it's allogeneic or autologous, we focus on scale up or scale out. Automation is one of our major focuses during the development stage as well as process characterization depending on the phase of application.

CASE STUDY 1: MANUFACTURING OF CLINICAL-GRADE CELL THERAPY PRODUCTS FROM IPSCS

This first case study pertains to a directed differentiation process starting from human induced pluripotent stem cells (iPSCs), which is a highly complex process that requires very accurate control of each step. There are several factors involved in the manufacturing process including the quality of starting material, cellular microenvironment, cell–cell interaction, signaling factors, and other important parameters.

The entire process can be divided into four major steps:

- Manufacturing of GMP-grade iPSCs;
- Expansion of iPSCs to create sufficient number of cells prior to the differentiation;
- Directed differentiation process;
- Characterization and testing of the final product (and intermediate materials) depending on the direct differentiation process.

For this particular process, some of the best practices we have implemented during our process development and optimization steps include the development of a robust cell culture system for generation of human iPSCs and expansion of pluripotent stem cells including iPSCs and human embryonic stem cells (hESCs) as well as development of bioreactor protocols for expansion pluriptent stem cells in 3D.

Our focus has been on establishing a high quality iPSC using our GMP manufacturing process and to that end, developing a comprehensive iPSC characterization platform. Having established both 2D and 3D bioreactor systems, this enabled directed differentiation of iPSCs into different lineages. Depending on the application, different strategies can be taken to optimize the manufacturing process, some of which are highlighted below.

FIGURE 1

Best practices in process development and characterization of cell and gene therapy products.

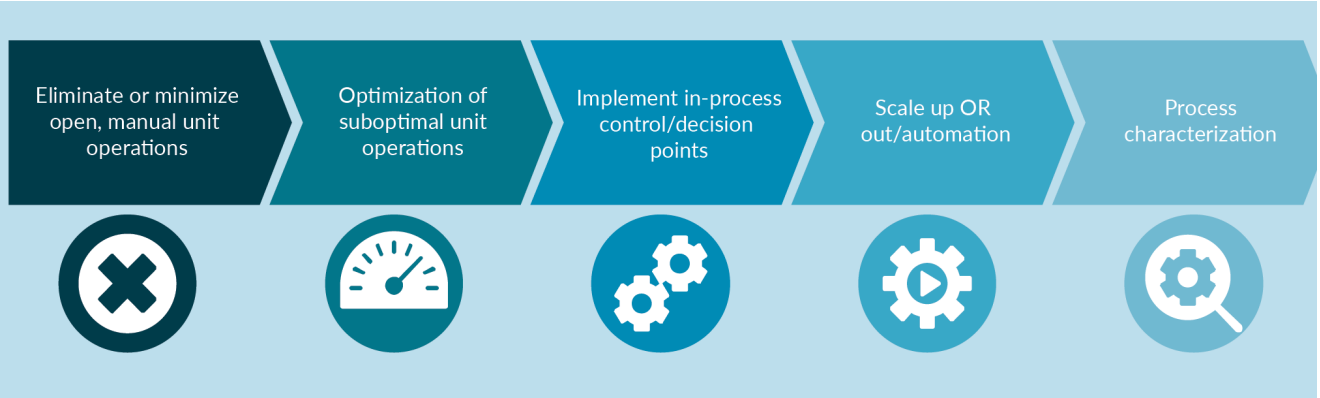

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TABLE 1

Lonza generated multiple fully characterized iPSC lines.

Assay	Objective	Evaluation criteria	Category	Tested iPSC line
Pluripotency markers	Identity and purity	SSEA-4 >70%, Tra-1-60 >70%, Tra-1-81 >70%, Oct3/4 >70%; Purity: CD34 <5%	Release assay	All lines
Karyotype analysis	Safety	46, XX or 46, XY	Release assay	All lines
Mycoplasma testing	Safety	Negative	Release assay	All lines
Sterility testing	Safety	Negative	Release assay	All lines
Endotoxin testing	Safety	Standard QC release (<0.5 EU/ml)	Release assay	All lines
Vector clearance	Safety	No trace of episomal plasmid DNA detected	Release assay	All lines
STR genotyping	Purity and identity	STR Profile of starting population and iPSC line are identical	Release assay	Al lines
Cell count and viability	Viability	% viability >50; minimum cell number/vial	Release Assay	All lines
Viral panel testing	Safety	Standard MCB Release Panel	Release Assay	LiPSC-GR1.1
Characterization assays				
EB formation	Identity and potency	Detection of at least one marker per germ layer	FIO*	All lines
Gene array analysis	Identity	Clustering with established hPSCs	FIO*	All lines
Colony morphology	Identity and purity	Characteristic morphology of culture/colonies; lack of sponta-neously differentiated cells	FIO*	All lines
Post-thaw plating	Thawing efficiency and viability	20+ colonies/vial (after 7 days or 50% confluency)	FIO*	All lines
HLA typing	Identity	HLA-A, B, C, DRB1 and DQB1Type	FIO*	All lines
CGH+SNP microarray	Identity	Amplifications and/ or deletions of specific genes	FIO*	LiPSC-GR1.1 and ER2.2
Whole genome sequencing	Identity	HiSeq X Human Whole Genome Sequence	FIO*	LiPSC-GR1.1 and ER2.2

(Baghbaderani *et al.* 2016; *Stem Cell Reviews and Reports*).

L7™ cell culture system: a defined & cGMP compliant cell culture system for manufacturing of human PSCs

In order to establish a robust and reproducible cell therapy manufacturing process, it is essential to use a high quality starting material, in this case iPSCs, and with that in mind we established a GMP manufacturing process using a defined and cGMP-compliant L7™ cell culture system. This has enabled the generation of iPSCs under GMP guidelines, the details of which have been published previously [1].

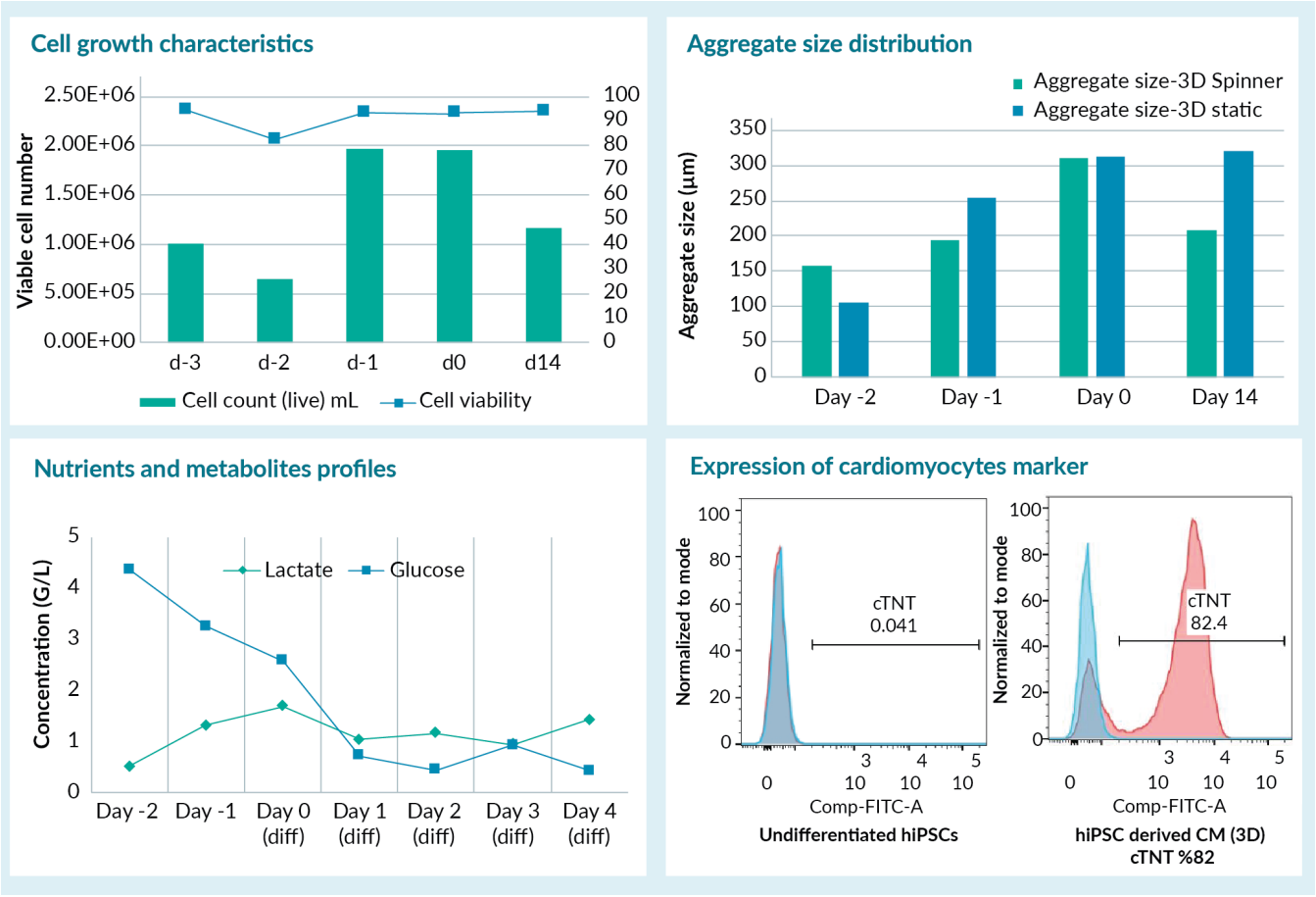
Our group has extensively focused on the development of a series of analytical methods that are required for release and characterization of iPSCs, as shown in Table 1 [2].

In the next step we have shown that iPSCs generated in our cell culture system and manufactured under GMP can be used in the directed differentiation process for different clinical applications and hold the potential to differentiate into all three main lineages: ectoderm, mesoderm and endoderm.

To help address some of the current manufacturing challenges associated with the use of traditional, open and manual 2D unit operations (which may lead to major quality, quantity and efficiency concerns), we considered it essential to establish a computer-controlled 3D bioreactor system. In order to develop a 3D bioreactor based process, we focused on

FIGURE 2

Directed differentiation process optimization in 3D culture for cardiomyocytes.



several important process parameters, including inoculation condition, hydrodynamics of the bioreactor, process control, expansion, feeding strategies.

We took a step by step approach towards the scale up of the iPSC-derived processes, from 2D cell culture systems into 3D suspension; first moving into a spinner flask, and then into a larger scale computer controlled bioreactor.

We demonstrated our approach towards scale up and establishing a 3D process by focusing on iPSC differentiation into cardiomyocytes using a protocol reported in the literature [3]. First, we optimized the differentiation process in 2D conditions before moving to 3D. Next, we optimized the cell culture and directed differentiation conditions in spinner flask to enable the transition of the process into a 3D environment. We demonstrated that the iPSCs derived under GMP conditions can be differentiated into cardiomyocytes in 3D as supported by cellular characterization studies. We also evaluated various process parameters, including aggregate size distribution in 3D, as well as cell metabolite and nutrient composition and used these parameters to optimize the process and control critical quality attributes of the process. Some of the process variables and target criteria including the cell viability, key surface marker expression are exhibited in Figure 2.

From a scale-up perspective, we then focused on incorporating novel bioreactor technologies into the manufacturing process and direct differentiation moving from small uncontrolled 3D, spinner flasks into larger scale computer controlled bioreactors. For instance, we used computational fluid dynamics (CFD) modelling in scale up [4]. Using this method we took into consideration the geometry of the

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vessel, and more accurately, evaluated the hydrodynamics of the bioreactor, allowing scale up of the process from small spinner flasks into the larger scale 3D bioreactor.

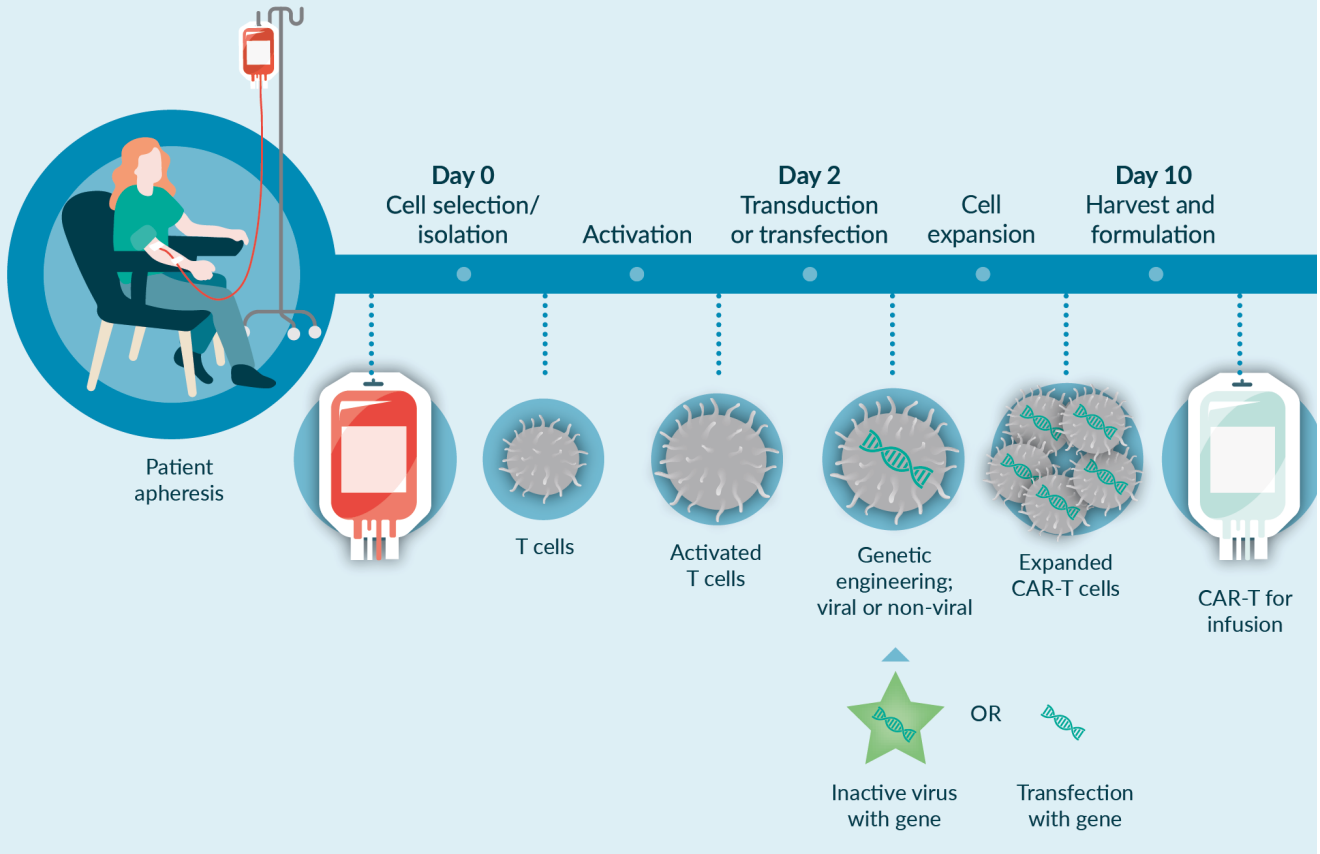
In conclusion, we have been able to show that iPSCs generated under cGMP conditions could be differentiated into all three lineages successfully in 2D and 3D, and these processes can be established and scaled up as needed depending on the mode of culture [3]. Our most recent publication details the CFD modelling we've used for the development of scalable cell therapy manufacturing process [4].

CASE STUDY 2: MANUFACTURING OF CLINICAL-GRADE CAR-T PRODUCT

For a CAR-T-based application, the manufacturing process is complex involving many unit operations in different combinations. A current challenge with this type of autologous cell manufacturing process is that many of these unit operations are open and manual, and use uncontrolled culture conditions, increasing risks of contamination and driving up costs of manufacturing. To fundamentally address these challenges, automation may be a feasible approach to replace open manual steps with more automated approaches. The automation can be a modular (i.e., focusing on one or more unit operations of the manufacturing process, for instance cell isolation step) or end-to-end approach (i.e., focusing on all of the unit operations in the entire manufacturing process from isolation to fill and finish), the latter of which would be our preference. Some of the unit operations that may need specific attention include apheresis product processing and cell isolation, ficollation or density based selection, magnetic selection of specific cell

FIGURE 3

Case study 2: manufacturing of clinical-grade CAR-T product.



types, and/or buffer exchange into culture medium. For instance, Cell isolation is the first step in the manufacturing process, and is often a manual unit operation. Our recommended approach is to move this manual unit operation into automated closed system modalities to remove the inherent risks associated with the operator dependent manipulations and reduce labor-intensive procedures. However, the current most prevalent approach to automation is modular, owing to a lag in development of appropriate end-to-end, fully closed, automated platform technologies.

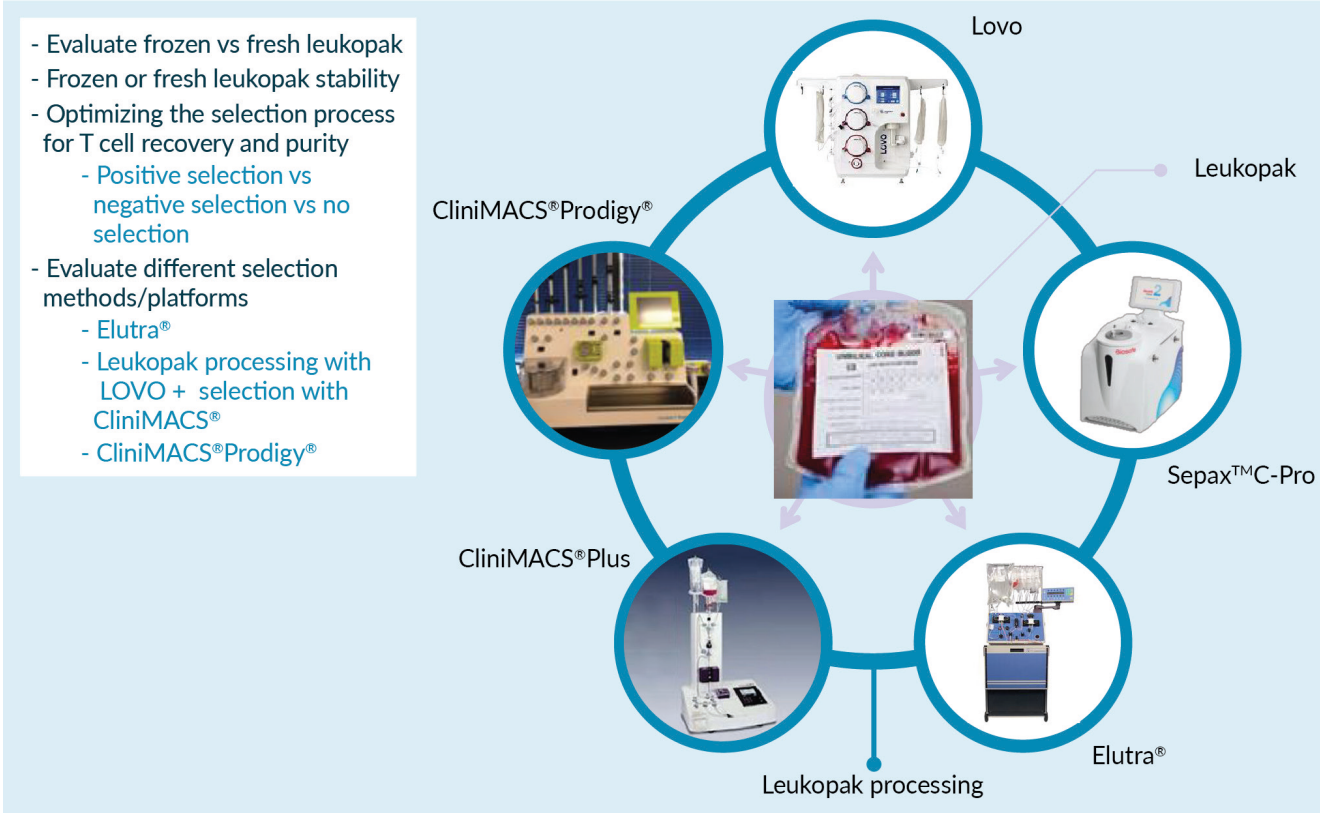
In addition to automation, process optimization and elimination or reduction of suboptimal unit operations with more robust and optimized process steps require specific attention. Figure 3 highlights the main steps of a typical CAR-T manufacturing process, from receiving starting material leukopaks, onto cell isolation, activation, transduction, expansion, harvest, and final fill and formulation. Depending on the status of the baseline process, and considering the target criteria for the process and these unit operations, there could be several opportunities for optimization across the workflow.

In order to optimize the unit operations (for example, cell isolation), one can run various scenarios and perform a wide range of optimization studies. These could include the evaluation of fresh versus frozen starting materials, the selection strategy (i.e. positive selection or negative selection), and also using different technologies and platforms during the isolation as highlighted in Figure 4.

Depending on the approach we take from automation perspective, and technologies that are implemented in the process, the outcome of the process development and target criteria (e.g., for process recovery) could be different. The decision on the best process development

FIGURE 4

Leukopak processing and selection of T cells.



Unit operations optimization.

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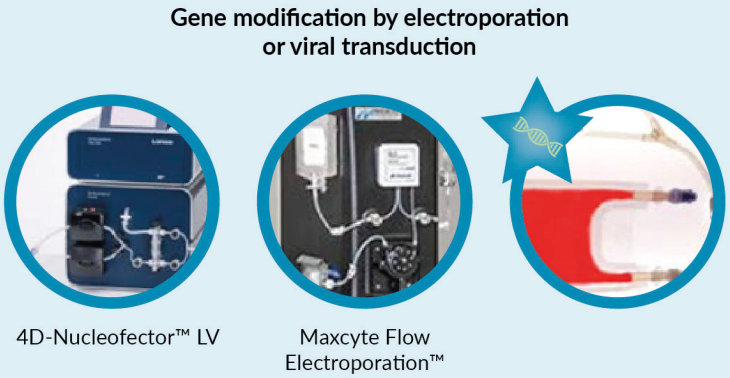
FIGURE 5

Gene delivery.

- Optimize for efficient CAR transfection or transduction of T cells
- Evaluate different gene delivery platforms
  - 4D-Nucleofector™ LV unit
  - Maxcyte Flow Electroporation™
- Mode of gene delivery optimization
- Priming cytokine evaluation with goal of improving transfection
- Transduction medium optimization for better recovery of T cells



Unit operations optimization.

strategy must be based on a phase appropriate, risk assessment, and evaluation of quality, safety, reproducibility, productivity and cost considerations.

Gene delivery is also another critical unit operation in the manufacturing of a CAR-T product. There are two main strategies for gene delivery: viral transduction or non-viral transfection. For each strategy, different process optimization and technologies (see Figure 5) can be evaluated during the development and process optimization phase.

Different gene modification technologies can be used for gene editing step. Lonza 4D-LV nucleofection system has been used in different CAR-T applications, and we have performed optimization and scale up studies moving from Lonza 4D Nuecleofector system to our larger scale (LV Nuceleofector) automated system while using different starting materials and gene delivery systems.

When using viral methods for gene delivery, the optimization of viral modification system may focus on initial titration study (to understand the transduction efficiency) and then performing titration study using multiple experimental conditions (for example, different combinations of media to enhance the efficiency of transduction).

Perhaps one of the most important steps in the development of a commercially viable manufacturing process is to pay specific attention to process analytics, in-process control and in-process monitoring, and implementation of appropriate control strategy in the process. Some of the suggested in-process controls for CAR-T applications include understanding the composition of the starting material (Box 1), monitoring changes in the composition following isolation, activation, before and after transduction or transfection (depending on the method of gene editing).

We strongly recommend defining the process control strategy and defining the input and output of each unit operation to ensure adequate control of the manufacturing process. Some proposed process controls for CAR-T cell therapies include:

- CD3, CD4 and CD8% of the starting material (apheresis);
- Post-selection T cell recovery;
- Post-selection cell purity (CD3%) and phenotype (memory subsets);

- Post-selection Impurities (RBC, platelets, tumor cells);
- Transduction, transfection or transposition efficiency;
- Monitoring for memory phenotype, activation state and exhaustion markers during expansion;
- Cell count and cell viability throughout culture and downstream processing;
- Process residual clearance.

BOX 1

Characterization of starting material.

- Total nucleated cell count/WBC
- Cell size
- Starting material composition:
  - Diff count (lymphocytes, monocytes, granulocytes)
  - Hematocrit
  - T cells, B cells, NK cells, NKT cells
  - CD4/CD8 ratio – ratio can be re-adjusted to get defined CAR-T product
  - Memory phenotype
  - Tumor burden

In summary, to meet the growing demands as more cell and gene therapies move towards commercial-scale manufacture, it is important to implement proper process and assay development strategies, as well as using innovative technologies to establish a robust and reproducible automated closed system. Implementation of appropriate product release and characterization is going to be the key to maintaining the critical quality attributes of the final product. Finally, a phase appropriate development approach is needed to adjust the process and assay requirements for clinical and commercial applications.

FEATURING THE TOP 5 QUESTIONS FROM OUR LIVE WEBINAR AUDIENCE

You mentioned in your presentation a desire to move towards automating classically manual processes. But how do you go about automated density gradient processes for cell isolation?

At the moment there are a wide range of technologies available for automation of different unit operations including cell isolation step. First, it's important to understand what is the final product quality requirement, or for each unit operations what would be the input and output quality attributes considering the process parameters. For example, if you are performing a study to evaluate processing of the starting material, then you should have a good understanding of the final volume, concentration, cellular compositions requirements. Then, using a wide range of technologies that are available, for instance Lovo Cell Processing, Elutra® Cell Separation System, SepaxTM Cell Processing Instrument, you would need to focus on the outputs and the quality attributes, incorporate appropriate technology (if possible through side-by-side comparability testing), and eventually optimize the process.

What is the largest size currently of 3D bioreactor for iPSC differentiation?

Currently most of the iPSC-based therapies are in early clinical stage, and based on my experience and understanding, the majority of applications may not need scale beyond 3 L bioreactors. We have actually worked with 50 L bioreactors for iPSC-based applications and I understand that as we move forward into clinical phases, larger scale bioreactor processes may be needed. So, we need to work based on a phase appropriate approach during the process development step and pay specific attention to bioreactor configurations, the scalability of a specific platform,

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scale, and clinical versus commercialization demands to properly choose the appropriate bioreactor technology and scale for each phase.

What do you see as the biggest roadblocks for cell & gene therapies to transfer from the lab to the market?

In my opinion, there are multiple challenges including GMP compliance (for instance, availability of GMP compliant materials) and understanding the critical steps of the process, and establishing a strong relationship between critical materials, critical process parameters, and critical quality attributes.

Another challenge is related to the assays that need to be implemented into the process. Depending on the applications, it is important to have a clear view of the type of assays and analytical methods that can be available to properly characterize starting materials, intermediate products, and also the final products.

Also, when these processes move to clinical manufacturing, harmonization and standardization of the approach for implementing new technologies and development of manufacturing processes need to be addressed.

In your opinion what do you think of the current state of assays we have available? Do you see this as being quite a significant bottleneck?

Absolutely! We know that the field of cell and gene therapy is growing, and also the need to develop new emerging assays is evolving. There are some characterization tools, analytical methods, that can be used, for instance flow cytometry based assays, molecular biology based assays, or cell-based assays. But the question we would need to answer is how we could incorporate emerging assays and technologies, for example whole genome sequencing, into manufacturing platforms.

At the moment, we proposed to use some of these assays in the characterization of starting materials, for instance iPSCs. If these methods could be qualified or validated, they could be used in the future quality control and release of the final product. This will allow improving product characterization and better control around the quality of the cell therapy products.

Can you speak about scaffold requirement in 3D bioreactors?

The use of scaffolds purely depends on the application. We know that the cellular microenvironment is important (*in vitro*) as we are trying to mimic the *in vivo* environment of the cells and maintain the therapeutic impact of the cell therapy products. Depending on the application, whether we want to minimize the immune rejection or whether we want to boost the therapeutic efficacy, we might need to use a scaffold and/or other biomaterial as a hybrid transplantation, along with the cell therapy product.

The type and implementation of scaffolds in 3D bioreactors will be largely dependent on the type of application, and how the cells are essentially inducing the therapeutic effect after the transplantation as well as strategies around control of immune rejection. The biomaterials field is significantly evolving, and it's an important concept that will require attention in particular regarding the choice of materials, cell–cell interaction and cell-scaffold interaction, impact of the hydrodynamics of the bioreactors on the scaffolds and cells, cell format in the scaffolds (single cells vs cell aggregates), and the cellular therapeutic impact after transplantation into the patients.

AFFILIATION

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AUTHORSHIP & CONFLICT OF INTEREST

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CELL & GENE THERAPY INSIGHTS

RESEARCH ARTICLE

Cost analysis of vein-to-vein CAR T-cell therapy: automated manufacturing and supply chain

Adriana G Lopes, Rob Noel & Andrew Sinclair

A cost model was described using Biosolve to measure the impact of automation and scale-out of a CAR-T manufacturing process, and associated supply chain. The partially automated CAR-T manufacturing process was the most economical configuration by CoGs analysis. However, after scale-out and inclusion of the impact of time by NPC evaluation, the manual CAR-T process became the most cost-effective option to meet an annual demand of 5,000 patients. Key process cost drivers were identified as the viral vector required to introduce CARs into T-cells and QC tests. A sensitivity analysis was undertaken to aid a manufacturer on whether to produce or undertake the latter in-house or outsource. Local and centralized CAR-T supply chain models to treat a peak demand of 5,000 patients were also compared. The cost of treatment to the patient, including supply chain costs, was estimated to be between $78k–$93k. A sensitivity analysis of the supply chain models showed the impact of resource availability, patient demand and lengthening the bottleneck on estimated costs.

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INTRODUCTION

CAR T-cell therapies

Autologous T cells expressing a chimeric antigen receptor (CAR) therapies are now a reality with the release of commercial products such as CD19-targeted CAR T-cell therapy Kymriah® from Novartis and Yescarta® from Kite Pharma (Gilead Company) [1]. These therapies are currently the leading technology in the Advanced therapy

medicinal products (ATMP) space with sales list prices ranging from $300k to $500k [2,3] that result in a market forecasted to value $8.5B by 2028 [4].

The high price tag associated with autologous cell therapies is derived from their curative potential within their target market, but also from their high manufacturing costs, specialized infrastructure and staffing requirements [5–7]. Manufacturing of CAR T-cells involves multiple complex manipulation steps requiring highly skilled and experienced staff in a cleanroom environment, following Good Manufacturing Practices (GMP). The resulting cost remain a significant challenge and concern to developers, and are critical to eventual market access [8,9]. The importance of such evaluation was seen with the first autologous prostate cancer vaccine, Sipuleucel-T (Provenge®), that was prevented from sustaining a marketing license by relatively high upfront costs and by limited manufacturing capacity [10].

The cost for these autologous cell therapies is also influenced heavily by the complex supply chain logistics and the need to scale-out, rather than scale-up production. These are significant challenges that cannot be quantified without detailed studies or models, to understand and target the areas where cost reductions can be made. There is a scarcity of published information available in this area with existing studies concerning either autologous [5–7,11] or CAR-T allogeneic cell therapies [12,13]. Allogeneic manufacturing is similar to traditional biologics due to the potential for creation of a master cell bank and the use of scalable technologies for mass production [7]. Autologous cell therapies represent a completely different manufacturing model from this and there has been little comprehensive analysis on manufacturing and supply chain costs to date.

In this paper Biosolve Process (Biopharm Services, UK) software was used together with Orchestrate (Access, UK) scheduling tool to model the costs associated with both autologous CAR-T manufacturing and related supply chain options. Similarly to our previous published work, the impact of automation introduction was evaluated on the cost of goods (CoGs) estimate of CAR-T manufacturing processes [5,6]. For the automated CAR-T manufacturing process, the costs associated with supply chain options were added to the overall cost estimation. The sensitivity of key identified cost drivers, risks and benefits was assessed during a manufacturing commercialization plan to treat 5,000 patients using local or centralized processing centers.

Manufacturing process automation?

Automation is positioned to have a major impact in cell bioprocessing and should be implemented early in clinical development to avert high-risk late-stage process modifications. Automation plays a key role in supporting product quality through increased robustness and consistency, resulting in decreased risk of batch failure through contamination, and reduced direct labor costs. Despite these advantages, several leading cell therapy developers are opting to delay implementing automation until their second-generation product [4]. Pre-sterilized, closed, single-use flow paths form an essential part of the automated system package.

For most autologous cell-based therapies, including for CAR T-cell manufacturing, the process starts from the collection of peripheral blood mononuclear cells (PBMCs) from the patient, commonly achieved by a leukapheresis process. Collected apheresis product from the single patient can be processed in various ways depending on the level of process automation. Manual processing uses typical laboratory cell culture systems, manual separation and cell washes, and open manipulations under a biological safety cabinet (BSC) throughout. The introduction of automation would enable the combination of several manual steps into one closed system. The greater the implementation of automation, the smaller is the operator interaction, and hence variability and potential for failure.

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Typically, the production of CAR T-cells involves the isolation of PBMCs followed by activation, genetic modification and expansion of modified T-cells to target numbers. Available automated systems provide a separate level of containment, as they rely on single-use systems, and can perform automated cell culture, fractionation, enrichment, washing and isolation. Activation and expansion of patient PBMCs with anti-CD3 and anti-CD28 monoclonal antibodies was explored in this paper. The genetic modification of T-cells with CARs was assumed to be achieved by means of a viral vector, which was followed by expansion of cells to generate therapeutic doses of the modified CAR T-cells. The processing of source material into PBMCs, as well as the final CAR-T product, were assumed to be cryopreserved, allowing time for product release testing and more flexibility for further manufacturing process and supply chain planning [14–16].

Centralized or local supply chain?

Patient therapy requires careful scheduling between treatment center and manufacturing site. The location of both treatment center and the manufacturing site is also influenced by the product shelf life and the preservation method utilized. A therapy with a limited shelf life, which includes several types of autologous therapies, may require a distributed model in which manufacture occurs in multiple local centers near treatment centers [17]. Alternatively, it may require the movement of patients to a treatment center close to a single centralized manufacturing site [18].

The centralized model consists of a single site responsible for the manufacture of the cell therapy product, which is then delivered to the clinical treatment center for administration, normally located within an hospital setting. One manufacturing site is attractive because it can be streamlined, can be automated and more economical, and it is easier to ensure product consistency and quality. However, initial capital expenditure to set up a single manufacturing facility can be high and estimated patient demand may be overestimated. Moreover, centralized manufacturing strategies depend upon a complex array of transport and logistical services including cryopreservation and specialized airfreight, which need to work in tangent with both manufacturing and clinical sites.

Alternatives to centralized manufacturing models include strategies for cell production and processing at or near patients point-of-care. It is argued that autologous cell-based therapies benefit particularly from multicenter localized manufacturing solutions, particularly if bioprocessing can be confined to dedicated and contained automated systems installed within an hospital setting [19]. According to Haddock *et al*. multicenter manufacturing in hospitals, using automated systems in a local supply chain network, would minimize risks associated with transport, labor intensity and costs, as well as improve the robustness and scalability of CAR-T cell manufacturing, and is expected to be the key to treating increasing patient numbers. Currently, though, the number of clinical sites working to GMP is low and so some autologous therapies are being manufactured at central facilities. Distributed manufacturing models are subject to substantial comparability requirements, where centers must demonstrate the precise replication of products between centers but can offer logistical advantages [17].

METHODS

CAR-T manufacturing assumptions

The CAR-T manufacturing process model built in Biosolve Process (Biopharm Services, UK) was based on the automated 6-day GMP compliant procedure for production of CD19 CAR-T cells [15]. The autologous CAR T-cell manufacturing process started with the receipt of the collected apheresis product from a single patient and finished with formulation and cryopreservation of modified T-cells into vials. It included the generation

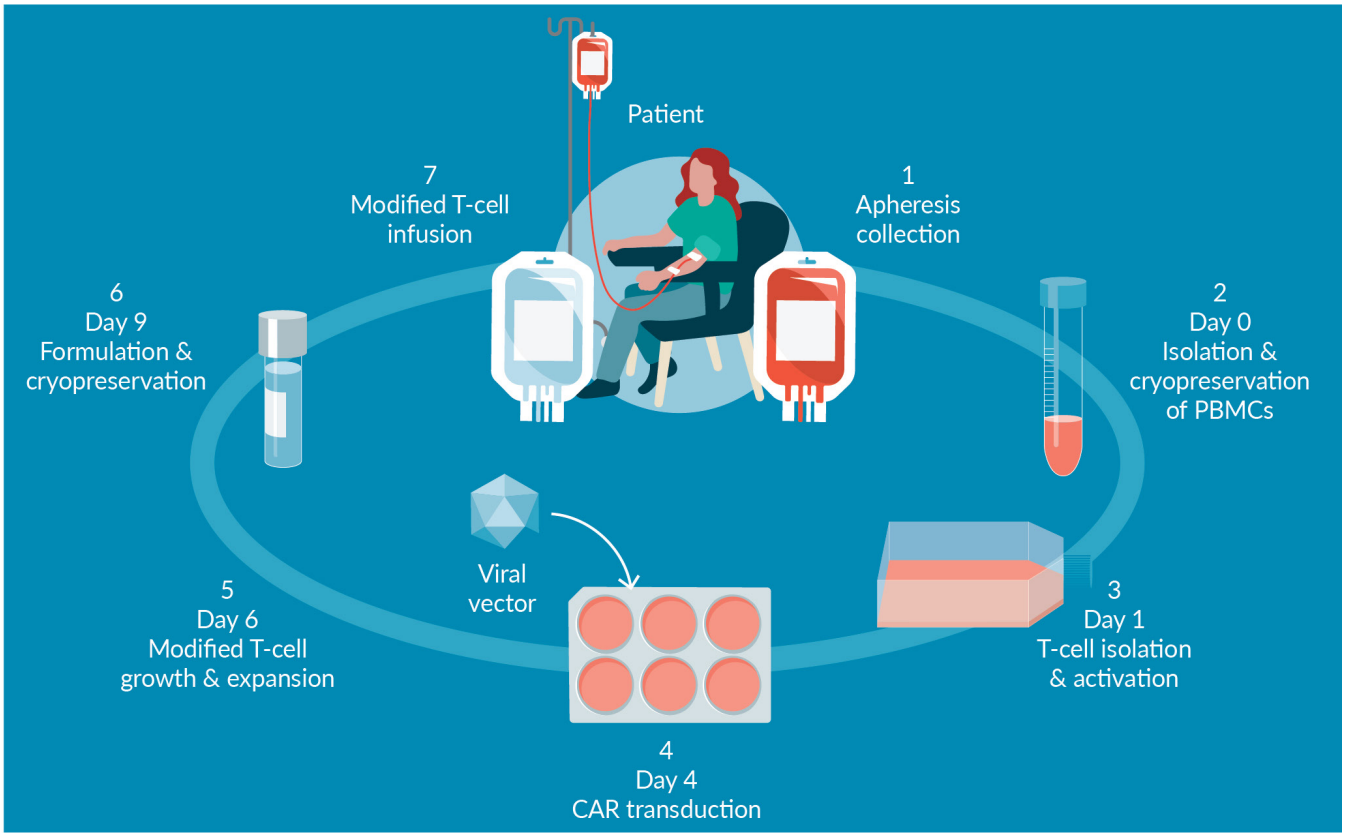
and cryopreservation of PBMCs from the apheresis material, as described previously [16]. The general sequence of process steps that described the entire configured CAR-T process is shown in Figure 1 and the associated Biosolve Process model assumptions are summarized in Box 1 [15,16,20].

Increasing levels of automation where introduced to the typical manual operations model [5,6]. The manual open manipulations carried out within a BSC, using traditional laboratory equipment such as incubators, bench top centrifuges, among others, were partially or fully replaced by self-contained automated systems that relied on single-use assemblies, and that combined several manual steps into one stand-alone, product dedicated system. Table 1 presents the manual CAR-T manufacturing process steps and highlights areas of the process where suitable automated closed systems were introduced to replace manual operations and achieve partial or fully automated CAR-T processes. To achieve a partially automated process, manual operations were replaced by a selective cell isolation and automated washing systems such as the Sepax® (BioSafe SA), PureCell Selectl™ (Pall Corp.), COBE® 2991 Cell Processor (Terumo BCT), or the RoboSep™ (StemCell™ Technologies). For the fully automated CAR-T process, the previous systems were used for the PBMC generation and programmable automated cell separation, washing, expansion and concentration platforms were used thereafter; for example, the Quantum® Cell Expansion System (Terumo BCT), Cocoon™ (Octane Biotech), DynaMag™ CTS™ (Life Technologies) or the CliniMACS (Miltenyi Biotec).

In comparison with other biologics, autologous cell therapy manufacturing is a very manual and labor-intensive process,

FIGURE 1

CAR-T manufacturing process schematics.



Started with receipt of apheresis material and finished with vial filling and cryopreservation of modified CAR T-cells.

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BOX 1

Summary of assumptions used for CAR T-cell manufacturing process in *Biosolve Process* (Biopharm Services, UK), with increasing levels of automation.

CAR-T all processes:

- Autologous (patient-to-patient) CAR-T Process [15,16]
- Starting material: 200mL of whole blood
- Titre: 2×10^{10} cells/L
- Lentiviral vector price (cost per CAR-T batch): $US 300[1]
- Equipment, consumables, materials based on one batch/patient
- Capital based on floor area classification
- Media prep basis: per unit op
- Headcount: 2 operators, 1 supervisor, 1 QA and 2 QC
- QC testing: raw material testing (leukapheresis cells and viral vector), in-process cell counting/viability at each process step, and CAR-T product release testing
- Final CAR-T product formulated into vials and cryopreserved

Manual process:

- 10% batch failure (*45 process steps*, Table 1)
- Typical lab equipment: BSC, incubator, bench-top centrifuges, water bath, controlled rate freezer, pipette aid, micropipettes
- Lab consumables: pipettes, centrifuge tubes, well plates, T-flasks
- Area classification B throughout process[2]

Partially automated:

- 5% batch failure rate (*23 process steps*, Table 1)
- Equipment: introduction of culture bags for cell culture [20], and automated system for cell isolation and wash, and tube welder
- Lab consumables: introduction of bags and tubing sets
- Area classification B and C throughout process[3]

Fully automated:

- 3% Batch failure rates (*11 process steps*, Table 1)
- Equipment: introduction of automated system for cell separation, wash, expansion & concentration
- Lab consumables: introduction of bags and tubing sets
- Area classification B and C throughout process[3]

[1]Obtained from Biosolve Process (Biopharm Services, UK) for lentivirus vector production, results not shown.
[2]Open manipulations undertaken in BSC.
[3]Introduction of automated/closed systems, assumed to require reduced area classification.

comprising many handling steps (e.g., density gradient cell processing, washing, feeding and so on) that require considerable interventions from skilled operators. Products may be rejected due to the variability of apheresis starting material, contamination or human error, which vary from patient-to-patient, operator-to-operator and with the cell expansion procedures used. Contamination is a concern due to length of culture time and the increased number of manual interventions i.e. manual washes, feeds and cell manipulations in open culture settings, that occur within a manufacturing process. Additionally, process consistency across multiple patients, manufacturing operators and process steps is difficult, as well as compliance to established product quality standards [21]. Based solely on the number of manipulations that occurred within each different process,

TABLE 1

CAR-T manual manufacturing process details and areas where automation systems were introduced to achieve partial or full automation.

No.	Process stage	Unit Op Name	Conc (#/L)	Yield (%)	Duration (hr)	Vol in (L)	Vol out (L)	Cells in	Cells out
1	Upstream	Whole Blood	2.0E+10	100	0.0	0.2	0.2	0.0	4.0E+09
2	Upstream	PBMC Isolation & Cryopreservation – Transfer & Dilution	9.9E+09	99	0.7	0.2	0.4	4.0E+09	4.0E+09
3	Upstream	PBMC Isolation & Cryopreservation – Transfer & Addition	7.2E+09	99	0.4	0.4	0.5	4.0E+09	3.9E+09
4	Upstream	PBMC Isolation & Cryopreservation – Centrifugation I	1.4E+11	95	0.7	0.5	0.0	3.9E+09	3.7E+09
5	Upstream	PBMC Isolation & Cryopreservation – Re-suspension I	5.7E+09	99	0.7	0.0	0.7	3.7E+09	3.7E+09
6	Upstream	PBMC Isolation & Cryopreservation – Centrifugation II	1.1E+11	95	0.6	0.7	0.0	3.7E+09	3.5E+09
7	Upstream	PBMC Isolation & Cryopreservation – Re-suspension II	5.3E+09	99	0.7	0.0	0.7	3.5E+09	3.5E+09
8	Upstream	PBMC Isolation & Cryopreservation – Centrifugation III	1.0E+11	95	0.5	0.7	0.0	3.5E+09	3.3E+09
9	Upstream	PBMC Isolation & Cryopreservation – Re-suspension III	6.5E+10	99	0.7	0.0	0.1	3.3E+09	3.3E+09
10	Upstream	PBMC Isolation & Cryopreservation – Centrifugation IV	1.2E+12	95	0.5	0.1	0.0	3.3E+09	3.1E+09
11	Upstream	PBMC Isolation & Cryopreservation – Re-suspension IV	6.2E+10	99	0.5	0.0	0.0	3.1E+09	3.1E+09
12	Upstream	PBMC Isolation & Cryopreservation – Centrifugation V	1.2E+12	95	0.5	0.0	0.0	3.1E+09	2.9E+09
13	Upstream	PBMC Isolation & Cryopreservation – Formulation & Fill	2.1E+11	99	0.7	0.0	0.0	2.9E+09	2.9E+09
14	Upstream	PBMC Isolation & Cryopreservation – Freezing	2.1E+11	100	0.7	0.0	0.0	2.9E+09	2.9E+09
15	Upstream	T Cell Selection & Activation – Thawing	1.6E+11	80	0.6	0.0	0.0	1.4E+09	1.1E+09
16	Upstream	T Cell Selection & Activation – Transfer I	3.0E+10	99	0.4	0.0	0.0	1.1E+09	1.1E+09
17	Upstream	T Cell Selection & Activation – Centrifugation I	3.5E+12	95	0.5	0.0	0.0	1.1E+09	1.1E+09
18	Upstream	T Cell Selection & Activation – Re-suspension I	2.1E+10	99	0.7	0.0	0.1	1.1E+09	1.0E+09
19	Upstream	T Cell Selection & Activation – Centrifugation II	2.0E+12	95	0.5	0.1	0.0	1.0E+09	9.9E+08
20	Upstream	T Cell Selection & Activation – Re-suspension II	2.0E+10	99	0.8	0.0	0.0	9.9E+08	9.8E+08
21	Upstream	T Cell Selection & Activation – Plating I	1.3E+09	99	0.9	0.0	0.7	9.8E+08	9.7E+08
22	Upstream	T Cell Selection & Activation – Incubation I	1.3E+09	100	2.4	0.7	0.7	9.7E+08	9.7E+08
23	Upstream	T Cell Selection & Activation – Wash & Addition	1.0E+09	80	1.0	0.7	0.7	9.7E+08	7.8E+08
24	Upstream	T Cell Selection & Activation – Incubation II	1.6E+09	150	48.4	0.7	0.7	7.8E+08	1.1E+09
25	Upstream	T Cell Selection & Activation – Harvest	1.65E+09	99	0.4	0.7	0.7	1.2E+09	1.2E+09
26	0Upstream	T Cell Selection & Activation – Centrifugation III	3.0E+10	95	0.5	0.7	0.0	1.2E+09	1.1E+09
27	Upstream	T Cell Selection & Activation – Re-suspension III	3.1E+09	99	0.8	0.0	0.4	1.1E+09	1.1E+09
28	Upstream	T Cell Selection & Activation – Centrifugation IV	5.9E+10	95	0.5	0.4	0.0	1.1E+09	1.0E+09
29	Upstream	Transduction – Plating I	4.7E+10	99	0.9	0.0	0.0	1.0E+09	1.0E+09
30	Upstream	Transduction – Centrifugation I	7.5E+10	95	1.9	0.0	0.0	1.0E+09	9.7E+08
31	Upstream	Transduction – Incubation I	7.5E+10	100	24.4	0.0	0.0	9.7E+08	9.7E+08
32	Upstream	Transduction – Plating II	3.7E+10	70	0.9	0.0	0.0	9.7E+08	6.8E+08
33	Upstream	Transduction – Centrifugation II	5.9E+10	95	1.9	0.0	0.0	6.8E+08	6.4E+08
34	Upstream	Transduction – Incubation II	5.9E+10	100	24.4	0.0	0.0	6.4E+08	6.4E+08
35	Upstream	CAR-T Cell – Harvest	6.0E+10	99	0.4	0.0	0.0	6.4E+08	6.4E+08
36	Upstream	CAR-T Cell – Centrifugation I	1.1E+12	95	0.5	0.0	0.0	6.4E+08	6.1E+08
37	Upstream	CAR-T Cell – Re-suspension I	1.2E+10	99	0.8	0.0	0.1	6.1E+08	6.0E+08
38	Upstream	CAR-T Cell – Centrifugation II	2.3E+11	95	0.5	0.1	0.0	6.0E+08	5.7E+08
39	Upstream	CAR-T Cell – Re-suspension II	4.0E+10	99	0.7	0.0	0.0	5.7E+08	5.6E+08
40	Upstream	CAR-T – Incubation I	6.0E+10	150	72.4	0.0	0.0	5.6E+08	8.5E+08
41	Upstream	CAR-T Cell – Centrifugation III	1.1E+12	95	0.5	0.0	0.0	8.5E+08	8.0E+08
42	Upstream	CAR-T Cell – Re-suspension III	5.7E+10	99	0.7	0.0	0.0	8.0E+08	8.0E+08
43	Upstream	CAR-T Cell – Centrifugation IV	5.4E+11	95	0.5	0.0	0.0	8.0E+08	7.6E+08
44	Upstream	Final Product – Formulation & Fill	2.0E+10	99	0.8	0.0	0.0	7.6E+08	7.5E+08
45	Upstream	Final Product – Freezing	2.0E+10	100	0.7	0.0	0.0	7.5E+08	7.5E+08

Dotted line: separates PBMC generation from CAR-T process; Gray areas: selective cell isolation and automated washing system; Red boxes: programmable automated cells separation, washing, expansion and concentration platform.

the manual manufacturing model required approximately 3 times more hands-on operations (45 unit operations; Table 1) than a traditional biologics process (average of 15 unit operations). Given that traditional biologics manufacturing processes batch failure rates are estimated at 3% [22] and applying the previous calculated figure for the CAR-T manufacture, the manual manufacturing model assumed a failure rate of 10%. The 10% failure rate of manual process was then halved based on the number of removed manual interventions. As a result, the introduction of automation reduced of the original 45 steps for the manual CAR-T process, to 24 and 10 process steps, with a resulting 5% and 3% batch failure rates for partially and fully automated processes, respectively. The latter fully automated process failure rate matched a traditional biologics process, where process steps are generally with automated systems and require fewer manual interactions.

It is assumed that the introduction of automation systems into the CAR-T process did not result in additional process failures by the use of validated tube welding/fusing technologies for connections/disconnections, and also the use of validated suppliers for the single-use manifolds used for single-patient processing, de-risking potential for leaks.

Capital estimates of the different CAR-T manufacturing process configurations was based on the sum of the floor area occupied by each specific equipment used for processing i.e. standard laboratory equipment and automated systems alike, multiplied by a cost per meter squared based on the cleanroom grade where these systems were located.

The cost of the viral vector used for introduction of CARs into the T-cells was assumed to be $300 per CAR-T process batch. This value was obtained using Biosolve Process model for an in-house lentiviral suspension culture production of 200L scale, within a traditional stainless-steel facility (results not shown). The cost per gram for the viral vector would be much higher if manufactured in cell factories or if purchased as an outsourced raw material.

A minimum of two personnel was assumed to be required for all GMP processing. For a single product, the production operations team was composed of 3 people, which included 2 operators and one supervisor. The quality team responsible for QA, QC and Microbiology testing required 3 people.

For all processes, the costs associated with quality control (QC) testing included starting material, in-process tests and the CAR-T-Cell-product release testing. Starting materials included the receipt of collected apheresis material, the cryopreserved PBMC and the viral vectors. These tests included cell concentration, viability, identity, sterility, endotoxin, mycoplasma, adventitious agents, immunoassay, RCL assay, vector titer and sequence, among others [15].

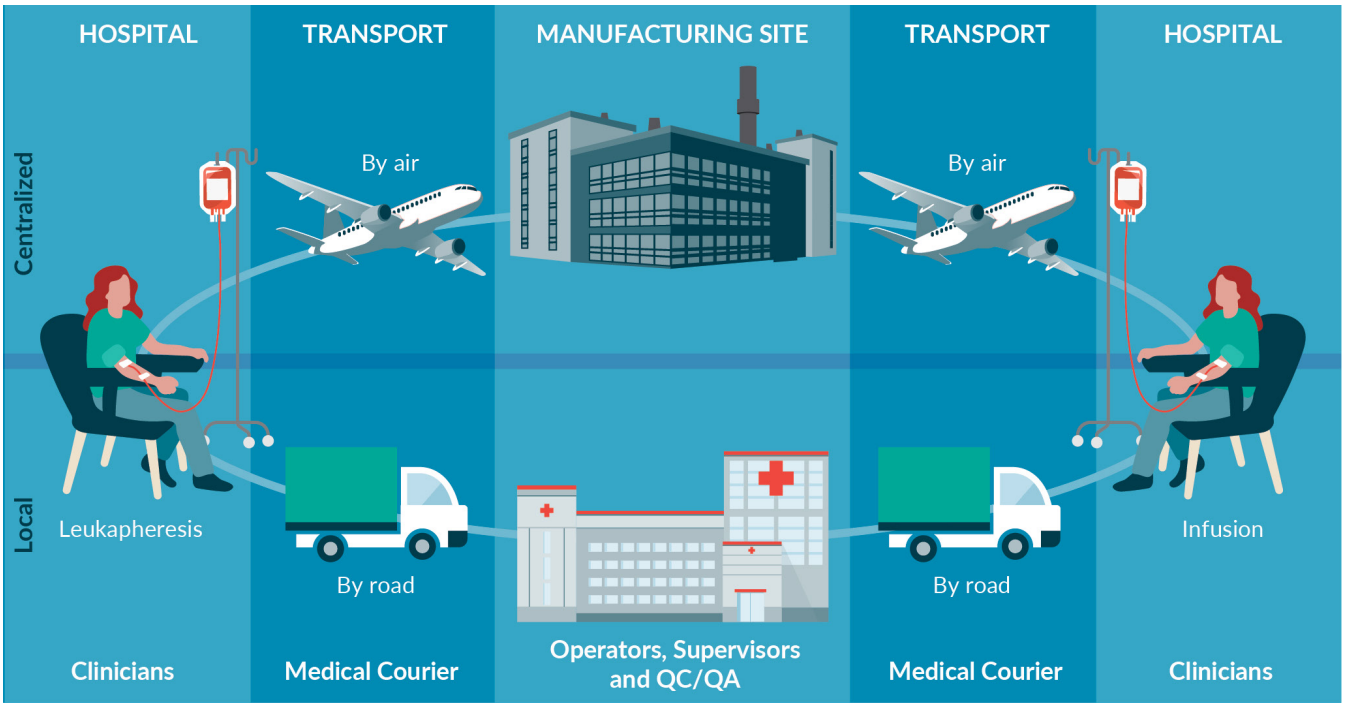
CAR-T supply chain assumptions

The supply chain model defined in this paper could be described by a vein-to-vein autologous CAR T-cell process that started with the collection of cells from the patient and finished with the re-infusion of modified CAR-T cells product into the same patient. The leukapheresis process was assumed to take place at a local treatment center situated within an hospital. Collected apheresis product from the single patient was then used within an automated CAR-T manufacturing process. The collected apheresis material and final CAR-T product were assumed to be cryopreserved prior to transportation from the treatment center, to and from the manufacturing site. The location of the manufacturing site was the subject of further analysis and could be either centralized to a single site or local, close to the patient treatment center. The sequence of process steps that described these CAR-T supply chain models are shown in Figure 2 and an outline of the assumptions made during the CAR-T supply chain analysis are summarized on Box 2 [4,23,26].

The current approach being taken by existing cell therapy products, and the one explored in this paper, was that the same

FIGURE 2

Local and Centralised CAR-T Supply chain pathways.



Started with the collection of blood via leukapheresis from a single patient, followed by transport to and from manufacturing site, and back to treatment centre, for re-administration into the same patient. The manufacturing of CAR-T could be undertaken locally within an hospital, and require transportation by road, or centralised to a single site where transportation would be mainly undertaken by air travel. The type of labor category required for each stage of the supply chain was chosen from a pool of clinicians, medical couriers and manufacturing personnel.

patient's apheresis cells were frozen and shipped to the manufacturing facility where they were expanded, purified and cryopreserved into vials, before being shipped back to the hospital. In the hospital they were stored and delivered to the patient [19]. The transporting of cryopreserved material between sites reflects the current centralized practices in Novartis' Kymriah® supply chain, as its' FDA approved application describes, with validated shipping of both leukapheresis and final product material to their respective sites [24]. Once manufactured, it was assumed that the finished product would be shipped and stored in a qualified dry-shipping unit, namely in a dedicated vapor-phase dewar, by common carrier to the clinical site for administration [25]. A supplementary QC test was added post-thawing and before re-infusion of same patient with engineered T-cells.

Two models for supply chain networks were evaluated: distributed local supply chain comprising of local networks that were limited to point of care, situated within an hospital setting; and a centralized regional supply comprising of a network within a single country, such as for example the US, or within a common area such as Europe. Besides Novartis, centralized manufacturing supply chain networks are also currently being utilized by Kite Pharma. Their current manufacturing facility located in LA has been reported to have 4041 m^2, utilizes 16 treatment centers across the US, and targets 5000 patient therapies per year [26]. From an online source that outlined the treatment centers where patients can receive Yescarta, 13 different hospitals in a range of States were identified [27] and their distance from the LA manufacturing site was used to calculate an average transport time of 4 hours by air. These details were used as the basis for the centralized model.

The local model assumed that manufacture would be undertaken within an hospital setting. From the NHS England UK website,

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BOX 2

Summary of assumptions used for supply chain of CAR T-cell therapy for centralized and local models

CAR-T Supply Chain – all models:

- Vein-to-vein: from leukapheresis to re-infusion into same patient **(Figure 2)**
- QC testing: Included all QC testing stages as outlined in **Box 1**, and additional tests conducted prior to re-infusion of final CAR-T product into patient
- QA/QP release: Included additional 7 days post manufacture, allocated for CAR-T product release to the patient. Also included QP[1] cost per batch of $10,000 [4]

Leukapheresis:

- 8 apheresis machines per treatment center located within an hospital
- 6 hours duration which included leukapheresis, processing and packing ready for transportation
- Work undertaken by clinicians[2]

Transportation:

- CAR T cell therapy was transported frozen and transport cost comprised mainly of the cost of the cryogenic storage system used for cryopreserved material transport
- A dedicated storage unit was used for product storage
- A medical courier is required to hand-carry and accompany the cryopreserved product throughout the entire transportation from manufacturing site to patient. Courier yearly salary assumed to be $US 33,172 [23]

Cryopreservation and thawing:

- Both leukapheresis sample and CAR-T product were cryopreserved before transportation
- Thawing required post-transportation: pre-manufacture and prior to re-infusion into same patient. Duration of 20 minutes
- A final QC testing was required post-thawing and before administration of CAR-T product back into patient

Manufacture:

- Fully automated autologous CAR-T manufacturing[3]
- Number of automated systems: estimated based on the floor area required to accommodate a particular manufacturing scale

Labor:

- Labor pool comprised of Clinicians, Medical couriers, manufacturing personnel composed of Operators and Supervisors, and Quality personnel with QA and QC
- The number of manufacturing supervisors and QA personnel were estimated from the Operator and QC numbers
- Supply chain operating 24/7

Local Supply Chain Model:

- Decentralized and local supply chain comprising of local networks that were limited to point of care located within an hospital setting
- Number of treatment centers: 1 per manufacturing unit
- Number of automated systems: 25[4] per manufacturing unit
- Transport: 20 min by road travel

Centralized Supply Chain Model:

- Centralized regional supply comprising of a network within a single country such as the US or Europe
- Number of treatment centers: 16 [26] per manufacturing unit
- Number of automated systems: 325[5] per manufacturing unit
- Transport: 4h by air travel

[1]Required for product release within the European Union.
[2]Yearly salary assumed to be the same as manufacturing supervisors in Biosolve Process (Biopharm Services, UK) cost database.
[3]Results obtained from Biosolve Process (Biopharm Services, UK).
[4]Based on an estimated 74m^2 clinical production laboratory located within an hospital.
[5]Based on the number of automated systems required to meet a maximum of 10,000 patient demand per year.

9 hospital-situated centers provided CAR-T treatment in 2018 [28]. Great Ormond Street Hospital in London reported clean room capacity for CAR-T processing. For the purposes of the present local supply chain model, a 74 m^2 cleanroom capacity was assumed, based on the average size of a clinical production laboratory. The travel time between the production room and the treatment of patients was assumed to be 20 minutes.

Further constraints were added to both models, such as the use of 8 apheresis machines per administration/treatment center, based on the number employed at the Children's hospital of Philadelphia, for apheresis utilized for CAR-T processing [29].

The costs associated with the type of transportation used and associated fuel consumption were not taken into consideration in the present analysis.

RESULTS & ANALYSIS

CAR-T manufacturing process with increasing levels of automation

Table 2 outlines key CAR-T process metrics and Table 3 presents the breakdown of costs associated with manual and automated operations of the CAR-T manufacturing process. For each case the results are based on a single manufacturing line, representing the worst-case value. If the capacity of the facility was increased to accommodate multiple lines, this would have the effect of reducing the batch cost achieved by sharing the fixed facility costs.

As seen on Table 2 the manual operation resulted in the lowest cost of the facility with $1.22M, and an increasing level of automation increased the manufacturing facility cost to between $1.36M to $1.63M, depending on adoption of automation. A reduction of both the overall process area and the surrounding cleanroom environment grade from B to C could be seen with automation adoption. The savings in moving to grade C cleanroom space brought about by increased automation was however not enough to nullify the associated increased equipment costs associated with introduction of automation. The partially automated configuration conferred the greatest process flexibility, enabling the highest batch throughput of 41 yearly, and the lowest cost of $76 per million cells. The fully automated configuration had the less flexibility, derived from the fact that a single automated system, comprised of several processing steps, was locked for longer, creating a longer process bottleneck and resulting in reduced batch annual throughputs of 24. Manual processing was depended upon the turn-around of a single-patient dedicated cleanroom and as a result had the lowest throughput of 14 yearly batches. Considerations regarding the implementation of partial or full automation in a CAR-T process have been previously discussed [21]. In this study it is also suggested the lack of flexibility of full automation and highlights the potential for introduction of new process bottlenecks.

As seen on Table 3, the partially automated process achieved the lowest CoGs per batch (or per patient) at $83k, while the fully automated process achieved the highest at $118k, with the manual process in between at $85k. Materials (39–51%) followed by Labor (32–36%) cost categories were the biggest drivers of the manufacture of CAR-T cell therapy. Material costs could be broken down further into: Direct Raw Materials, Media and Buffers used for processing, and in supporting QC tests, among others. For CAR-T manufacture, QC tests and Direct Raw Materials (RM) had the highest impact upon the overall Materials costs. QC tests included starting material testing (leukapheresis cells, PBMC cells and viral vector), in-process control of cell counting/viability at key process steps, and the finished CAR-T product release testing. Particularly relevant to the Raw Materials cost was the viral vector used for introduction of CARs into T-cells. These trends were to some extent in line with a recent CAR-T manufacture cost estimation where second to labor fixed costs were the viral vector cost and yearly batch throughput [11].

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TABLE 2

Key process metrics for CAR-T manufacture with increasing levels of automation.

Parameters	Manual	Partially automated	Fully automated
Total installed capital (million USD)	1.22	1.36	1.63
Process area (m^2)	30	27	25
Proportion of C grade area	17%	33%	56%
Batch failure rate	10%	5%	3%
Throughput (batches/yr)*	14	41	24
Cost per million cells (USD)	113	76	160

*Batches per year calculation based on process bottleneck:
Manual process – dedicated cleanroom availability, i.e., the length of entire batch process.
Automated processes – dedicated equipment availability, i.e., longest process step.

It would be expected that the introduction of automated systems would result in a reduction of the labor impact upon overall batch costs. This is the case as there is a reduction in man hours required. This assumes that the same labor pool could handle more automated machines without the need to increase headcount, until it reached a limit, as outlined in the following section for CAR-T process scale-out. Even though 30% less man hours were required to support a fully automated CAR-T process, the smaller number of batches attained per year (Table 2), resulted in higher cost of labor per batch and hence higher cost per batch/patient. Being a fixed cost, labor requirement depended heavily on the number of batches generated on a yearly basis. Automated equipment was tied up during long incubation times, creating a process bottleneck for the fully automated CAR-T process, resulting in a reduction to 24 batches compared to the 41 batches per year achieved by the partially automated configuration. Our previous publication showed attempts to remove this bottleneck by increasing the number of automated systems that could work in parallel [6]. Using the same methodology, if capacity was doubled (i.e. 84 batches per year), the initial capital investment would rise to 2 million USD and the resulting cost per patient (per batch) would be reduced to 96,634 USD (results not shown). In this instance, the fully automated process would remain the less competitive scenario. In the next section, variation of labor headcount will be explored further during CAR-T process scale-out and within the full CAR-T supply chain, as well as automated systems resource requirements.

Finally, the increased contribution of Capital charge and Consumables cost categories with increased automation levels is related to the introduction of expensive automated systems that relied on bags and tubing systems.

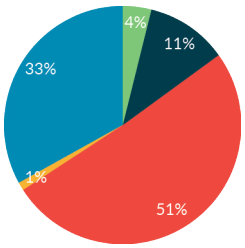
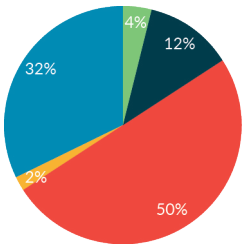
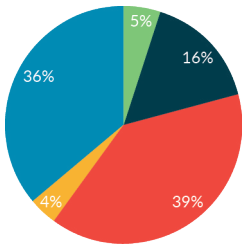
CAR-T manufacturing process sensitivity analysis: costs of QC testing & viral vector, modified T-cell expansion step duration & batch failure rate

The sensitivity of the two main Materials costs – QC tests and the viral vector cost – were further analyzed. The viral vector cost was varied by 10-fold and 50-fold from the original cost $300 per CAR-T batch to reflect the implication of manufacturing in-house or through a contract manufacturing organization (CMO). The impact of QC costs when varied by 50% and 100% was also assessed, as these may also be undertaken in-house or be outsourced to third party company, depending on the manufacturer's capabilities and expertise.

Given the high contribution of Labor upon CoGs, the impact of variation of Labor numbers was measured. A potential decrease in headcount associated with implementation of fully automated systems was evaluated by reducing the total personnel requirements to a minimum of 2 Operational and 2 Quality

TABLE 3

Summary of installed capital and CoGs breakdown per batch/per patient of CAR T-cell processing with increasing levels of automation.

US dollars	Manual	Partially automated	Fully automated
Equipment (total)	182,724	272,185	403,002
Capital charge	9,387	9,938	18,843
Materials	43,718	42,174	45,991
Media	1,397	1,484	1,183
Buffers	802	780	744
Direct RM	1,732	2,459	7,621
Brought WFI/PW	0.26	0.29	0.16
QC tests	39,786	37,451	36,443
Consumables	757	1,659	4,377
Culture flasks	70	–	–
Pipettes	568	104	4
Tubes and vials	105	43	34
Bags and bottles	8	1,061	419
Other	6	451	3,920
Labor	27,904	26,435	41,712
Process	11,205	10,615	16,749
Quality	12,441	11,787	18,598
Indirect	4,258	4,034	6,365
Other	3,062	3,155	5,762
Insurance & other	1,185	1,174	2,010
Waste	9	3	2
Maintenance	469	497	942
Utilities	1,399	1,481	2,808
Total	**84,827**	**83,362**	**116,685**
CoGs distribution			

Capital charge (facility and equipment)
Media, growth factors, supplements, starting materials, buffers, brought WFI/PW, QC tests
Bags, pipettes, tubes, vials, culture flasks, bottles, etc.
Process (operators and supervisors), quality (QA and QC)
Insurance, maintenance, waste treatment

personnel. An increased headcount to meet increased labor demands when a cleanroom was running at full capacity of 9 personnel (discussed in more detail in the following NPC section) was also evaluated for all process configurations.

The expansion step of the modified T-cells was required to generate the final target CAR-T therapeutic dose. This step was highly variable depending upon the apheresis product health, the number of starting cells, the protocols and proliferation methods used [30,31]. Being one of the longest process steps (of 72 h for manual process), it was also the process bottleneck, directly affecting the overall annual batches that could be achieved within a certain facility configuration. Based on reported data, the modified

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T-cell proliferation step was extended to up to 14 days.

As reported previously, batch failure rate had a significant impact upon the final cost of cell therapy [5]. Failure rates between 2–14% have been reported from clinical trial data for manual CAR-T cell therapy processing [32,33]. Higher batch failure rates associated with manual processing emphasizes the necessity for implementing closed automated systems for whole bioprocessing, as batch failure means patients fail to receive treatment. A lower range of 1% was modelled for the fully automated operations, representing when equipment failure was the only reported reason for process failure. This is supported by the 99% success rate reported for Kite pharma manufacturing [27]. Novartis has experienced an approximately 9% failure rate for the manufacturing of their CAR-T therapy Kymriah® [34]. The impact of failure rate ranging from 1% and 15% was evaluated in the present work, as outlined in Table 4. Figure 3 presents the impact of chosen criteria upon the CoGs per patient (equivalent to per batch).

As can be seen in Figure 3, the manual and partially automated processes show the same sensitivity to the main cost variables. Introduction of fully automated operations however reduced the sensitivity of the process to these variables, with exception of the failure rate. An increase in failure rate from 3% (baseline) to 5% (high) resulted in 50% increase in CoGs for the fully automated process. For the manual and partially automated processes an increase from 10% (baseline) to 15% (high) and from 5% (baseline) to 10% (high), respectively, only resulted in a 6% increase in the costs of the CAR-T therapy. As automated systems were dedicated to several steps at only one time, their lack of flexibility meant that batch failure had a greater impact upon costs. Only the fully automated process scenario included the introduction of an automated system for the modified T-cell expansion i.e. the process bottleneck, which explained the greater impact batch failure rate had upon this process configuration. A failure of delivery of a batch meant no therapy reached the patient, which would mean the re-ordering of another round of treatment, and in the worst case meant the death of the patient. It is therefore imperative to use models that allow the analysis of different process configurations, reduction of associated batch failure rate and strategies for de-bottlenecking.

The modified T-cell proliferation duration had the largest impact upon the overall CoGs of all processes. For the manual and partially automated processes an increase in the duration of this step resulted in a 50–150% increase in the costs for the patient. The impact of extension of this step was reduced for the fully automated processes to 10–90%. This was a similar effect to the one stated previously whereby a longer bottleneck had the biggest impact upon the resulting CoGs of the CAR-T process. The higher batch failure rates of 5% and 10% associated with the manual and partially automated processes respectively, drove the costs of these processes down even further, when compared to the fully automated manufacture, with a lower 3% batch failure.

When looking at the impact of the costs of key Materials, it was not surprising that reducing the viral vector costs had less of an impact than the QC tests, as the latter was the biggest cost driver in this category (Table 2). A variation of QC costs by 50% and 100% resulted in a ±23% variation of the total CoGs for both the manual and partially automated processes, respectively, and a ±16% variation

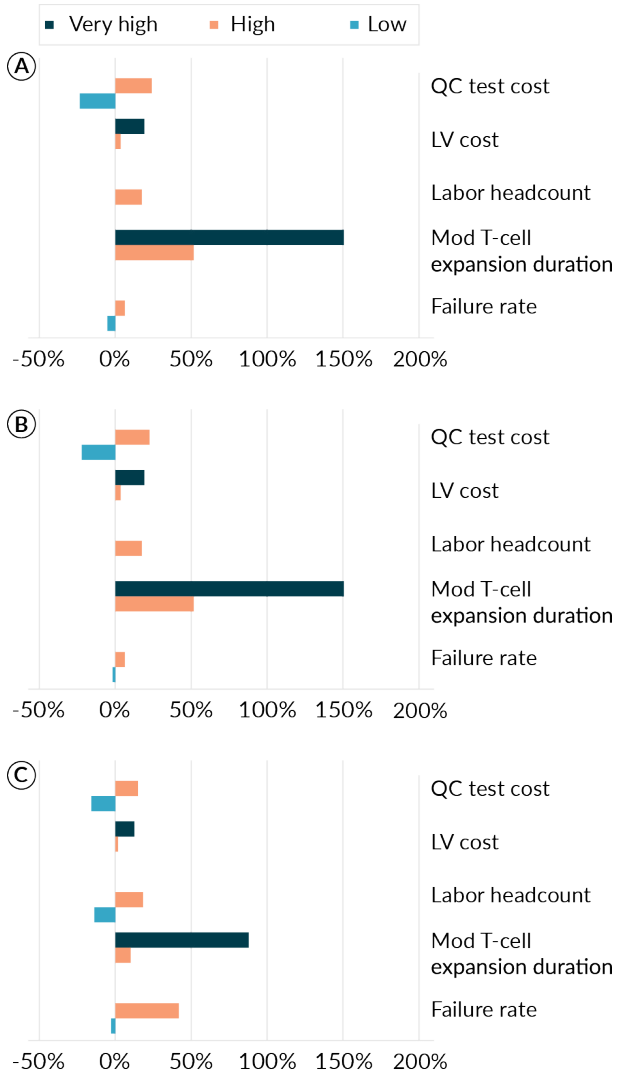
TABLE 4

Failure rate ranges used for sensitivity analysis of CAR-T processes with different levels of automation.

Process/failure rate range	Low	Baseline	High
Manual	5%	10%	15%
Partially automated	3%	5%	10%
Fully automated	1%	3%	5%

FIGURE 3

Sensitivity of selected parameters on CoGs per batch (per patient) of CAR-T processes with increasing level of automation.



(A) Manual; **(B)** Partially automated; and **(C)** fully automated CAR-T manufacturing process. Variables used for sensitivity analysis (ranges): QC test costs (50 and 100%); LV cost (10- and 50-fold); Labor Headcount (4* and 9 total personnel); Modified T-cell expansion step duration (up to 14 days); and Batch Failure rate **(Table 3)**. Variation ranges: Low (light blue), High (orange) and Very High (dark blue). * 4 personnel headcounts assumed to be achieved only with fully automation.

of CoGs for the fully automated process. Increasing 10 and 50 times the viral vector cost resulted in a 3% and 19% increase in CoGs per batch, respectively. The fully automated process had similar trend with exception of the 50-fold increase, which resulted in a lower 13% impact upon the cost of the final therapy. As with traditional biologics, the decision on whether to do it in-house or outsource, depended on the phase of development the product was at. Early stage phase would most likely outsource both QC and/or the generation of clinical therapy material, but at commercial stage would most likely be undertaken in-house with the use of outsourced capabilities for specific QC assays and for managing manufacturing capacity. In addition to this, for cell therapy processes, speed of response and capacity leverage were also very important factors. Managing several autologous therapies in parallel increases the need to outsource both part of QC and/or manufacturing capacity to respond to increased patient peak demands or derived from the increased inherent risk of batch failure.

Finally, when assessing the sensitivity of total Labor headcount for Figure 3 configuration A, B and C the resulting variation in CoGs was similar in the three cases (between 13–19%). For the three cases Labor ranged 33–36% of overall CoGs. As Labor had a similar contribution as Materials category, it was expected that the sensitivity analysis would yield comparable results. This is an important factor when considering the potential of decreased headcount associated with implementation of automation. A reduction of headcount to levels below 6 people (baseline level) could be potentially achieved in parallel with the phasing in of fully automated systems. In this case a minimum of 4 people would be required with one operator and QC in the cleanroom supporting automated processing operations, with both supervisor and QA covering breaks and conducting batch inspections/release as appropriate. A reduction of personnel required for automated processing would make fully automated costs more competitive at a final CAR-T treatment (per batch) to USD 101,117.

Scale-out of CAR-T manufacturing capacity

Besides the CoGs analysis, the potential commercial value of a CAR-T therapeutic

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opportunity can be further evaluated to understand the interplay between initial capital investment, operating costs and the amount of product generated on a per year basis, across the product lifecycle. The impact of these factors can be considered through the calculation of the Net Present Cost (NPC). This approach has been previously used to provide a quantitative understanding of the financial benefits and to guide the decision making of which technology options to evaluate and/or further develop towards implementation in the manufacturing of both traditional biologics and autologous cell therapies [6,35]. The most favorable financial option corresponded to the manufacturing process, option or technology that achieved the lowest value of NPC. In this study, the NPC was calculated using Biosolve Process (Biopharm Services, UK), based on the assumptions outlined in Box 3. The NPC for the alternative manufacturing processing options, namely manual, partial and fully automated processes provided information of when to make investment decisions as the product moved through the clinical phase changes, and as additional capacity was built.

To ensure that cell therapies can meet the increasing demand as they move through the clinical stages, suitable scalable manufacturing processes must be developed, either before or during the drug clinical assessment phase, with the final process option being used to produce batches for Phase 3 and carried forward to supply commercial product. Alternative scale-up/scale-out approaches were undertaken, depending on manufacturing model i.e. manual or automated, as shown in Figure 4. It was assumed that for each manufacturing model, the processing suite area was kept the same throughout, as detailed in Table 2. Additional production capacity was achieved by increasing the number of equipment until a maximum quantity was reached (Figure 4B), followed by addition of new processing suites (Figure 4A & C). A processing suite operating with the maximum number of equipment would have an increase in personnel by: 2 Operators and 1 QC (Box 1). As an example, the scale-up of the manual process could only be achieved by scaling-out additional processing areas. Due to the use of open manipulations, dedicated processing rooms were required for each batch. As one processing suite was dedicated to one patient (batch) at the time, the number of batches that could be achieved was based on availability of processing rooms. In this case, the personnel required within a team was kept the same throughout scale-out.

For automated processes, scale-up could be firstly achieved by an increase in the number of dedicated automated systems. At maximum equipment capacity, it was assumed that additional personnel (numbers as specified above) would be required. When the maximum capacity of a processing room was reached, then scale-up was achieved by acquiring a new processing suite. In this case one equipment rather than the processing suite was dedicated to the product of one patient at the time, which resulted in more flexibility. As an example, 100 patients in a Phase 1 clinical trial, would require 2 cleanroom facilities based on a manual process in order to separate each patient batch. If automated systems were implemented, only one clean room suite would be required, with an increased number of equipment.

The capital inputs used for NPC calculation were obtained from Biosolve Process model for each CAR-T process configuration, as outlined in Table 2. Cost of additional equipment and/or manufacturing units to meet increasing patient demands were added to the models as required.

This scale-up/scale-out approach was applied to build additional capacity as each process moved through clinical Phases 1, 2 and 3 until product launch/manufacture, and

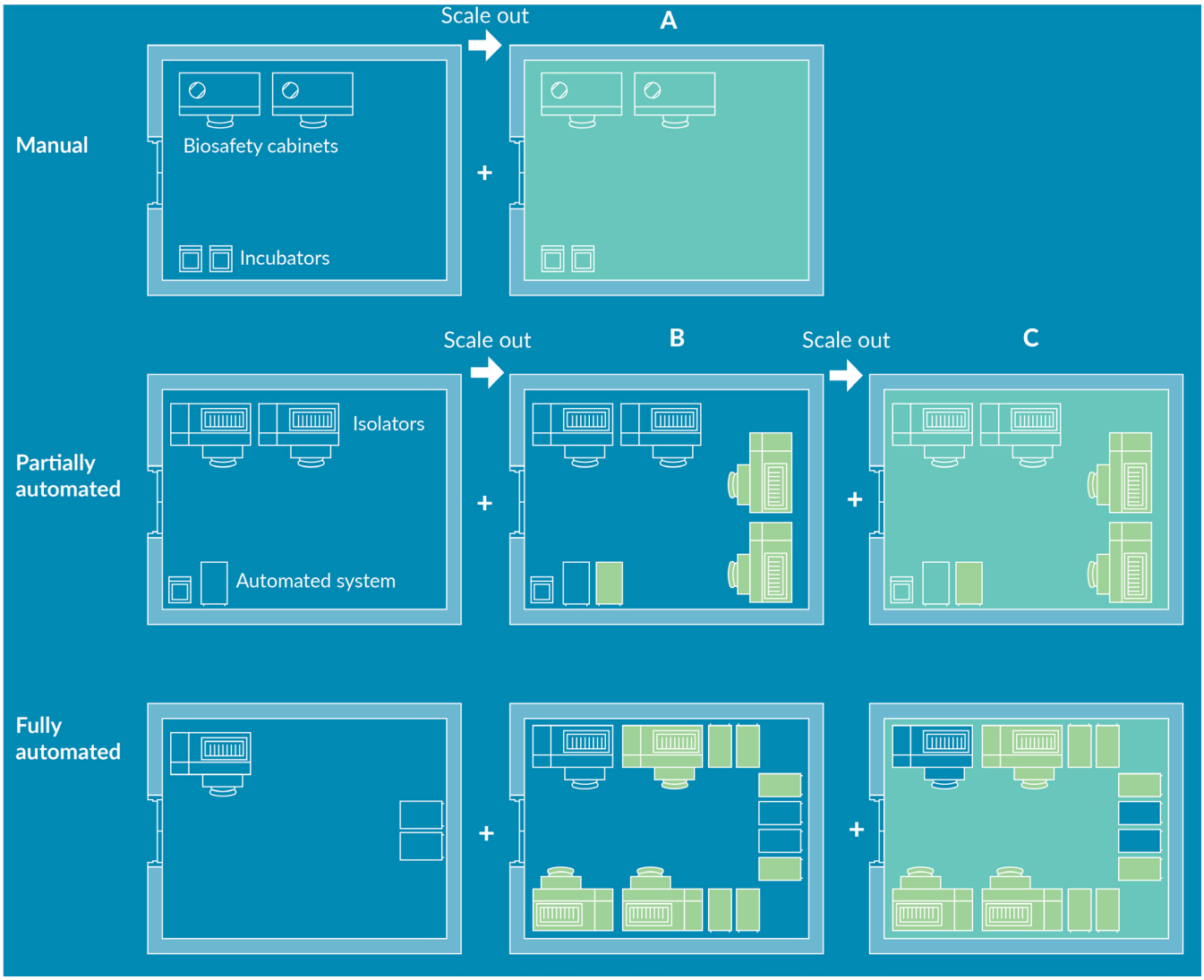
BOX 3

Net Present Cost (NPC) assumptions for calculation.

- Project life: 15 years
- Real discount rate: 10%
- Inflation rate: 3% (from year 1)

FIGURE 4

Scale-out approach of manual, partially and fully automated CAR-T processes.



(A) Additional Processing suite containing required equipment (turquoise); **(B)** Increase in the number of equipment within the same processing suite (green) until a maximum amount is reached; **(C)** Additional processing suite (turquoise) and associated equipment (green) to achieve maximum throughput.

was used to calculate NPC as a function of the number of patients per year, as shown in Figure 5. Here it could be seen that the manual CAR-T process configuration resulted in more favorable economic analysis through NPC evaluation to meet under 6,500 patients per year. For a yearly patient demand over 6,500 patients the partially automated process became the most cost-effective process configuration. These results are in line with the previous CoGs analysis, whereby the partially automated process followed by the manual CAR-T process resulted in the cheapest associated manufacturing costs [5,6]. When incorporating the impact of scale-out to meet increasing patient demands and time to build additional capacity by the aid of an NPC analysis, the partially automated process previously identified as having the cheapest associated manufacturing costs by CoGs analysis, was shown to only become a feasible option when targeting over 6,500 patients annually.

As seen in Figure 5, to supply cell therapy product to a target 5,000 patients, introduction of automation resulted in 6% and 46%

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increased NPC when compared with manual processing, for partially and fully automated processes respectively. As the processes were scaled-up further to meet an even higher demand of 10,000 patients, the partially automated scenario became the most attractive investment proposal to meet projected patients demands throughout the product lifecycle, under the current assumptions. For larger patient populations, the partially automated system configuration allowed for greater flexibility to increase capacity within a manufacturing facility and hence resulted in more favorable economic analysis by both COGs analysis and through NPC evaluation. While more capital was needed to be spent up front, the operating cost savings and the flexibility to manufacture many products within the same processing suite, resulted in reduced NPC. The manual process was the most cost-effective option for a relatively smaller patient target and their dependence on scale-out of processing rooms to meet increasing demand resulted in a proportional increase in NPC, and showed the limitations of GMP facilities with many separated processing suites, particularly when moving towards commercial manufacture. The fully automated process was found to be the least attractive proposition by both CoGs and NPC analysis. The higher initial investment cost required, the increased associated manufacturing costs and the lack of flexibility derived from the 'locking' of automated systems for longer periods of time, resulted in less overall capacity. Even if the capacity of the fully automated CAR-T process was doubled and labor requirements were reduced to a minimum, as shown previously, the savings on NPC as patient demand increased were not significant, given the current assumptions.

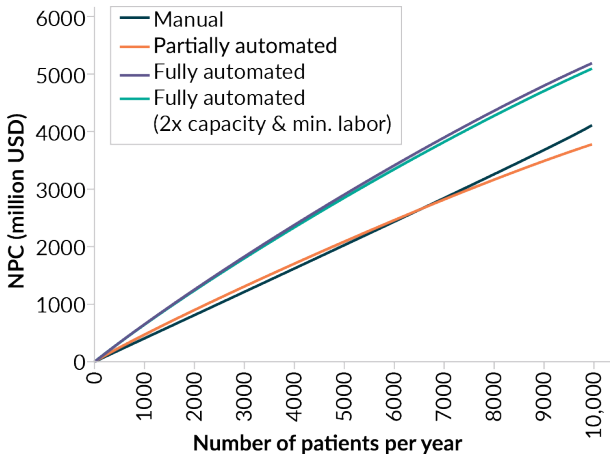
Local & centralized CAR-T supply chain models to reach 5,000 patients

Table 5 presents a summary of the results and key metrics used to compare between the local and centralized supply chain models for a CAR-T therapy. The doughnut charts within this table show the CAR-T supply chain cost breakdown for each of the cost categories outlined previously for CoGs analysis, and an additional transport category.

Orchestrate scheduling tool (Access, UK) was used to estimate peak labor requirements for the entire supply chain process, chosen from a limited defined pool of clinicians, medical couriers, operators and QC personnel. 24/7 operations were assumed, based upon characteristic working hours of hospitals, commercial manufacturing sites and transportation services that support the supply chain network. Given the sensitive nature of these therapies associated with their limited shelf-life, and the emergency associated with these treatments, derived from the critical stage of most patients, working 24 hours a day was considered a requirement to supply such cell therapies. The information obtained from the scheduling tool was used to understand the utilization profiles of the different groups of personnel across the pathways in the local and centralized supply chains. The peak labor requirements were baselined against the outputs of Biosolve Process (Biopharm Services, UK) for a CAR-T manufacturing process

FIGURE 5

Net Present Cost (NPC) of CAR-T processes with increasing levels of automation to meet higher patient demands.

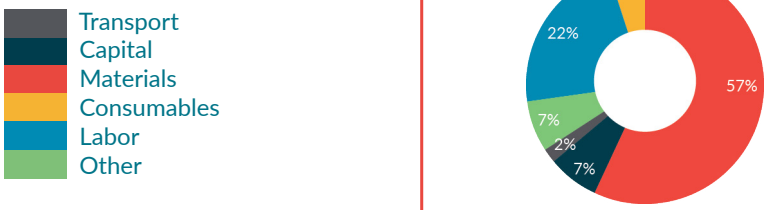


CAR-T manufacture: Manual (blue line), partially automated (orange line), fully automated (purple line) and fully automated with double capacity and minimum labour requirements (green line).

TABLE 5

Summary key metrics of the local and centralized CAR-T supply chain models to meet 5,000 patient peak demand.

Supply chain	Local		Centralized	
No. manufacturing units	13		1	
No. automated systems	325		325	
No. treatment centers (hospitals)	13		16	
No. apheresis machines	104		128	
Transport duration (min)	20		240	
Target no. patients	5,000		5,000	
Labor headcount*				
Clinicians	117		128	
Medical couriers	104		128	
Operators	650		650	
Supervisors	65		65	
QC	624		325	
QA	364		358	
Overall no. of personnel	1,924		1,654	
Facility				
Max. No. batches per year	10,257		10,264	
Total facility floor area (m^2)	10,702		7,682	
Total process floor area (m^2)	961		961	
Total installed capital (USD, million)	$328		$277	
Costs (USD million)	**Total**	**Yearly**	**Total**	**Yearly**
Transport	$6.31	$7.69	$3.75	$7.70
Capital	$28.32	$58.11	$23.87	$49.00
Materials	$236.73	$485.64	$236.73	$485.98
Consumables	$21.88	$44.89	$21.88	$44.92
Labor	$90.26	$190.41	$75.27	$160.99
Other	$28.81	$59.10	$28.81	$59.14
Total cost	$413	$846	$391	$808
Total cost incl. QP release	$463	$896	$441	$858
Costs distribution				



Summary	Local	Centralized
Cost per treatment (USD)	$82,480–82,600	$78,200–78,720
Cost per treatment incl QP release (USD)	$87,355–92,600	$83,592–88,200
Supply chain cycle (days)	15	15

* Peak demand of 5,000 patients at only one time.

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utilizing a single manufacturing line within a local setting. Finally, the results of peak labor demand were employed for the calculation of total Labor costs. The present analysis did not include the optimization of labor, its operational productivity or facility capacity. Any additional risks of failure associated with local production, due to more variation within the various manufacturing facilities and manual labor pool, was not considered in this work.

A baseline peak demand of 5,000 patients and associated peak labor requirements were modelled to allow for 'worse-case' scheduling across both supply chain networks. Increased capacity requirements were also considered in subsequent sensitivity analysis.

From the labor numbers shown in Table 5, to carry out the processing of a peak of 5,000 target patient batches, 650 operators would be required within both supply chain models. The quality personnel required to support QC testing and QA product release were estimated to be of 988 for the local model, and of 683 for the centralized supply chain. The number of clinicians responsible for the collection of apheresis material and re-administration of final CAR-T therapy, varied between 117 for local manufacture, and 128 for the centralized model. A maximum number of 104 and 128 medical couriers would be required to transport cryopreserved apheresis cells and final frozen CAR-T product, from and back to the treatment center, within the local and in the centralized network, respectively. These results showed an increase in the number of personnel required for duties outside manufacturing, from the local supply chain network to the centralized model. This was due to the higher number of apheresis machines and longer travelling times required by the centralized network. The effect of having a single manufacturing unit enabled only Quality labor resource to be used more efficiently in the centralized supply chain, to meet a similar target of 5,000 patients. For manufacturing operations, the number of automated systems was the same for both models resulting in identical requirements for manufacturing personnel.

Kite Pharma has reported having future capacity for 900 jobs at their centralized facility in LA to meet 5000 patients per year [36]. Given that the present centralized model had approximately double capacity than the Kite Pharma of 10,269 patients annually, and assuming that of the reported values approximately 75% corresponded to manufacturing and quality personnel, then these numbers are aligned with present study.

Table 5 shows the estimated cost of the local and centralized facilities with similar capacity. To meet the maximum throughput as the centralized supply chain model of approximately 10,260 patients per year, 13 local manufacturing units would have to be built. As a result, a higher total investment of $328M would be required for the local model, compared with $277M for the centralized supply chain. The total installed capital investment was calculated based on cleanroom areas required for processing which were in turn derived from the equipment used and their associated footprint. The total installed capital cost differential resulted from the increase in the total facility footprint from 7682 m^2 for single site to 10,702 m^2 for the total of the 13 local units. The initial investment of the local model however could potentially be reduced by spreading costs of building new manufacturing centers across several years, which would comprise of less risk and more flexibility to respond to increasing future patient demands.

When analyzing the total supply chain costs to meet the same peak target patient pool of 5,000, costs were decreased from $413M to $391M, as the supply chain was moved away from local manufacture. It could also be seen a decrease in the values of all cost categories, except Materials and Consumables. Here there was no change due to comparable target number of batches/patients for the two supply chain configurations. From the distribution of costs across the different categories shown in the graphs presented in Table 5, once more Materials was the predominant cost driver accounting for 57% and 61% of local and regional overall supply

chain costs, respectively. A similar impact was observed for labor costs with a contribution of between 19% and 22% of overall cost of both supply chain networks.

When the costs associated with the different supply chain models were translated in terms of the total cost per treatment (per patient), the centralized model remained the option with the lowest cost. A final estimated cost of between $78–78k in the US and $84–88k in Europe could be achieved by the centralized supply chain model, compared with $82–83k in the US and $87–93k in Europe for the local network. The differential cost between the US and Europe was derived from the fact that the latter included QP final therapy release costs.

Finally, the supply chain cycles for both models were estimated to be completed within 15 days. This was in line with the reported Kite pharma turnaround time of 14 days with a capacity to produce 5,000 patient specific therapies per year [29].

CAR-T supply chain sensitivity analysis: resource availability, patient demand and CAR-T & modified T-cell expansion step duration

The impact of resource variations such as the pool of personnel and a pre-established number of automated systems available for manufacturing, had upon the total supply chain costs was evaluated. The maximum labor availability for each labor category was set at 100 and 1,000 for the local and centralized networks, respectively. A 50% and 100% variation were analyzed for the availability of personnel for each supply chain model. The number of leukapheresis and automated systems was also varied from the original numbers set out in Table 5. A range of 4 to 16 apheresis machines, and 12 to 50 automated systems per manufacturing unit were made available for the sensitivity analysis of the local supply chain. For the centralized network, a range of 80 to 275 leukapheresis machines and 200 and 700 automated systems were applied.

The impact of a variation in patient target demand was expected to have a significant impact upon the overall supply chain costs. Target patients' values were varied between 2,500 and 10,000 patients for both supply chain models. For the localized network, it meant a change in the number of manufacturing units required to meet new target demands. The centralized model was assumed to remain the same, with no reduction of capacity as demand decreased.

Finally, an increase in the duration of the modified T-cell proliferation step was evaluated. The sensitivity of costs to the extension of cell expansion length for up to 14 days was assessed.

Figure 6 presents the different graphs showing the impact of chosen criteria upon key metrics, as well as the final cost of treatment per patient, for both local and centralized supply chain models.

Figure 6A presents the impact of resource availability upon the cost of CAR-T therapy per patient. Here an increase in resource availability in terms of equipment numbers (leukapheresis machines and automated systems), and labor headcount, resulted in an increase of the final supply chain cost. The same trend was seen for both supply chains, whereby more equipment available required more personnel to operate them, and hence drove costs up to $112k per patient for the local model, and an increase to $106k of the total cost per treatment for the centralized model.

As can be seen in Figure 6B, a variation in target patient demand had no significant impact on the final cost of CAR-T therapy for the local supply chain. The peak labor requirements to meet the revised target patient demands had no change for the local model as this model was assumed to be running continuously at peak labor capacity. However, for the centralized network, a significant increase to 3034 total personnel headcounts were required to meet a higher peak demand of 10,000 patients, which resulted in an

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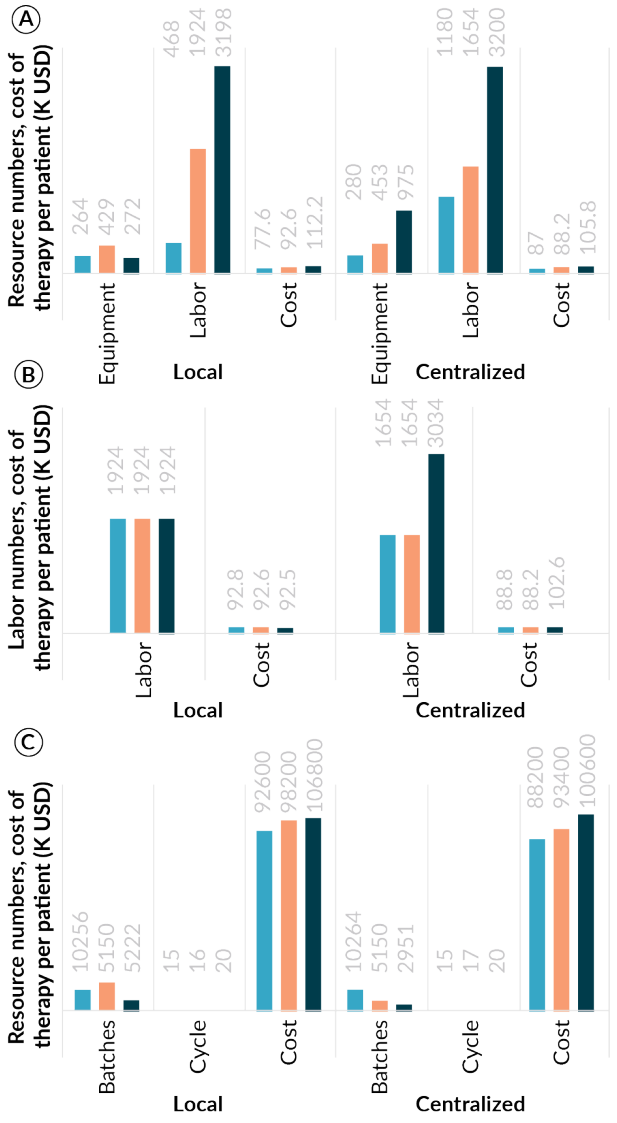
FIGURE 6

CAR-T Supply Chain Sensitivity Analysis.



The impact of the following variables upon cost of CAR-T therapy per patient (USD), namely: (A) Resource Availability (equipment and labor); (B) Patient demand (2,500 and 10,000); (C) Modified T-cell Expansion Duration (up to 14 days). Variation ranges: Low (light blue), Baseline (orange) and High (dark blue).

increased overall cost of therapy to $103k per patient.

Finally Figure 6C shows that there was a gradual increase in the cost of CAR-T per treatment of both the local and centralized supply chain models when the length of the modified T-cell expansion step was extended. Both local and centralized models saw a similar increase in their supply chain cycles from 15 to up to 20 days. This was in line with reported data for commercial CAR-T therapies. Kymriah® (Novartis) cryogenic logistics between a centralized manufacturing facility and its network of 35 treatment sites across the US was set to be achieved in a 21 days cycle from patient-to-patient [1]. Kite Pharma were targeting for vein-to-vein median durations of approximately 17 days with their CAR-T therapy [22].

Both networks saw a 6–15% increase in the cost per patient to up to $107k for local and to $101k for the centralized model. The extension of modified T-cell proliferation step also impacted upon the maximum number of batches it could be achieved within a year and the supply chain cycle duration. As this step was also the manufacturing and supply chain process bottleneck, an increased duration resulted in reduced maximum annual throughput. Even with an excess capacity, an extension of proliferation step by 14 days (Very high) resulted in a reduced throughput of 2951 patients per year, and therefore affected the ability of the centralized manufacturing model to meet the original 5,000 patient target demand. Under the same circumstances, the local network was able to meet the 5,000-patient demand by increasing the number of manufacturing units from 13 to 23.

CONCLUSIONS

In the present work the costs associated with CAR-T manufacture have been estimated using Biosolve Process model and were shown to reflect published data as well as current CAR-T state of play. The major key cost drivers of the autologous CAR T-cell therapy were found to be QC analytics and viral vector starting material, both of which could be outsourced costs or could be developed and managed in-house. The impact of potential labor reduction associated with introduction of automation was evaluated and resulted in a 10% overall cost savings. This emphasises that the introduction of automation is highly dependent upon the process, existing bottlenecks and available systems suitability. Further sensitivity analysis highlighted the

importance of patient (batch) throughput on overall cost of therapy. The effect of long process steps being 'locked' in dedicated fully automated units, such as during the final expansion of modified T-cells, limits facility efficiency creating manufacturing risk. This highlights the need for a radically different approach to manufacturing. Partial automation overcomes some of these whereby several process steps are undertaken by different specialized automated systems that can be integrated and work in parallel. The flexibility conferred by partial automation was found to result in a reduction of the cost associated with CAR-T processes and was shown to be the key for reaching increased patient populations.

Preliminary analysis was performed to compare supply chain models based on local hospital setting versus regional centralized manufacturing to reach a peak demand of 5,000 patients. The fully automated process was utilized in each scenario. Centralized manufacturing was found to be more efficient and presented lower patient costs but was more at risk of demand fluctuations. The importance of debottlenecking the process was illustrated for both supply chains variants.

The present paper highlighted the value of building cost models to provide insight into CoGs, lifecycle and supply chain costs for these expensive cell therapies. Key factors could be identified early, guiding where the industry needed to focus to improve areas such as process development, manufacturing planning and supply chain management. These dynamic tools allowed feasibility assessments to be made regarding the viability of alternative technologies and process options; to guide a future commercial strategy by estimation of costs associated with the scale-out to meet increased patient demand; and outline turn-around-times and resource capacity for future capacity requirements. The model inputs were based on published data and represent a starting point for further analysis of autologous cell therapy manufacture and supply chain optimization.

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